Bioelectrochemistry 80 (2011) 151–154

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Sensitive lactate determination based on acclimated mixed bacteria and palygorskite co-modified oxygen electrode

Jing Chen *, Yeling Jin

College of Bioengineering and Chemical Engineering, Jiangsu Provincial Key Laboratory of Palygorskite Science and Applied Technology, Huaiyin Institute of Technology, Huaian 223003, PR China

ARTICLE INFO

Article history:
Received 11 January 2010
Received in revised form 10 July 2010
Accepted 15 July 2010
Available online 23 July 2010

Keywords:
Lactobacillus
Oxygen electrode
Lactate
Acclimation
Palygorskite

ABSTRACT

A sensitive bacteria biosensor was prepared for the detection of trace lactate. The sensitive bioelement, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* mixed cultrue, and palygorskite, a perfect matrix for bacteria, was co-immobilized on the surface of oxygen electrode. The biosensor possesses fine selective specificity, good sensitivity and longer operational life time, which were due to the mutual help relationship of symbiotic bacteria and 240 days acclimation with lactate as the carbon source. Hydrodynamic amperometry, an advanced electrochemical method, is suitable for on-line monitoring the concentration change of dissolved oxygen that is closely accompanied with the metabolism of lactate. Electrochemical data show that the current is very sensitive to the changes of the concentration of lactate. The response current was linear with lactic acid concentration in the range from 0 to 300 μmol L^{-1}, where the response time is no more than 240 s ($R = 0.9952$), and the sensitivity was 1.87 mA mol^{-1} L. Experiments show the biosensor is also very useful for long time on-line monitoring of lactate, such as fermentation progress.

1. Introduction

Simple, reliable and rapid monitoring of lactate in complex media has been desired continuously in many areas, such as clinical analysis, sports medicine, food industry and biotechnology. Blood lactate levels can predict multiple organ failure, including shock, respiratory insufficiencies, and heart or liver diseases [1,2]. Continuous real-time monitoring of metabolites with portable instruments represents a significant advance in clinical, sport medicine and fermentation technology [2,3]. Besides, lactate determination is a very useful way to detect the microbial contamination because the microbial contamination of food always results in lactate fermentation [4]. The on-line detection, control and fault analysis on lactate will play much greater role than past with the development of ideal analysis method of lactate.

There are some physical methods for lactate measurement, such as conductivity measurement [5], near-infrared spectroscopy [6], HPLC [7], spectrophotometry [8] and chemiluminescence [9]. However, it is still the main way to employ various enzymes in the analysis of lactate, such as NAD^+-dependent lactate dehydrogenase [10], lactate oxidase [11] and flavocytochrome b_2 [12], because of the high selectivity of the bio-recognition element. All of these enzymes catalyze the oxidation of L-lactic acid to pyruvic acid with the reduced form of nicotinamide adenine dinucleotide (NADH) or hydrogen peroxide (H_2O_2) being the signaling molecule for the detection. Nevertheless, these methods have some disadvantages: (1) The interfering substances in the sample can introduce unacceptable analytical errors; (2) Time-consuming assays, usually requiring a protein-free filtration or other pretreatments, cannot meet the practical requirements of rapid simultaneous measurement. Thereafter, enzyme-based amperometric biosensors have been developed during the past decades considerably, due to not only their high selectivity and the sensitivity of electrochemical signal transduction, but also their simple sample preparation and rapid portable analysis. However, enzyme-based amperometric biosensors often suffer from bad reproducibility, low stability, and expensive cost.

It is promising to use plant tissues, animal tissues or bacteria as the bio-recognition element because of some obvious advantages: no need of adding co-factors, no need of isolation and purification of enzymes, low analytical cost and probable increase of enzyme stability since the enzyme is retained in its natural environment. Poor selectivity is the main disadvantage of tissue or bacteria biosensor. However, microbial acclimation or genetic recombination can improve the analytical performance of the relevant bio-recognition element to get high selectivity and wide detection range greatly. Many studies have focused on the feasibility of various tissues [9] and bacteria [13–16] as the bio-recognition elements of lactate. *Lactobacillus bulgaricus* and *Streptococcus thermophilus* have perfect symbiotic relationship, have always been mixed culture and widely used in the dairy industry. In this paper, the two bacteria, as recognition element, were mixed and acclimated to endue the biosensor with better selectivity, sensitivity and long operational lifetime [17].

* Corresponding author. Tel.: +86 517 83591044; fax: +86 517 83591190.
E-mail address: chenjing6910@163.com (J. Chen).

1567-5394/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bioelechem.2010.07.004

The reactions involve in the analysis of lactic acid are the following:

$$\text{Lactate} + O_2 + H_2O \rightarrow \text{Pyruvate} + H_2O_2 \quad (1)$$

$$\text{Pyruvate} + O_2 \rightarrow CO_2 + H_2O \quad (2)$$

The response current of oxygen electrode depends on the consumptions of dissolved oxygen by the bacteria, which is proportional to the concentration of lactic acid.

The sensitivity of biosensor also depends on the immobilization technology, where the immobilized materials are very important [18–20]. Palygorskite, a hydrated magnesium aluminum silicate, is a natural one-dimensional (1D) nano-material. Bradley proposed the ideal structure of palygorskite early in 1940 [21]. Because of its unique physical and chemical properties, palygorskite is receiving more and more attention in many areas, such as inorganic–organic polymer hybrids, nano-fillers, fertilizer suspensions, drilling fluids, oil refining, catalysts, catalyst supports, pharmaceutical industry and so on [22–24]. Many national pharmacopoeias have accepted palygorskite [25]. In addition, its fine biological affinity makes it a good carrier for cell immobilization [26]. Therefore, there are three reasons that we choose palygorskite as the matrix of enzyme immobilization: (1) moderate absorptive capacity; (2) fine biological affinity; (3) unique one-dimensional (1D) nano-structure, which can significantly reduce the shrinkage cracks of the modified membranes just like carbon nanotubes. Our experiments proved that the *lactobacillus*/palygorskite biosensor is very successful in lactate measurement.

2. Experimental

2.1. Chemicals

Palygorskite (from Jiuchuan clay company, Jiangsu, China) was first soaked in water for 24 h. Then the mixture (the concentration of palygorskite is about 5%) was dispersed by magnetical stirring and ultrasonic wave for 3 h. After that the colloid suspension was centrifuged and the supernatant (about 1/2 of volume) was collected. By repeating the above processes three times relatively pure palygorskite can be obtained. Then the pure palygorskite was re-suspended in water with the concentration of 5 mg mL^{-1}. Lactate assay kits were purchased from Eton Bioscience, Inc. Beef paste and peptone were of reagent-grade purchased from Shanghai Fortune Biotech Ltd, and all the other chemicals used in this study were of analytical grade from Shanghai Chemical Reagent Co., Ltd. All solutions were prepared with double distilled water. The buffer system (pH = 6.8, PBS) was 0.1 mol L^{-1} KH_2PO_4-Na_2HPO_4. Basal medium (1000 mL): bacto-peptone 10 g, beef extract 3 g, NaCl 5 g. Inorganic salt medium (1000 mL): $(NH_4)_2SO_4$ 1 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $Na_2HPO_4 \cdot 7H_2O$ 0.05 g, KCl 0.1 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, and the total amount of lactose and lactic acid was fixed on 15 g. Milk samples were purchased from local markets and blood samples were collected from a local hospital. All samples were used directly without further treatment. Samples were sealed and kept at 4 °C before analysis for no more than 3 days.

2.2. Cell culture and preparation of biofilm

Both *L. bulgaricus* and *S. thermophilus* were isolated from yogurt and identified according to Berger Microbial Identification System. They were then mixed and cultured at 1:1 ratio. The mixed bacteria were incubated in basal culture medium under aerobic condition for 12 h at 37 °C with shaking at 150 rpm. The acclimation process includes two steps: (1) gradually increasing the ratio of inorganic salt medium to basal medium; (2) after acclimation to inorganic salt medium, gradually increasing the ratio of lactic acid to lactose where the total amount of lactic acid and lactose fixed at 15 g / 1000 mL. Then after 240 days of acclimation, the mixed lactobacillus got their fine selective specificity to lactate while retaining their powerful metabolism vitality in high concentration of lactic acid (about 1.5%). The acclimated cells were cultured under the same condition as above, then harvested and washed twice with 0.1 mol L^{-1} PBS at room temperature. The bacterial suspensions used for the experiments were fixed on 0.5 g wet cells in 1 mL PBS solution.

100 μL of the bacterial suspension and equal volume of palygorskite suspension were mixed. A certain volume of the above mixture was cast onto a piece of acetyl cellulose membrane filter (with pore diameter of 0.2 μm, purchased from Shanghai Biochemical Co., Ltd.), dried at ambient temperature for 30 min. If not used immediately, the biofilm was sealed and stored in refrigerator at 4 °C.

2.3. Experiment procedure

The biofilm was fixed on the surface of an integrated oxygen electrode (including an Au cathodic electrode, an Ag anodic electrode, and an Ag/AgCl reference electrode) with an 'O' ring just before an electrochemical experiment. The polarization potential was −0.7 V. The current measurement was carried out by Dissolve Oxygen Analysis Instrument (JPSJ-605, Shanghai Jingke, China), The whole procedure was performed in the detection chamber containing 20 mL of buffer solution, stirred with a magnetic bar. A constant temperature-bath was used to maintain the experiment temperature at 35 ± 0.2 °C unless stated otherwise. When the current output of the oxygen electrode reached a steady state, the sample was added into the buffer solution. The current output of the oxygen electrode decreased and reached a new steady state after addition of sample. The difference between the steady state currents before and after addition of the sample was taken as the response current.

3. Results and discussion

3.1. The effect of acclimation

After acclimation, the mixed bacteria were able to grow well in inorganic salt medium at higher lactate concentrations (1.5%). Fig. 1 shows the effect of acclimation on the calibration curve of the biosensor. The response current of the electrode made by acclimated bacteria is about double that of the electrode by plain bacteria. Thus, higher sensitivity and wider linear range of the biosensor on lactic acid should be attributed to the acclimation.

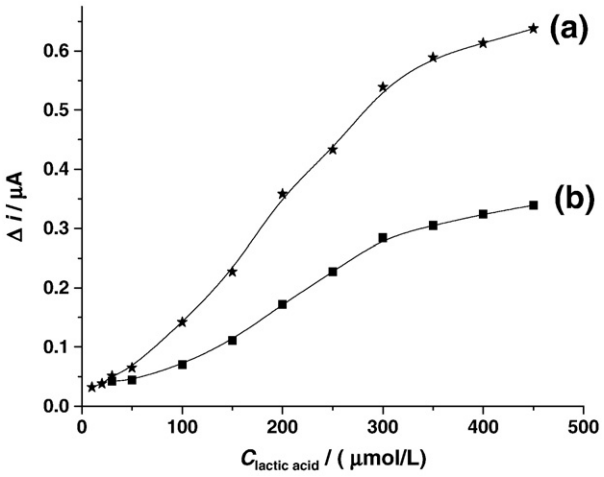

Fig. 1. Current changes vs. the concentration of lactic acid: (a) the biosensor made from acclimated bacteria; and (b) the biosensor made from non-acclimation bacteria. Buffer: 0.1 mol L^{-1} PBS (pH 6.8). Analytical temperature: 35 ± 0.2 °C. Stirring speed: 150 rpm.

The acetate cellulose membrane can act as the barrier of ascorbic or uric acid, keeping them from the electrode surface. Therefore, those compounds do not affect the biosensors fabricated with acetate cellulose membrane [27]. Besides, lactic acid, glucose and ethanol, which are present in biological samples widely, are also explored on their effects on lactate analysis (Fig. 2). Fix the concentrations of all analytical substrates on 100 μmol L^{-1}, the response current of the biosensor to lactate is 44.8 times that of ethanol, and 11.9 times that of glucose, which proves that these interfering compounds do not affect lactate detection of the biosensor.

3.2. The effect of temperature

Temperature plays an important role in most enzymatic relative reactions, so the effect of temperature on the sensitivity of the biosensor is studied in the range from 15 to 45 °C. Experiment revealed that a rise of temperature will increase the biosensor sensitivity and response current (Fig. 3). The sensitivity of the biosensor at 35 °C is 1.87 mA mol^{-1} L, and that at 25 °C is 0.44 mA mol^{-1} L. Because the increase of the response current in optimum temperature that ranges from 35 °C to 45 °C is very small, and high temperature has adverse effects on the stability and accuracy of the biosensor, 35 °C is chosen as the analytical temperature.

3.3. Response time

The steady state current decreases after the addition of lactic acid and reaches a new steady state in 40 s for 10 μmol L^{-1} lactic acid, 60 s for 50 μmol L^{-1} lactic acid, and 220 s for 250 μmol L^{-1} lactic acid. The response time to reach 95% steady state was approximately 225 seconds, so the measuring time with the biosensor is less than 4 min, which is shorter than that of commercial testing kits (10 min), and the best response time of a similar microbial electrode (about 5 min) [28]. The response time of the biosensor depends on the concentration of lactic acid and the diffusion barrier of the biofilm, so the biosensor response is under diffusion control.

3.4. Characterization of the biosensor performance

Fig. 4 shows the dependence of current response on lactate concentration, and the linear relation is between 0 and 300 μmol L^{-1} of lactate, the sensitivity of the biosensor is 1.87 mA mol^{-1} L in that condition. The relative standard deviation (RSD) is 3.2% for measurement of a standard sample (120 μmol L^{-1}) by six different modified

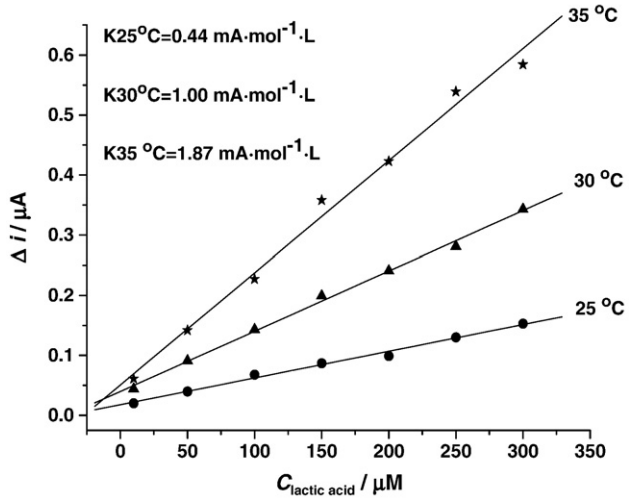

Fig. 3. The calibration plots of current changes vs. the concentration of lactic acid at different temperatures. Other experimental conditions are same as in Fig. 1.

electrodes, which is reasonably satisfactory. The measurements on five different concentrations (50, 100, 150, 200 and 250 μmol L^{-1}) are performed on three separate days, where the reproducibility calculated on the three slopes is 4.1% (n = 3).

Fig. 5 shows that the biosensor can work well and give correct response signal for at least 13 h, so it is possible for the biosensor to be used in an on-line analysis, such as monitoring the progress of fermentation in factory. The biosensor, having been used for short-time analysis, can recover its analytical ability after re-immersing in blank buffer solution for 3 min, and can be reused for up to 15 measurement since the response current of the 15th measurement was no less than 90% of the initial one as shown in the inset of Fig. 5. Besides, the biofilm can keep 93% of its activity after being sealed and stored in the refrigerator at 4 °C for 46 days.

Further studies have proven that the biosensor can also be used in the analysis of milk products and blood samples successfully. The results agreed well with that measured by commercial lactate testing kit. The regression equation between the results obtained by commercial testing kit (x-axis) and those by the biosensor (y-axis) for the sample of milk products (six samples) is y = 1.017 x − 0.1311 with R of 0.9786. Analytical results for the six blood samples are

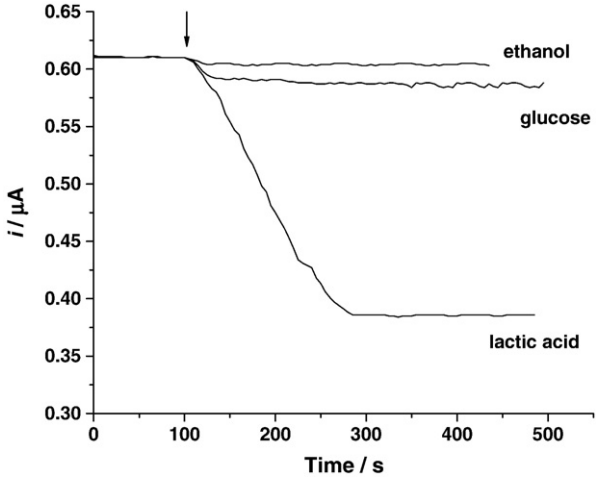

Fig. 2. Current response of the biosensor upon addition of different substrates. Ethanol, glucose and lactic acid are fixed on 100 μmol L^{-1} (final concentration) respectively. Other experimental conditions are same as in Fig. 1.

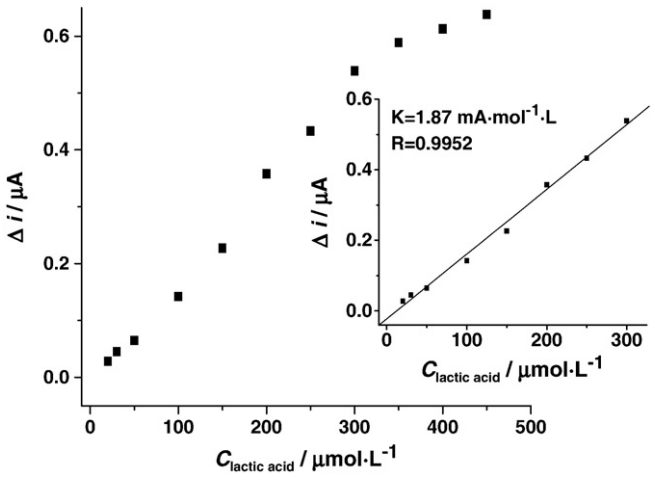

Fig. 4. Dependence of response current on the concentration of lactic acid. The inset is the calibration plot of the biosensor, with a linear regression equation of y = 0.02281 + 0.00187 x (R = 0.9952.). Other experimental conditions are same as in Fig. 1.

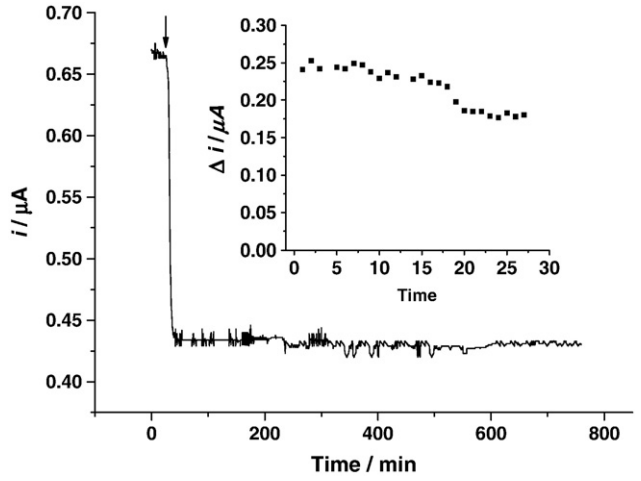

Fig. 5. Signal response of the biosensor to 170 μmol L^{-1} lactate on continuous application. The inset shows the dependence of the response currents on the reuse times of one biosensor (n = 3).

Table 1
Comparative investigation of lactate concentrations for different blood samples by the biosensor and a commercial lactate testing kit (n = 3).

Analysis method	The lactate concentration of sample (mmol L^{-1})					
	1	2	3	4	5	6
The biosensor	0.36	1.24	1.59	0.75	0.74	0.87
Commercial lactate testing kit	0.34	1.25	1.54	0.73	0.71	0.87

shown in Table 1. The results show that the present biosensor is practical and accurate for commercial application.

Compared with kidney tissue [9], *Escherichia coli* [13,14] and yeasts [15,16], the perfect symbiosis and long time acclimation brought high analytical performance to the biosensor, such as better selectivity and long time on-line analysis. Besides, the working stability of oxygen electrode and absence of redox mediator made the biosensor very suitable for long time on-line monitoring of lactate, which cannot be done by the biosensor aided with the redox mediator [15,16].

4. Conclusions

The biosensor, based on acclimated *lactobacillus* and palygorskite co-modified oxygen electrode, performed very well in lactate analysis. The two main achievements of this work, better selectivity and long time on-line analysis, were very meaningful for practical application. The correct selection and proper treatment of the bacteria were the first one, where the perfect symbiosis of *L. bulgaricus* and *S. thermophilus* and long time acclimation of the bacteria endured the biosensor with better selectivity, sensitivity and long operational lifetime. The second one, Palygorskite is a good matrix for bacteria immobilization, which is also important to ensure the perfect performance of the biosensor. Further studies indicate that the biosensor could be used for on-line analysis of lactate and its assay in for example milk products and blood samples.

References

[1] P.B. Oliver, Lactic acidosis, Am. J. Med. 48 (1970) 209–225.
[2] A. Poscia, D. Messeri, et al., A novel continuous subcutaneous lactate monitoring system, Biosens. Bioelectron. 20 (2005) 2244–2250.
[3] M. Esti, M.C. Messia, et al., Curd-ripening evaluation by flow injection analysis of L-lactic acid with an electrochemical biocell during mozzarella cheese manufacture, J. Agric. Food Chem. 44 (1996) 3102–3107.
[4] F. Palmisano, M. Quinto, et al., Flow injection analysis of L-lactate in milk and yoghurt by on-line microdialysis and amperometric detection at a disposable biosensor, Analyst 126 (2001) 866–870.
[5] T. Payot, M. Fick, On-line estimation of lactic acid concentration by conductivity measurement in fermentation broth, Biotechnol. Techniques 11 (1997) 17–20.
[6] M.G. Macedo, M.F. Laporte, C. Lacroix, Quantification of exopolysaccharide, lactic acid, and lactose concentrations in culture broth by near-infrared spectroscopy, J. Agric. Food Chem. 50 (2002) 1774–1779.
[7] A.M. Girelli, E. Mattei, Application of immobilized enzyme reactor in on-line high performance liquid chromatography: a review, J Chromatogr. B 819 (2005) 3–16.
[8] C.I. Li, Y.H. Lin, et al., Sol–gel encapsulation of lactate dehydrogenase for optical sensing of L-lactate, Biosens. Bioelectron. 17 (2002) 323–330.
[9] F.Q. Wu, Y.M. Huang, C.Z. Huang, Chemiluminescence biosensor system for lactic acid using natural animal tissue as recognition element, Biosens. Bioelectron. 21 (2005) 518–522.
[10] M. Zayats, A.B. Kharitonov, et al., An integrated NAD^+-dependent enzyme-functionalized field effect transistor (ENFET) system: development of a lactate biosensor, Biosens. Bioelectron. 15 (2000) 671–680.
[11] R. Garjonyte, Y. Yigzaw, et al., Prussian Blue and lactate oxidase-based amperometric biosensor for lactic acid, Sensor. Actuat. B 79 (2001) 33–38.
[12] O. Smutok, G. Gayda, et al., A novel lactate-selective biosensor based on flavocytochrome b2 from methylotropic yeast *Hansenula polymorpha*, Biosens. Bioelectron. 20 (2005) 1285–1290.
[13] S.G. Ignatov, J.A. Ferguson, D.R. Walt, A fiber-optic lactate sensor based on bacterial cytoplasmic membranes, Biosens. Bioelectron. 16 (2001) 109–113.
[14] J.M. Dong, J.S. Taylor, et al., Three overlapping lct genes involved in L-lactate utilization by *Escherichia coli*, J. Bacteriol. 175 (1993) 6671–6678.
[15] R. Garjonyte, V. Melvydas, A. Malinauskas, Mediated amperometric biosensors for lactic acid based on carbon paste electrodes modified with baker's yeast *Saccharomyces cerevisiae*, Bioelectrochemistry 68 (2006) 191–196.
[16] R. Garjonyte, V. Melvydas, A. Malinauskas, Amperometric biosensors for lactic acid based on baker's and wine yeast, Microchim. Acta 164 (2009) 177–183.
[17] K. Lee, J. Lee, Y.H. Kim, et al., Unique properties of four lactobacilli in amino acid production and symbiotic mixed culture for lactic acid biosynthesis, Curr. Microbiol. 43 (2001) 383–390.
[18] H. Tap, P. Gros, A.M. Gué, An amperometric silicon-based biosensor for D-lactate, Sensor. Actuat. B 68 (2000) 123–127.
[19] D.A. Chen, Y.B. Cao, et al., A BOD biosensor based on a microorganism immobilized on an Al_2O_3 sol–gel matrix anal, Bioanal. Chem. 372 (2002) 737–739.
[20] K. Kriz, L. Kraft, et al., Amperometric determination of L-lactate based on entrapment of lactate oxidase on a transducer surface with a semi-permeable membrane using a SIRE technology based, biosensor. Application: tomato paste and baby food, J. Agric. Food Chem. 50 (2002) 3419–3424.
[21] W.F. Bradley, The structural scheme of attapulgite, Am Miner. 25 (1940) 405–410.
[22] J.H. Huang, Y.F. Liu, et al., Effect of attapulgite pore size distribution on soybean oil bleaching, J. Amer. Oil. Chem. Soc. 84 (2007) 687–692.
[23] S.D. Miao, Z.M. Liu, et al., Ionic liquid-assisted immobilization of Rh on attapulgite and its application in cyclohexene hydrogenation, J. Phys. Chem. C 111 (2007) 2185–2190.
[24] J.H. Huang, Y.F. Liu, X.G. Wang, Influence of differently modified palygorskites in the immobilization of a lipase, J. Mol. Catal. B. 55 (2008) 49–54.
[25] The United States pharmaeopoeis 30th ed, the united states pharmacopoeial convention.
[26] J.M. Xu, W. Li, et al., Direct electrochemistry of cytochrome c on natural nano-attapulgite clay, modified electrode and its electrocatalytic reduction for H_2O_2, Electrochim. Acta 52 (2007) 3601–3606.
[27] A. Poscia, M. Mascini, et al., A microdialysis technique for continuous subcutaneous glucose monitoring in diabetic patient (part 1), Biosens. Bioelectron. 18 (2003) 891–898.
[28] G. Kenausis, Q. Chen, A. Heller, Electrochemical glucose and lactate sensors based on "wired" thermostable soybean peroxidase operating continuously and stably at 37 °C, Anal. Chem. 69 (1997) 1054–1060.