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Development of a conductometric phosphate biosensor based on tri-layer maltose phosphorylase composite films

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ABSTRACT

A conductometric biosensor for phosphate detection was developed using maltose phosphorylase (MP) from recombinant *Escherichia coli* immobilized on a planar interdigitated electrode by cross-linking with saturated glutaraldehyde (GA) vapour in the presence of bovine serum albumin (BSA). The process parameters for the fabrication of the mono-enzymatic sensor and various experimental variables such as the enzyme loading, time of immobilization in saturated GA vapour, working buffer solution and temperature were investigated with regard to their influence on sensitivity, detection limit, dynamic range, operational and storage stability. The biosensor can work well at the temperature between 20 °C and 50 °C, and reach 90% of steady-state conductance in about 10 s. The sensor has two linear ranges, one is from 1.0 μM to 20 μM phosphate with a detection limit of 1.0 μM, and the other is between 20 μM and 400 μM phosphate. When stored in citrate buffer (0.1 M, pH 6.0) at 4 °C, the biosensor showed good stability over two months. No obvious interference from other anionic species like SO_4^{2-}, Cl^-, NO_3^-, NO_2^- and HCO_3^- was detected. The biosensor is suitable for use in real water samples.

1. Introduction

Phosphate is an essential nutrient for the growth of aquatic plants. Because of its widespread presence in detergents and fertilizers, phosphate concentration can be so high that it leads to the eutrophication of lakes and rivers [1]. In clinical diagnosis, the determination of phosphate levels in body fluids can provide useful information about several diseases, the energetic state of cells and bone function [2]. Additionally, an excess diet of phosphate in food products will affect human health [3]. Thus, phosphate determination is also relevant to food quality control.

Spectrophotometric methods, based on colorimetric detection, have been widely applied for the determination of phosphate. However, these methods are tedious and time consuming [4,5]. Moreover, expensive instruments, well-trained operators and carcinogenic chemicals, most commonly molybdenum and antimony tartrate, are needed in these analyses [6]. Ion-selective electrodes based on tin complexes and metallic wiring have also been developed

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for phosphate analysis, but both methods suffer from low selectivity and poor stability of the ion-selective membrane [7,8]. Consequently, it is necessary to develop a sensitive, reliable, stable and cost-effective phosphate sensor that can instantly analyze the phosphate concentration.

Biosensors for the determination of phosphate are normally based on multi- or mono-enzymatic reactions where phosphate acts as inhibitor or substrate [6]. The importance of enzyme-based amperometric biosensors has increased considerably during the past decades due to the high selectivity of the biorecognition element and the sensitivity of the electrochemical signal transduction [1]. A four-enzymatic sensor for phosphate detection was reported using maltose phosphorylase (MP), acid phosphatase (AP), glucose oxidase (GOD) and mutarotase (MR) with the following reaction sequence [9].

$$\text{maltose} + \text{phosphate} \xrightarrow{\text{MP}} \beta\text{-D-glucose-1-phosphate} + \alpha\text{-D-glucose} \quad (1)$$

$$\beta\text{-D-glucose-1-phosphate} \xrightarrow{\text{AP}} \beta\text{-D-glucose} + \text{phosphate} \quad (2)$$

$$\alpha\text{-D-glucose} \xrightarrow{\text{MR}} \beta\text{-D-glucose} \quad (3)$$

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{GOD}} \beta\text{-D-glucose acid} + H_2O_2 \quad (4)$$

$$H_2O_2 \xrightarrow{-2e} 2H^+ + O_2 \quad (5)$$

The combination of the former two enzymes generates two glucose molecules per reaction cycle and recycles one molecule of phosphate, and the oxidation of glucose is catalyzed by the last enzyme after its mutarotation. The formation of hydrogen peroxide during the enzymatic reaction can be monitored electrochemically at a platinum electrode. Based on this study, both tri- and bi-enzymatic sensors for phosphate detection have been described. Karube and coworkers [10] reported a chemiluminescence flow-injection analysis biosensor in which MP, MR, GOD, and *Arthromyces ramosus* peroxidase were used to build up a reaction system. The response for this system was linear in a range between 10 nM and 30 μM phosphate. Mousty et al. [11] used a simple method to fabricate an amperometric phosphate biosensor containing MP, MR, and GOD with a linear range of 1–50 μM. Hüwel et al. [12] successfully applied MP and GOD as the bioelements of a simple bi-enzymatic sensor, in which the first enzyme consumed phosphate as a cosubstrate and yielded a product that was a substrate for the second enzyme.

However, more enzymes involved in the sensor system have been leading to more non-specific response due to the presence of substrates for the next enzymes [13]. Furthermore, the stability of each enzyme caused fluctuations in the sensor performances. For example, in the phosphate detection system consisting of nucleoside phosphorylase and xanthine oxidase, the degradation of inosine often restricted the reliability of phosphate analysis [14]. For this bi-enzyme system, no linear range was found even though it sensed phosphate [14,15]. In addition, it is sure that multi-enzyme systems are rather complicated and expensive.

On the other hand, for the past five years, conductometric biosensor devices have been reported increasingly [16–18]. Conductance measurements involve the electrical resistance determination of a sample solution between two parallel electrodes. Conductometric biosensors present some advantages as follows: (a) the planar conductometric electrodes are simple and relatively cheap which suits well for miniaturization and large scale production, and are therefore very promising for practical use; (b) the applied voltage can be sufficiently small to minimize significantly the sensors power consumption; (c) a large spectrum of analytes of different nature can be determined on the basis of various reactions and mechanisms; (d) conductometric biosensors are the most suitable for integration since they do not require any reference electrode and the conductometric transducers can be manufactured using simple thin film technology [17,19]. To our best knowledge, no previous work describing the immobilization of enzymes for phosphate detection on an electrode surface resulting in conductometric biosensors has been reported to date.

In this paper we present a mono-enzymatic biosensor for phosphate analysis based on MP immobilized on a conductimetric transducer. The principle of the detection is based on the fact that many biochemical reactions in solution produce changes in the electrical resistance (reciprocal conductance). The subsequent local changes of conductance inside MP-based membrane are dependent on the reaction (1), and thus the conductometric phosphate biosensor is conceived. The use of MP is advantageous because mono-enzyme involvement reduces interferences from the sample constituents [20]. In addition, the buildup of the biosensor requires an optimized procedure for enzyme immobilization leading to an optimized performance of the conductometric phosphate biosensor.

2. Experimental

2.1. Reagents

Maltose phosphorylase (EC 2.4.1.8) from recombinant *Escherichia coli* (15 U mg^{-1}) was obtained from Biozyme Laboratories Limited. Bovine serum albumin (BSA), aqueous solutions (25%, w/v) of glutaraldehyde (GA), and maltose were purchased from Sigma–Aldrich Chemie GmbH. Monopotassium phosphate (KH_2PO_4) was obtained from Merck. All other chemicals were of analytical grade. Millipore Milli-Q nanopure water (resistivity 18.2 MΩ cm) was used throughout for the preparation of solutions.

2.2. Apparatus

The measurement setup is shown in Fig. 1. The conductometric transducers (Fig. 1(a)) were fabricated at the Institute of Semiconductors Physics, Kiev, Ukraine. A pair of Au (150 nm thick) interdigitated electrodes were made by lift-off on a ceramic support (10 mm × 30 mm). A 50 nm thick intermediate Ti layer was used to improve the adhesion of Au to the support. The pads of the sensor chip were covered manually with epoxy resin for their insulation. Both the digit width and interdigital distance were 20 μm, and their length was about 20 μm. As a result, the sensitive area of each electrode was about 1.0 mm^2.

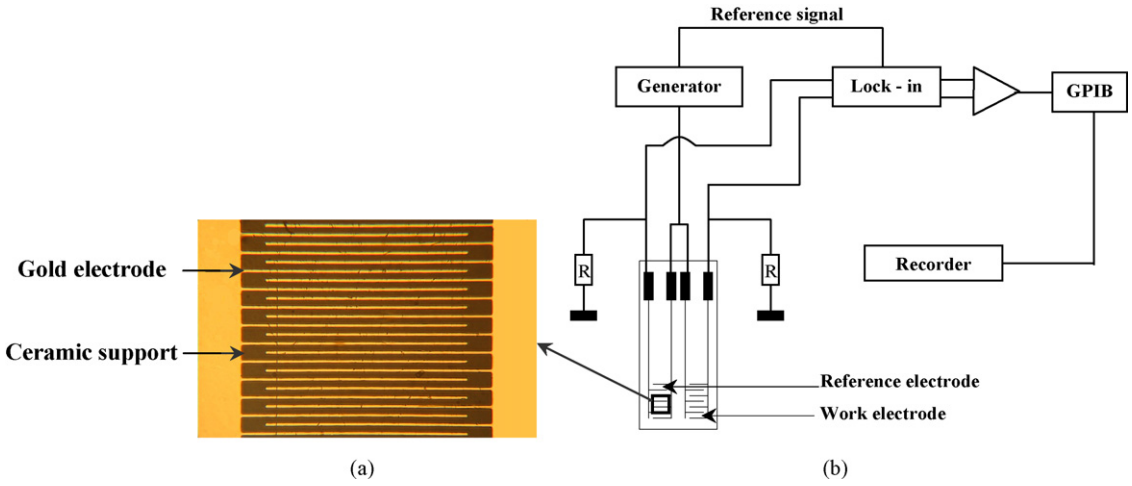

Fig. 1 – (a) Optical microscopy view of the conductometric electrode digits; (b) general view of the experimental setup. *Note*: "R" refers to standard resistance.

A low-frequency wave-form generator (Schlumberger, type 4431) was used to generate a sinusoidal wave with a frequency of 100 kHz and a peak-to-peak amplitude of 10 mV around a fixed potential of 0 V to each pair of electrodes, forming a miniaturized conductance cell.

2.3. *Biosensor preparation*

The enzymatic membrane was prepared on the electrode surface by cross-linking the enzyme with saturated GA vapour [18]. An enzymatic mixture (0.5 μL) of 10% (w/w) MP and BSA, 10% glycerol in 0.1 M citrate buffer (pH 6.0) was deposited on the sensitive area of the sensor using a drop method with a microlitre syringe, while another mixture (0.5 μL) of 10% (w/w) BSA, 10% (v/v) glycerol in 0.1 M citrate buffer (pH 6.0) was similarly deposited on the other electrode. The latter was considered to be the reference electrode. The enzymatic membrane consisted of three overlapping layers, each of which was placed in saturated GA vapour for 20 min followed by drying in air for 20 min at room temperature (about 25 °C). The prepared biosensors were stored in 0.1 M citrate buffer (pH 6.0) at 4 °C.

2.4. *Phosphate measurements*

All measurements were performed in daylight at room temperature in a glass cell. As shown in Fig. 1(b), the sensor chip was immersed in a measuring cell filled with 5 mL of magnetic-stirred citrate buffer (0.1 M, pH 6.0), containing 20 mM maltose and a specific mono-potassium phosphate concentration. After stabilisation of the output signal, sample concentrations (mono-potassium phosphate solution) were adjusted by adding defined volumes of an appropriate concentrated stock phosphate solution. The differential output signal between the working electrode (covered with the immobilized enzyme) and the reference electrode was logged by a SR 510 lock-in amplifier (Stanford Research System) and ΔS was calculated (=($S_n - S_0$), where S_n is the conductive value in the presence of phosphate and S_0 is the conductive value obtained in the absence of phosphate); the steady-state response of the biosensor was plotted as a function of the phosphate concentrations.

3. Results and discussion

3.1. *Optimization of the enzymatic membrane formation*

The performances of the biosensor, in terms of calibration range, detection limit, sensitivity and long-term stability, are strictly dependent on the quantity of loaded MP. As the sensitive area of the interdigitated electrode is only 1 mm^2, a volume over 0.5 μL of a drop of the enzymatic mixture went against the membrane formation (data not shown). Therefore, the amount of the loaded enzyme was controlled by modifying the quantity of the enzyme layers, while for each

Table 1 – Effect of the amount of MP loading on the performances of the conductometric phosphate biosensor

Number of layers	Response time (s)	Conductance response to 200 μM phosphate (μS)	Linear range (μM)	Detection limit (μM)
0	–	–	–	–
1	5	9.9 ± 0.4	14–260	6.0
2	8	21.8 ± 0.6	8.0–320	4.0
3	10	30.7 ± 0.6	1.0–400	1.0
4	35	10.3 ± 0.8	1.0–400	1.0
5	90	6.2 ± 0.3	1.0–320	1.0

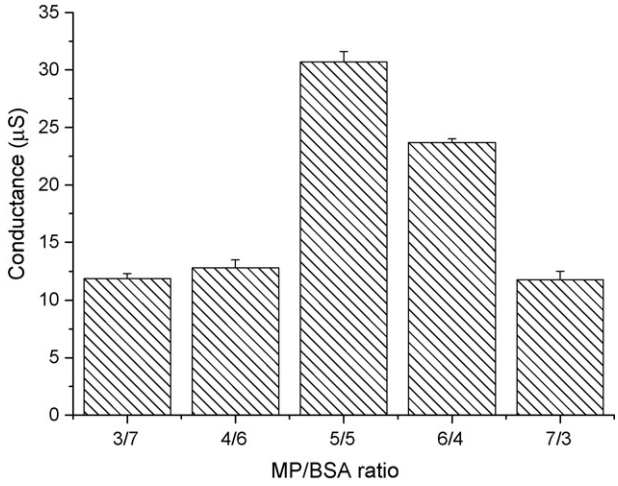

Fig. 2 – Dependence of the conductometric phosphate biosensor response on the MP/BSA ratio in the enzymatic membrane. The total amount of protein remains constant (10%, w/v). Measurements were performed in citrate buffer (0.1 M, pH 6.0) containing 20 mM maltose at room temperature with 200 μM phosphate.

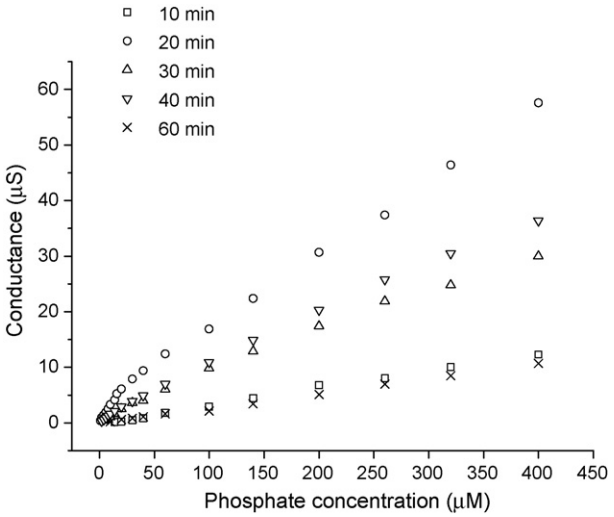

Fig. 3 – Dependence of the conductometric phosphate biosensor performances on the exposure time to saturated GA vapour. Measurements were performed in citrate buffer (0.1 M, pH 6.0) containing 20 mM maltose at room temperature.

layer the 0.5 μL of enzyme solution was dropped. As shown in Table 1, tri-layer is optimal for the electrode, and the biosensor response time, i.e. time necessary to reach 95% of the steady-state amplitude was about 10 s. The further increase in the layers might be leading to the increase of the diffusional resistance of the biochemical reaction signal arriving at the electrode surface and then to a longer response time of the biosensor. On the other hand, if enzyme quantity is too low, there are not enough enzyme molecules involved in the reaction which leads only to a slight conductance variation. A tri-layer enzymatic membrane can not only assure enough enzyme molecules immobilized, but also promote the device stability.

Note that without any enzyme, no signal was observed. This indicates that the detection process of the conductometric biosensor is phosphate dependent and enzyme-catalyzed.

The effect of the MP/BSA ratio in the enzymatic membrane on the biosensor response to phosphate was investigated (Fig. 2). It can be seen that the maximum of the enzyme activity in membrane can be achieved for the MP/BSA ratio equal to 1:1. The further increase in the ratio might lead to the enzyme molecules too tight and most of the active sites concealed. On the other hand, if the ratio is too low, the excessively dispersed enzyme molecules have less opportunities to contact with the reagents and then to take part in the biochemical reaction. In addition, a high concentration of BSA may effectively decrease the MP activity [21].

Considering the effect of exposure time to saturated GA vapour on the activity of MP composite film, the biosensor response to phosphate with different times of exposure is shown in Fig. 3. It can be seen that best results were obtained for 20 min of exposure. For longer exposure times, a dramatic decrease in response was found. This phenomenon may be related to the formation of a large number of covalent bondings between GA and the enzyme molecules, which lead to blocking enzyme active sites. Moreover, such dense membranes can also block the diffusion of substrates and products of the biochemical reaction. On the other hand, for a shorter exposure time (<20 min), the enzyme leakage through the membrane may take place due to an insufficient covalent bondings, and thus the stability of the biosensor becomes poor and the response of the sensor decreases strongly with the number of measurements [17,22].

3.2. *Influence of phosphate measurement variables*

Since the enzyme activity is strongly affected by the buffer solution pH, the influence of pH on the biosensor response to phosphate was examined in the presence of 200 μM phos-

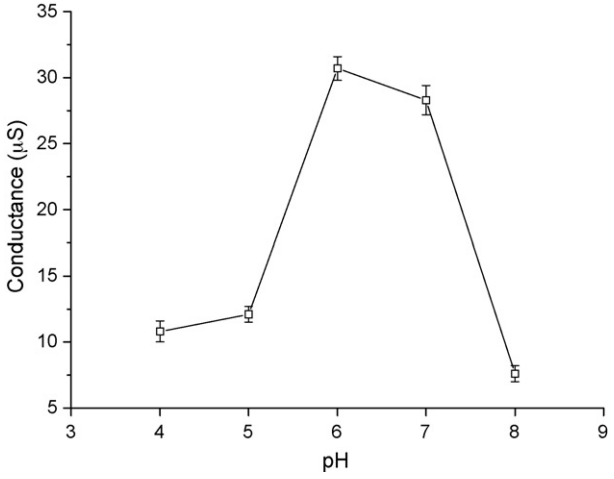

Fig. 4 – Influence of the buffer pH on the conductometric phosphate biosensor response. Measurements were performed in citrate buffer (0.1 M) containing 20 mM maltose at room temperature with 200 μM phosphate.

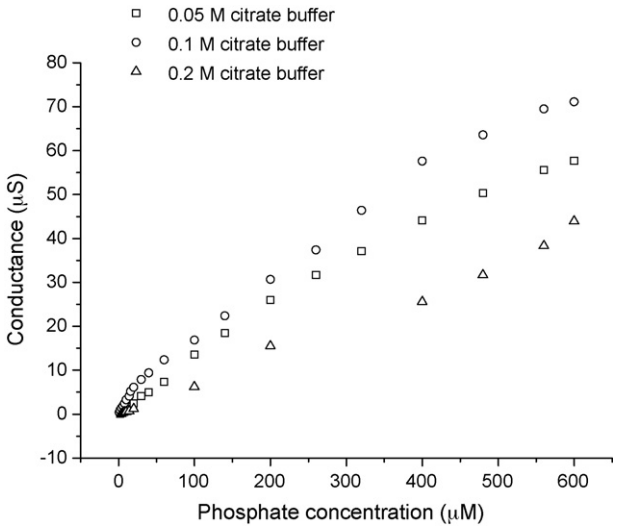

Fig. 5 – Influence of the buffer concentration on the conductometric phosphate biosensor response. Measurements were performed in citrate buffer (pH 6.0) containing 20 mM maltose at room temperature with 200 μM phosphate.

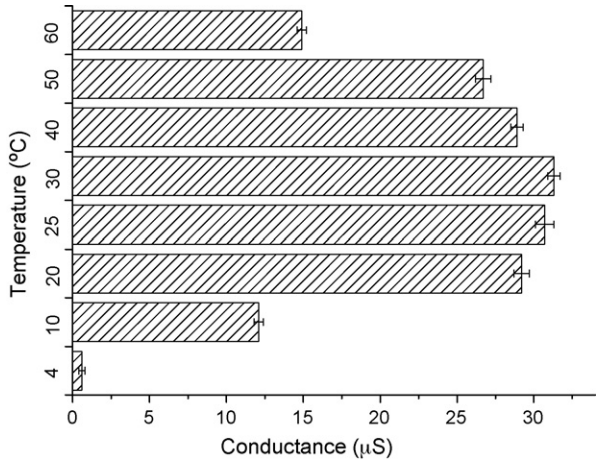

Fig. 6 – Influence of temperature on the conductometric phosphate biosensor response. Measurements were performed in citrate buffer (0.1 M, pH 6.0) containing 20 mM maltose with 200 μM phosphate. The optimum temperature value was 30 °C.

phate in 0.1 M citrate buffer (Fig. 4). The maximum response of the biosensor occurs at pH 6.0. This result is strongly correlated to those obtained using MP-based methods for the determination of phosphate [9,11]. This result indicates that the immobilization of MP in the GA cross-linking composite film has not modified the microenvironment of the enzyme. We then used the buffer solution of pH 6.0 throughout the experiments to obtain the maximum sensitivity.

The influence of the concentration of the buffer solution on the biosensor response is shown in Fig. 5. The highest sensitivity was obtained when the 0.1 M citrate buffer was used, which is also correlated with the results obtained using MP-based methods for phosphate detection [11]. Therefore, 0.1 M citrate buffer was used in the following experiments. Such dependence of the response value on the buffer concentration is due to a kind of "carrier-mediated" transport of protons out of the enzymatic membrane in the presence of mobile buffer species [23]. It means that when the buffer species are present in the solution, protons can associate with them and form "an additional channel" of their diffusion out of enzymatic membrane (the first one is the diffusion of protons as free ions) [22]. A decrease of the ionic strength would result in a weak buffer function, while an increase of the ionic strength would result in an increase of the general conductivity of the solution and a decrease of sensitivity of conductometric transducer [22].

Fig. 6 shows the influence of temperature on the sensitivity of the selected biosensor. Like most other enzymes, the activity of MP is related to temperature. It can be seen that the biosensor performs well at a temperature between 20 °C and 50 °C, below which MP cannot be activated completely, and over which MP denaturation most possibly take place.

Under the above optimized conditions, the calibration curve of the conductometric phosphate biosensor was obtained. As shown in Fig. 7, the sensor has two linear ranges, one is from 1.0 μM to 20 μM phosphate concentration with a detection limit of 1.0 μM, and the other is from 20 μM to 400 μM phosphate concentration. For the first linear section, the regression equation is ΔS (μS) = 0.182 + 0.298 [phosphate, (μM)], R^2 = 0.9942, and for the second section, the equation is ΔS (μS) = 3.820 + 0.133 [phosphate, (μM)], R^2 = 0.9993. The K_m of MP for phosphate was about 238 μM and the biosensor response time was about 10 s.

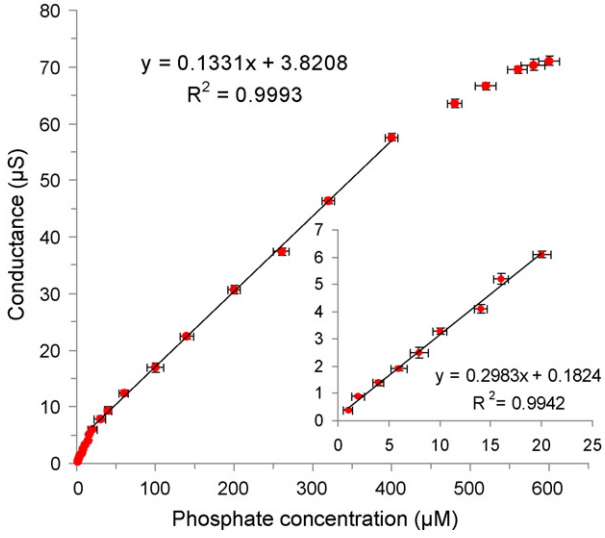

Fig. 7 – Calibration curve of the optimized conductometric phosphate biosensor. Measurements were performed in citrate buffer (0.1 M, pH 6.0) containing 20 mM maltose at room temperature. *Note*: The erect and horizontal bars refer to the standard deviations between measurements done with the same biosensor and between measurements done with different biosensors, respectively.

Table 2 – Analytical results of phosphate in real water samples ($n = 3$)

Water sample	Initial concentration (μM)	Added concentration (μM)	Obtained concentration (μM)	Recovery (%)
Saône River	1.4 ± 0.5	2.0	3.6 ± 0.4	113
Givors Entrée	91.6 ± 1.2	90.0	179.5 ± 1.6	98
Givors Intermédiaire	16.8 ± 0.6	20.0	38.0 ± 0.4	106

3.3. *Biosensor stability*

Long-term operational and storage stability is the key biosensor performance parameter. An exponential time decay of the enzyme activity caused by thermal or chemical inactivation of the enzyme has been reported by Renneberg et al. [20]. The conductometric phosphate biosensor was stored in 0.1 M citrate solution (pH 6.0) at 4 °C. In order to evaluate the long-term stability of the biosensor, the conductance at a fixed phosphate concentration of 200 μM was checked periodically with the same sensor (Fig. 8). The conductance response of the sensor was found to be fairly stable during the first week, and then to drop gradually with time. After two weeks, it maintained more than 70% of the initial response, and after three weeks there was a decrease of 50% from the initial response. Other experiments showed that the conductometric biosensor could be maintained at about 70% of the initial response for about two months when the checking were reduced to less than 5 times. The reduced sensor response may then be related to both the operation frequency and the lifetime of MP.

The possible interferences from other anionic species were also investigated. K_2SO_4, KCl, KNO_3, KNO_2 and $KHCO_3$ (final concentration 200 μM) were added into the detection system containing 200 μM phosphate, respectively. No obvious interference from the anions was found.

It is interesting to compare our rather simple and cheap conductometric phosphate biosensor with recently reported amperometric multi-enzymatic sensors. The linear range of the tri-enzymatic phosphate sensor developed by Mousty et al. [11] was 1–50 μM phosphate at 40 °C, while its response value sharply decreased to a half at room temperature. For the bi-enzymatic sensor developed by Ikebukuro et al. [14], it showed a narrow linear range between 0.5 and 10 μM phosphate, and was very sensitive to temperature. Kwan-Roger et al. [1] reported an amperometric phosphate biosensor based on immobilized pyruvate oxidase, which exhibited a linear range from 7.5 μM to 625 μM phosphate with a detection limit of 3.6 μM. However, the stability of the sensor was quite low when immersed in buffer and kept at 4 °C, with more than 50% of the enzyme activity lost by the second day. Moreover, it required the presence of many cofactors, such as thiamine pyrophosphate (TPP), flavine adenine dinucleotide (FAD) and Mg(II) for wakening its activity. While the low detection limit of our conductometric phosphate biosensor is comparable, we found a wide application range, instant response and a better stability of operation and storage. These advantages together with its simplicity, low cost, etc. would be considerably fit to widespread applications for on-site monitoring.

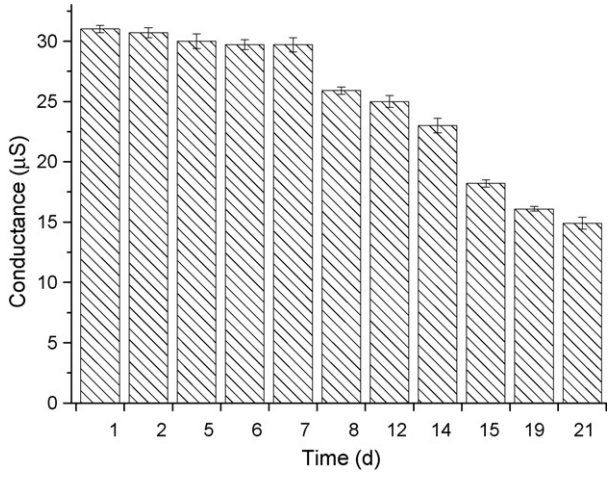

Fig. 8 – Storage stability of the optimized conductometric phosphate biosensor. Measurements were performed in citrate buffer (0.1 M, pH 6.0) containing 20 mM maltose at room temperature with 200 μM phosphate.

3.4. *Application*

The developed conductometric phosphate biosensor was used to determine phosphate in three real water samples using the method of standard addition, following the procedure described in Section 2.4. Before measurement, the samples were filtered through a PTFE membrane (0.45 μm). The addition reclaim method was adopted to confirm the reliability of the biosensor. The results were listed in Table 2. As shown in Table 2, the recoveries (98%–113%) for determination of phosphate in the samples are satisfactory. The results indicate the conductometric phosphate biosensor can be successfully applied to the determination of phosphate at concentrations normally presented in some water samples. Further practical use of this biosensor in determination of phosphate for on-site monitoring (river, lake, wastewater, etc.) is being investigated.

4. Conclusions

In the present paper, an original conductometric biosensor for phosphate determination has been developed. Three overlapping layers of maltose-phosphorylase-based composite film were immobilized by cross-linking with saturated GA vapour. The proposed conductometric biosensor is relatively reliable, inexpensive, and easy to operate. The conductometric biosensor has two linear ranges, one is from 1.0 μM to 20 μM phosphate concentration with a detection limit of 1.0 μM, and the other is from 20 μM to 400 μM phosphate concentration. No interference from other anionic species was detected. Finally, the conductometric biosensor exhibited a long-term storage and operational stability as well as a good thermal stability. Measurements in the real water samples were satisfactory.

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