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The measurement of alkaline phosphatase at nanomolar concentration within 70 s using a disposable microelectrochemical transistor

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Abstract

We report a new approach to the measurement of alkaline phosphatase concentration based on the use of a disposable poly(aniline) microelectrochemical transistor. The measurement is carried out in a two cell configuration in which the poly(aniline) microelectrochemical transistor operates in acid solution and is connected to the alkaline buffer solution containing the analyte by a salt bridge. Disposable microelectrochemical transistors were reproducibly fabricated by electrochemical deposition of poly(aniline) onto photolithographically fabricated gold microband arrays. Using these devices alkaline phosphatase was detected by employing *p*-aminophenyl phosphate as the substrate for the enzyme and using glucose and glucose oxidase to recycle the *p*-aminophenol generated upon enzyme catalysed hydrolysis of the phosphate. Recycling the *p*-aminophenol with glucose and glucose oxidase amplified the detection of alkaline phosphatase approximately tenfold. Using this approach we obtain linear calibration curves for alkaline phosphatase up to 5 nM within 70 s on single use devices.

Keywords: Alkaline phosphatase; Nanomolar concentration; Disposable microelectrochemical transistor

1. Introduction

In both medical and environmental monitoring there is an increasing demand for portable detection devices. Such devices should offer fast response times and operate with little or no maintenance. Commercially available disposable amperometric glucose sensors designed for home use are highly successful examples of this type of device [1,2]. They have response times of less than 30 s, they operate in whole blood without the need for sample preparation, and the part of the device that comes into contact with the sample is disposed of after use so there is no need for maintenance. However, although the first disposable electrochemical glucose sensors came to the market 20 years ago, only lactate and cholesterol point of care devices have followed. The trend to smaller sample volumes and multi-analyte measurements requires the use of smaller electrodes which in turn necessitates the measurement of smaller currents especially for analytes present at low (micromolar and lower) concentration. This introduces new problems. The measurement of small currents is subject to noise and the design of disposable electrode arrays and smaller electrodes is restricted by the cost and ease of manufacture. Mass manufacture, for example by screen printing, often demands the use of a planar electrode configuration and restricts the choice of reference electrode. These difficulties have led us to explore the use of microelectrochemical transistors [3] because these devices do not require a reference electrode, and because with the microelectrochemical transistor the reaction of the analyte switches the transistor "on" or "off" leading to significant signal amplification. This has the advantage that the measurement is less subject to noise because the current passing through the transistor is significantly larger than the current that could be generated by the direct redox reaction of the analyte.

Wrighton et al. [4–8] described the first microelectrochemical transistor in 1984 and went of to develop the technology in a series of publications. However these early devices had limited chemical selectivity. Matsue et al. [9] reported the first microelectrochemical enzyme transistor in 1991 and this was followed by examples of microelectrochemical transistors responsive to glucose,

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NADH, and hydrogen peroxide from our own group [10–12], all based on poly(aniline). The use of poly(aniline) restricted the use of the glucose responsive device to operation at pH 5.

Several approaches have subsequently been investigated in order to extend the microelectrochemical transistor function of poly(aniline) to higher pH, including very recently the chemical modification of the polymer [13]. We have investigated the use of composite films of poly(aniline) with a polyanion such as poly(vinylesulfonate) [12]. In these composites deprotonation of the emeraldine form can only occur if cations from the solution enter the film to maintain electroneutrality [14,15].

The problem is especially acute when one tries to design microelectrochemical enzyme transistors to operate with alkaline phosphatase because the pH optimum for the enzyme is pH 9.5 [16]. Nevertheless alkaline phosphatase is a very attractive enzyme to use because it is widely used as a label in immunoassays and a wide range of alkaline phosphatase labeled monoclonal antibodies are commercially available [17,18]. In a recent paper [19], we showed that by using a two cell arrangement in which a microelectrochemical transistor operating in acid solution was linked by a salt bridge to a collecting electrode in a buffer solution we could use a microelectrochemical transistor based on poly (aniline) to detect *p*-aminophenol in basic solution.

In this paper we use this same approach to measure alkaline phosphatase at pH 9.5. We report the detection capabilities of a disposable poly(aniline)-poly(vinylsulfonate) microelectrochemical transistor. We demonstrate the reproducible fabrication of these devices and show how they can be used to obtain a linear calibration for alkaline phosphatase from 0 to 5 nM, with measurement times of around 1 min.

2. Material and methods

2.1. Reagents

All aqueous solutions were freshly prepared using water purified by a Whatman RO 50 and a Whatman 'still plus' system. Sulfuric acid (Aldrich 97–99%), HCl (BDH, AnalaR), citric acid (Aldrich, Ultrapure grade), sodium di-hydrogen orthophosphate (BDH, AnalaR), di-sodium hydrogen orthophosphate 12 hydrate (BDH, AnalaR), TRISMA Base (SIGMA 99.9%), PBS-Tween 20, pH 7.4 (Sigma), glucose (BDH AnalaR) and KCl (BDH, AnalaR) were used as received. *p*-aminophenol (Aldrich 98%) was recrystalised and stored under argon before use. Aniline (Sigma) was distilled and stored under argon before use. Argon (99% from BOC) was used to sparge solutions to remove dissolved oxygen. Glucose oxidase (E.C. 1.2.4.4, type VII from *Aspergillus niger*, MW 186 000, 100 units mg^{-1} (pH 5), 271.2 mg ml^{-1}) stock solution was a gift. Glucose solutions were prepared 24 h before use and stored at 4 °C. Alkaline phosphatase EC 3.1.3.1 from bovine intestinal mucosa 2047 U/mg, $M_r = 140\,000$ was purchased from Fluka. *p*-Aminophenol phosphate was a gift from MediSense.

2.2. Apparatus and procedures

Electrochemical experiments were carried out using either a Ministat potentiostat (Thompson Electrochem) or an Oxford Electrodes portable potentiostat (Oxford Electrodes, model PP2) used in conjunction with an home made voltage follower, and recorded with an XY/t chart recorder (Gould, series 60 000), a Pharmacia LKB REC 102 Y/t recorder, and Keithley 175 A digital voltmeter. When required a large area platinum gauze was used as the counter electrode. All potentials are reported with respect to saturated calomel (SCE) reference electrode. pH measurements were made using a 145 pH ion selective electrode probe (Corning Science Products).

The polymer transistors were constructed on photolithography fabricated interdigitated gold electrodes, commercially available from Smart Microsystems (MB4000). These have four individually addressable gold electrodes each 10 μm wide and 2 mm long, with a gap of 10 μm between electrodes.

Electrodeposition of the poly(aniline) was carried out as follows. Two neighboring gold electrodes are shorted together and connected to the potentiostat as the working electrode. A gold wire was used as the counter electrode and the reference electrode was separated from the solution by a double frit containing 2 M sulfuric acid to prevent KCl contamination from the saturated calomel reference electrode. The polymerisation solution was 5 ml of 2 M H_2SO_4 containing 124 μl distilled aniline and 714 μl poly(vinylsulfonate). This stock solution was kept at 25 °C with a waterbath in order to maintain identical conditions at the start of each experiment. For each polymerisation experiment 10 μl of the aniline/poly(vinylsulfonate) solution was deposited on the gold electrodes and the counter electrode together with the reference electrode were brought up to make contact with the solution droplet using a micrometric screw clamp. The potential was switched from open circuit to 0.9 V vs. SCE and the current recorded as a function of time. The polymerisation charge used to prepare the devices was (500 ± 12) μC for a batch of 70 transistors. In each case the poly(aniline) film connects two of the neighboring gold electrodes totally covering the 10 μm gap between them.

2.3. Alkaline phosphatase measurements

The microelectrochemical transistor was successively set at 0 V vs. SCE for 1 min, 0.35 V for 1 min, and 0.2 V for 1 min in 2 M sulfuric acid. Meanwhile 100 μl of 0.1 M pH 9.5 Tris–HCl buffer containing 100 mM

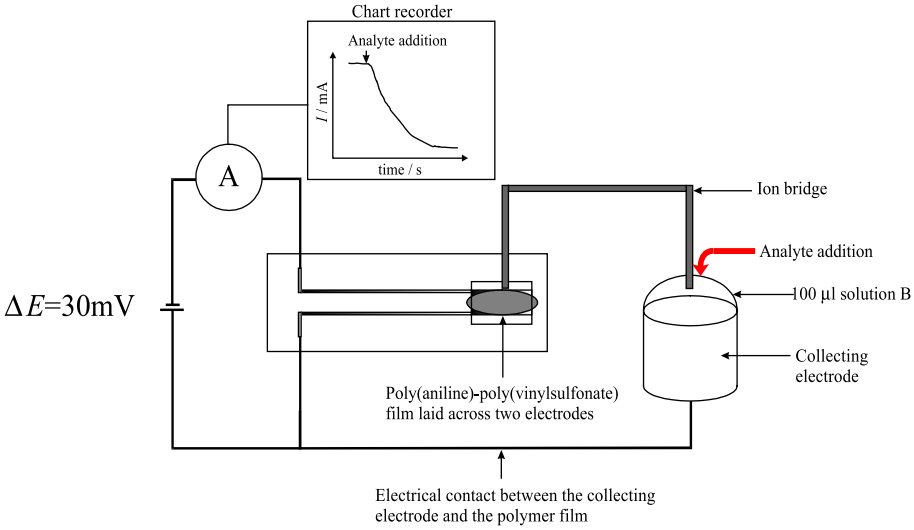

Fig. 1. The two cell arrangement used to make the measurements of alkaline phosphatase concentration. The poly(aniline)-poly(vinylsulfonate) film, deposited across the two gold microband electrodes, bathed in 2 M H_2SO_4 solution. Before stating the measurement the poly(aniline) is held at 0.2 V vs. SCE in 2 M H_2SO_4 using a conventional three electrode configuration. This sets the polymer in its conductive, emeraldine, state. A drain voltage of 30 mV is applied between the two gold microbands and the resulting drain current flowing through the microelectrochemical transistor measured by the ammeter and recorded on a chart recorder. The enzyme catalysed reactions take place in 100 µl of pH 9.5 Tris–HCl buffer containing 23 µM *p*-aminophenyl phosphate, 100 mM glucose and 38 µM glucose oxidase (solution B) held at 25 °C resting on a 1-cm^2 HOPG graphite electrode (basal plane). The collecting electrode is connected to one of the pair of microband electrodes across which the polymer is deposited. The detection experiment starts when alkaline phosphatase or *p*-aminophenol is added to the collecting electrode solution (solution B). The enzyme catalyses hydrolysis of the *p*-aminophenyl phosphate and results in reduction of the poly(aniline) with a consequent drop in the drain current measured by the ammeter.

glucose, 38 µM glucose oxidase and 20 µM *p*-aminophenyl phosphate (solution B) was thermostatted at 37 °C in a multiwell plate. Dilutions of alkaline phosphatase were prepared in pH 9.5 Tris–HCl buffer. Then with the microelectrochemical transistor set at 0.2 V vs. SCE, solution B was deposited on a 1-cm^2 graphite electrode connected to one of the microband electrodes of the transistor. A salt bridge was used to make contact between solution B and the sulfuric acid solution covering the polymer transistor (Fig. 1). A drain potential of 30 mV was then applied across the two gold microband electrodes of the microelectrochemical transistor and the drain current recorded as a function of time. A 1-µl aliquot of the alkaline phosphatase solution was added to solution B to start the measurement.

3. Results and discussion

3.1. Fabrication of the microelectrochemical transistors

The disposable transistors were prepared by electropolymerisation of aniline on commercial gold microband electrode arrays. Fig. 2 shows the gold microband electrodes as received from the supplier. The arrays comprise four individually addressable evaporated gold electrodes each 10 µm wide and 2 mm long separated by a gap between bands of 10 µm. For our experiments we used only two neighbouring electrodes. To check the microbands and to clean them before use we carried out cyclic voltammetry of each electrode in 2 M H_2SO_4 using the gold oxide reduction peak to assess the area of each microband. Immediately after the cyclic voltammetry in acid poly(aniline) was deposited onto the devices. The microelectrochemical transistors were all prepared by electropolymerisation of aniline at 0.9 V vs. SCE in 2 M sulfuric acid and the polymerization charge was recorded for each device to ensure reproducibility. Fig. 3 shows one of the transistors after the deposition of

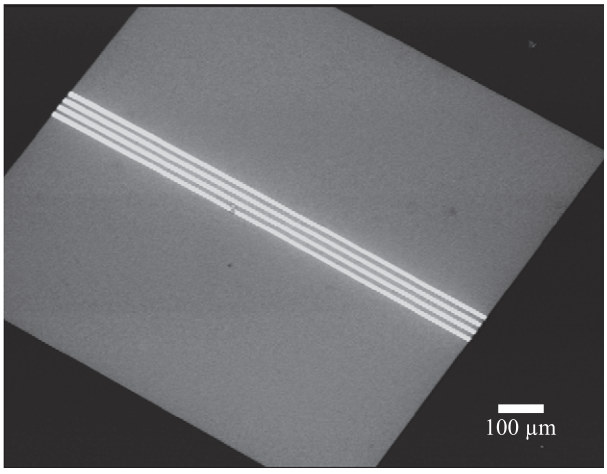

Fig. 2. A tilted SEM image of the photolithography fabricated four microband electrode array used to build the microelectrochemical transistor. Four gold bands can be seen, the darker area around the gold bands is due to the presence of a photoresist insulating film which defines the exposed length of the bands.

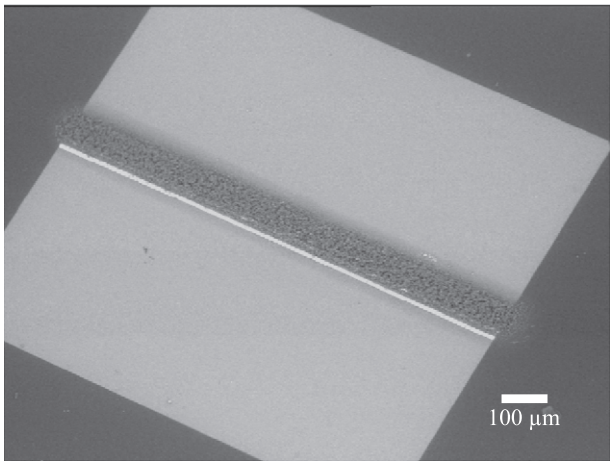

Fig. 3. A tilted SEM image of the poly(aniline)-poly(vinylsulfonate) coated microband array showing the polymer deposited between two neighboring gold electrodes. The poly(aniline) was deposited from 5 ml of a 2 M solution of H_2SO_4 containing 124 μl distilled aniline and 714 μl of poly(vinylsulfonate) at a potential of 0.9 V vs. SCE. The total polymerisation charge passed was 500 μC.

poly(aniline). From the micrograph we can see that the polymer completely covers the gap between the two electrodes and makes contact between them.

Fig. 4 shows an image of the polymer connecting the two gold electrodes at higher magnification. It is clear that the polymer structure is not uniform and this probably accounts for the slight variation in conductivity between devices. In order to assess the reproducibility of fabrication of the batch of 54 microelectrochemical transistors prepared for the experiments described below, the drain current before the addition of alkaline phosphatase was recorded for an applied drain voltage of 30 mV. From these measurements we found an mean drain current of 455 μA (corresponding to a resistance of 65.9 Ω for the device in the conducting, "on", state) with a standard error on the mean of 12 μA. Thus electropolymerisation onto the microfabricated electrode arrays shows reasonable reproducibility under these conditions.

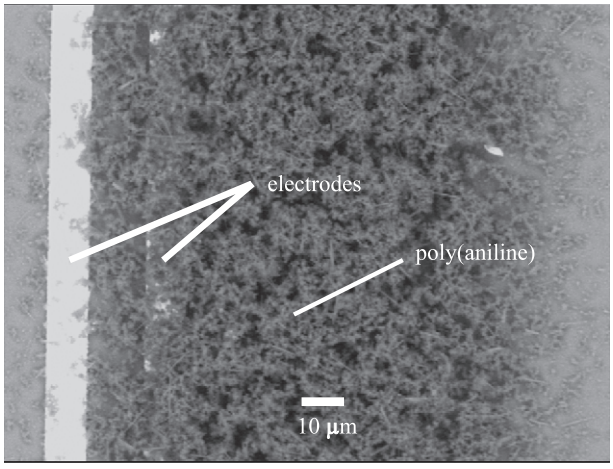

Fig. 4. Close up view of the polymer on the device shown in Fig. 3. The two 10 μm wide microbands which were left largely unconnected during the deposition can be seen on the right whilst the two coated microbands are concealed by the poly(aniline).

3.2. Switching of devices

The microelectrochemical transistor is in the "on" state when the poly(aniline) is in its oxidised, conducting, emeraldine form and in the "off" state when the poly(aniline) is in its fully reduced, insulating, leucoemeraldine form. In 2 M sulfuric acid when poly(aniline) is set at 0.31 V vs. SCE, the drain potential applied between the two contacting gold microband electrodes will result in a current flowing through the transistor because at 0.31 V in acid solution the poly(aniline) is in its conductive state. For the devices described here this drain current is in the mA range and can be readily measured with a simple ammeter. A very small reduction charge is required to reduce the small volume of polymer deposited on the device. Fig. 5 shows how the transistor switches from "on" to "off" as the poly(aniline) is reduced. The plot shows the resistance of the device as a function of the charge passed starting from the conductive state. From the figure we can see that the poly(aniline) remains fully conductive until a threshold is reached, and that then the resistance increases rapidly with further reduction. This property allows us to bring the transistor very close to the threshold without appreciable loss of conductivity, so that a very small reduction charge (of the order of 5–10 μC for the present devices) will cause the transistor to switch from fully conductive to fully insulating. This change in resis-

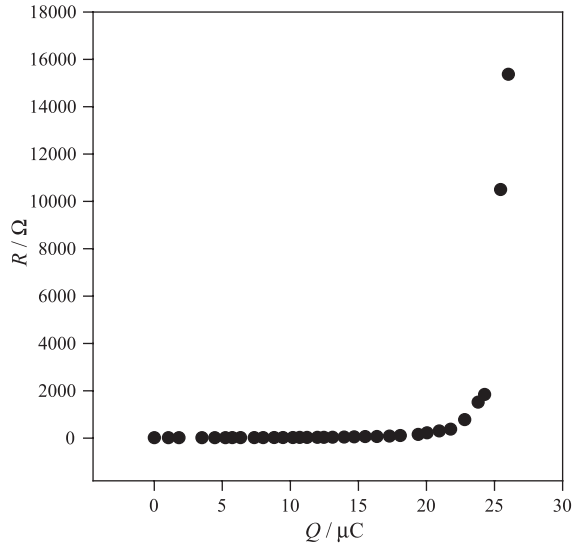

Fig. 5. Plot of the resistance of the poly(aniline)-poly(vinylsulfonate) film, calculated from the drain current, and plotted against the total reduction charge passed in reducing the film from 0.31 to 0.1 V vs. SCE. The charge passed to deposit the film was 0.7 mC.

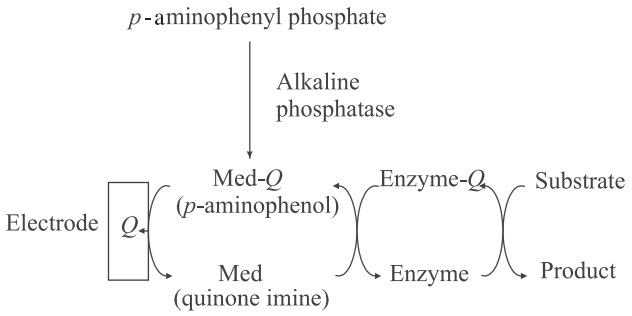

Fig. 6. Scheme for the detection of alkaline phosphatase. The enzyme hydrolyses *p*-aminophenyl phosphate to *p*-aminophenol, which is then oxidized at the collecting electrode. The reaction product quinine imine is recycled by reaction with glucose oxidase and glucose leading to an amplification of the signal.

tance is readily detected as a large change in the drain current flowing through the device.

3.3. Alkaline phosphatase measurement

Alkaline phosphatase was measured using *p*-aminophenyl phosphate as the substrate for the enzyme and using glucose and glucose oxidase to amplify the response. In these experiments the *p*-aminophenol generated by the alkaline phosphatase catalysed hydrolysis of *p*-aminophenyl phosphate acts as a mediator for the oxidation of glucose catalysed by glucose oxidase. This amplification strategy has been described by Thompson et al. [16] and is shown in the scheme in Fig. 6. The *p*-aminophenyl phosphate itself is electroinactive at low potentials and so does not interfere with the electrochemistry of the *p*-aminophenol. Once the *p*-aminophenol has been oxidised by reaction at the electrode the reaction product (quinone imine) is recycled to *p*-aminophenol by reaction with glucose oxidase and glucose thus regenerating the *p*-aminophenol and amplifying the signal for alkaline phosphatase detection. This assay method is highly sensitive to alkaline phosphatase concentration because the kinetics of both the *p*-aminophenol phosphate hydrolysis and the reaction of the quinone imine with glucose oxidase are fast. At pH 9.5 under our conditions we found that K_m and k_{cat} for the hydrolysis of *p*-aminophenyl phosphate catalysed by alkaline phosphatase were 1.32×10^{-4} mol dm^{-3} and 2.74×10^{3} s^{-1}, respectively, and that the rate constant for reaction of the quinone imine with glucose oxidase was 1.38×10^{5} mol^{-1} dm^{3} s^{-1} [20]. This is comparable with the work of Thompson et al. [21] who reported a value of K_m for value of K_m for the alkaline phosphatase catalysed hydrolysis of *p*-aminophenol phosphate of 56 μM in the same buffer.

The optimum pH for alkaline phosphatase activity is pH 9.5 but, as discussed in Introduction, this is incompatible with the requirements of the poly(aniline) based microelectrochemical transistor since an acidic solution required for it to operate. To overcome this problem we used the two-cell arrangement as shown in Fig. 1 and described in our earlier paper [19]. In this arrangement the microelectrochemical transistor and the alkaline phosphatase are in different solutions of different pH (2 M sulfuric acid and pH 9.5 buffer). Connection between the two solutions is established by a salt bridge. The collecting electrode (Fig. 1) provides a large surface area on which the *p*-aminophenol can react. The collecting electrode is polarised by the polymer and its potential is directly related to the potential of the polymer. Using this arrangement, a small volume of polymer (requiring a small switching charge and hence giving high sensitivity) can be used provided that the polymer redox change is sufficient to polarise the collecting electrode.

At the start of the detection experiment, the stability of the polymer is shown by the fact that the drain current remains constant. In control experiments we found that addition of glucose oxidase and glucose and did not alter the drain current. It is only when alkaline phosphatase is added to the buffer solution in the presence of *p*-aminophenyl phosphate that the drain current starts to fall. Upon addition of alkaline phosphatase to the buffer solution *p*-aminophenol is generated and this reacts at the collecting electrode leading to the reduction of the poly(aniline) film and thus causing the microelectrochemical transistor to switch from "on" to "off". Fig. 7 shows a typical set of drain current transients following addition of different concentrations of alkaline phosphatase. As the reaction proceeds the amount of *p*-aminophenol generated by hydrolysis of *p*-phenyl phosphate, and available to be

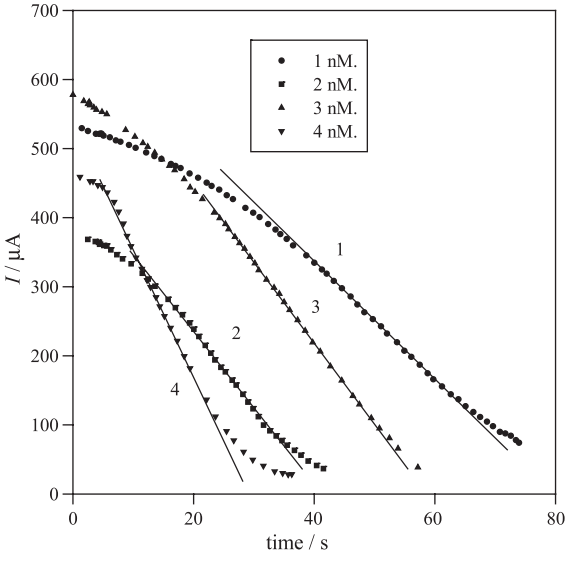

Fig. 7. Plot of the drain current for a 30-mV drain voltage following addition of different concentrations (1, 2, 3 and 4 nM) alkaline phosphatase. Before the measurement the polymer was set at 0.2 V vs. SCE. The polymerisation charge for each transistor is 500 μC. The sample was buffered at pH 9 with 0.1 M Tris–HCl buffer and each transient is obtained for a different device. The initial drain current value varies from one device to another but this does not affect the measured slope.

recycled by the glucose/glucose oxidase system, increases. At the same time the reaction of the *p*-aminophenol reduces the poly(aniline) and causes its potential, and hence the potential of the collecting electrode, to shift cathodic reducing the overpotential for *p*-aminophenol oxidation. These two effects account for the shape of the drain current transients seen in Fig. 7 for alkaline phosphatase concentrations down to 1 nM.

We also tested the sensitivity of the devices when there was no glucose oxidase added to the buffer solution, so that there was no recycling of the *p*-aminophenol. Under these conditions we found that the lowest alkaline phosphatase concentration that could be detected within 90 s was 10 nM (data not shown) indicating that the glucose/glucose oxidase recycling significantly improves the detection limit in these experiments giving approximately a 10-fold amplification of the signal.

In order to determine the alkaline phosphatase concentration from the transients shown in Fig. 7 we measure the slope of the linear part of the transient, this value is then plotted against the alkaline phosphatase concentration as shown in Fig. 8. From Fig. 8 we find the linear range of detection of our device to be from 0 to 5 nM alkaline phosphatase. Based on these results we choose to study the detection of alkaline phosphatase over the range 0–4 nM in greater detail (Fig. 9). For each concentration the response was separately measured four times using a new microelectrochemical transistor each time. Using our techniques we can reproducibly detect 1 nM alkaline phosphatase in 70 s, and 4 nM in less than 30 s. These results clearly demonstrate the speed and accuracy of this disposable device. The simplicity of the electronics required to carry out the measurement allows the entire instrument to be of a compatible size for point of care use. In addition, the analytical concentration range of the device can be adjusted for particular applications by varying the sensitivity of the microelectrochemical transistor, which is determined by the polymerisation charge used to deposit the poly(aniline) together with the dimension of the microband electrodes used.

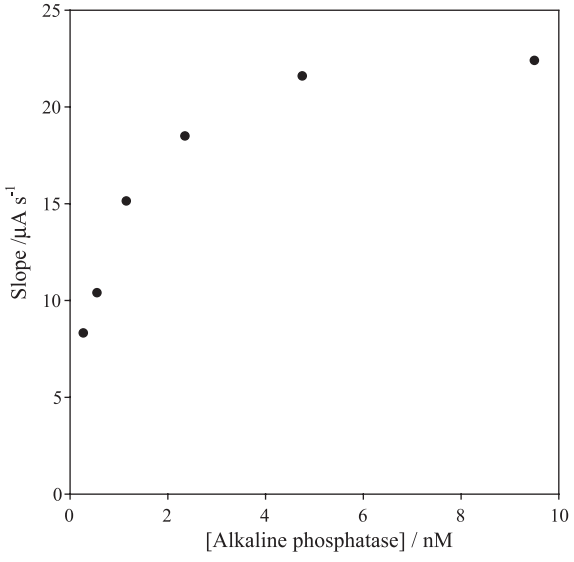

Fig. 8. Calibration results from 0 to 9.7 nM alkaline phosphatase. Each measurement was made with a different microelectrochemical transistor. The buffer solution (Solution B) contained glucose 100 mM, glucose oxidase 38 μM and *p*-aminophenyl phosphate 20 mM in pH 9.5 Tris–HCl 0.1 M. The time for the measurement is 70 s for 1 nM and 10 s for 9.7 nM.

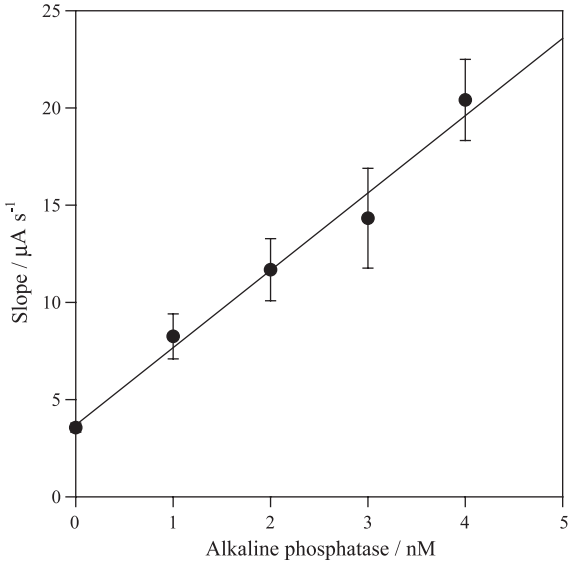

Fig. 9. Linear calibration curve for alkaline phosphatase. The measurements were carried using each microelectrochemical transistor only once with four replicate measurements taken for each concentration of alkaline phosphatase. The values for each concentration were measured on different days using freshly prepared solutions. The buffer solution (Solution B) was glucose 100 mM, glucose oxidase 38 μM, *p*-aminophenyl phosphate 20 mM in pH 9.5 Tris–HCl 0.1 M. The measurement time for 1 nM is 70 and 30 s for 4 nM.

4. Conclusions

In this paper we demonstrated the ability of microelectrochemical transistors to detect very low analyte concentrations (0–5 nM) in short times (~ 1 min). We have demonstrated that the reproducible fabrication of microelectrochemical transistors can be achieved by electrochemical deposition of poly(aniline) on to lithographically patterned gold electrodes on oxidised silicon substrates. By using a two-cell arrangements with a separate large area collecting electrode we have been able to combine the microelectrochemical transistor operating in acid solution with analyte detection at pH 9.5 in a separate solution. The disposable device reported here gives both an accurate and rapid response. The detection times and detection limits can easily be adapted to the desired type of sensing by modifying the size of the polymer transistor and the dimension of the electrodes on which it is electropolymerised.

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