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Biosensors and Bioelectronics 22 (2007) 3064–3071
BIOSENSORS & BIOELECTRONICS
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# Microfluidic conductimetric bioreactor

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Received 8 October 2006; received in revised form 19 December 2006; accepted 10 January 2007
Available online 16 January 2007

**Abstract**

A microfluidic conductimetric bioreactor has been developed. Enzyme was immobilized in the microfluidic channel on poly-dimethylsiloxane (PDMS) surface via covalent binding method. The detection unit consisted of two gold electrodes and a laboratory-built conductimetric transducer to monitor the increase in the conductivity of the solution due to the change of the charges generated by the enzyme-substrate catalytic reaction. Urea–urease was used as a representative analyte-enzyme system. Under optimum conditions urea could be determined with a detection limit of 0.09 mM and linearity in the range of 0.1–10 mM ($r = 0.9944$). The immobilized urease on the microchannel chip provided good stability (>30 days of operation time) and good repeatability with an R.S.D. lower than 2.3%. Good agreement was obtained when urea concentrations of human serum samples determined by the microfluidic flow injection conductimetric bioreactor system were compared to those obtained using the Berthelot reaction ($P < 0.05$). After prolong use the immobilized enzyme could be removed from the PDMS microchannel chip enabling new active enzyme to be immobilized and the chip to be reused.


*Keywords:* Microfluidic; Urea; Urease; Immobilization; Conductimetric; Bioreactor

## 1. Introduction

In recent years, there has been growing interest in the development of microfluidic lab-on-a-chip systems for analytical chemistry, biology, biomedical and clinical diagnostics applications and these have been a subject of several recent reviews (deMello, 2006; El-Ali et al., 2006; Psaltis et al., 2006; Srinivasan et al., 2004; Wang et al., 2006; Whitesides, 2006; Yager et al., 2006; Zhang et al., 2005). Microfluidic devices offer many potential advantages including reduced reagent consumption, smaller analysis volumes, faster analysis times, and increased instrument portability (Park et al., 2006; Pollack et al., 2002). Clinical diagnostics is one of the most promising applications for microfluidic biosensor system. Recent reports include microfluidic system for urea (Koh and Pishko, 2005; Satoh et al., 2005; Suzuki and Matsugi, 2005; Zhang and Tadigadapa, 2004), creatinine (Suzuki and Matsugi, 2005), glucose (Kurita et al., 2002; Lammertyn et al., 2006; Lv et al., 2003; Moser et al., 2002; Srinivasan et al., 2004; Zhang and Tadigadapa, 2004), glutamate (Hayashi et al., 2003; Moser et al., 2002), and nucleic acid (Kwakye et al., 2006).

For microfluidic biosensors various transducers have been used, for examples optical transducer based on absorbance (Srinivasan et al., 2004) or chemiluminescence (Lv et al., 2003; Marquette and Blum, 2004; Xu and Fang, 2004) and thermal transducer (Zhang and Tadigadapa, 2004). Microfluidic

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doi:10.1016/j.bios.2007.01.001

biosensors based on electrochemical detection have also been investigated using amperometric (Kwakye et al., 2006; Lammertyn et al., 2006; Yamaguchi et al., 2002) and potentiometric (Suzuki and Matsugi, 2005) principles. Conductimetric transducer, although has not been applied to microfluidic biosensor system, is another interesting approach because it can be very sensitive to either the change of charge on the ions or the dissociation of ions in the solution that were the product of the reaction. Although this transducer is not ion specific, but if it is used together with a biological element which catalyses a specific biochemical reaction and gives rise to conductivity change, this transducer can become a specific detection system (Wongkittisuksa et al., 2003).

In this work conductimetric detection was investigated as a possible transducer for microfluidic bioreactor. Urea, an important parameter in clinical analysis, was used as analyte to test this biosensor system. During the catalytic hydrolysis reaction of urea by enzyme urease ions are generated (reaction (1)) causing the increase in conductivity of the solution. This change in conductivity is directly related to urea concentration (Lee et al., 2000; Limbut et al., 2004; Thavarungkul et al., 1991, 1999; Thavarungkul and Kanatharana, 1994).

$$(H_2N)_2CO + 3H_2O \xrightarrow{\text{Urease}} 2NH_4^+ + HCO_3^- + OH^- \quad (1)$$

For the fabrication of microfluidic chip polymeric material, such as poly-dimethylsiloxane (PDMS) has emerged as a rapidly fabricate and inexpensive alternative in microfluidic biosensors applications (Karwa et al., 2005; Zhang et al., 2005; Yamaguchi et al., 2002). The surface of PDMS in its natural state is hydrophobic, and is not suitable for biomolecular immobilization. Therefore, a number of researches immobilized the biomolecules within the channel of a PDMS chip through other materials such as controlled-pore glass (CPG) (Lv et al., 2003; Xu and Fang, 2004), glass (Yamaguchi et al., 2002), sepharose beads (Marquette and Blum, 2004) and sol gel (Karwa et al., 2005). However, it is possible to reform PDMS surface to be hydrophilic by creating a thin layer of silicon dioxide on the surface with oxygen-plasma treatment or chemical treatment (Bhattacharya et al., 2005; Karwa et al., 2005) and uses these silanol groups to immobilize biomolecules. There were a few research works that immobilized biomolecules on reformed surface of PDMS microfluidic channel via covalent linking with methacrylate (GMA) photopolymer (Park et al., 2006) or 3-aminopropyl-triethoxysilane with a mixture of EDC/NHS (*N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride/*N*-hydroxysuccinimide) (Zhang et al., 2005). In view of this it would also be possible to use glutaradehyde to react with amine, modified by using 3-aminopropyl-triethoxysilane, on the surface of PDMS to covalently immobilized the biomolecules.

This report describes the development of a microfluidic bioreactor chip by immobilizing enzyme urease on the surface of glutaraldehyde activated-3-aminopropyl-triethoxysilane modified-PDMS microfluidic channel via covalent binding method and the use of a conductimetric measuring system for the detection of urea based on catalytic reaction of enzyme urease. The system was optimized and used to analyse urea concentration in real serum samples.

## 2. Materials and methods

### 2.1. Materials

Poly-dimethylsiloxane (PDMS) polymer (Sylgard® 184) was obtained from Dow Corning (USA). Glutaraldehyde 25% (USA) and 3-aminopropyltriethoxysilane (Switzerland) were obtained from Fluka. Urease (amidohydrolase EC 3.5.1.5 Type IX: from jack beans, 62,100 units/g solid) was obtained from Sigma (St. Louis, Missouri, USA). Urea ($NH_2(CO)NH_2$, AR Grade) was from Mallinckredt, (USA). All other chemicals used were of analytical grade. All buffers were prepared with distilled water treated with a reverse osmosis-deionized system. Before use, the buffers were filtered through an Albet® nylon membrane filter (pore size 0.20 μm) (Albet, Spain) with subsequent degassing.

### 2.2. Fabrication

Fabrication of the microfluidic system consists of two main parts, coating a perspex base with gold electrodes (Fig. 1a) and synthesizing a PDMS cast containing microchannel (Fig. 1b). Gold electrodes on a perspex base were fabricated by thermal evaporation (EDWARDS AUTO 306, UK). The perspex surface was covered with aluminum foil, exposing only the two strips that would be coated. The two electrodes were first coated with a 50 μm layer of chromium and then with a 200 μm layer of gold.

To synthesize a PDMS cast, a patterned silicon wafer was first created (Fig. 1(b1–b7)). Silicon wafer was cleaned by placing it in piranha solution (conc. $H_2SO_4$: 30% $H_2O_2$ = 4:1) for 30 min, followed by a distilled water rinse and cleaned by a plasma cleaner (PDC-32G, Harrick Scientific, USA) for 3 min. This was to remove any physical adsorption on the surface. The silicon wafer surface was dried on a hot plate at 150 °C for 30 min (Fig. 1(b1)). Then a 100 μm layer of SU-8 2100 photoresist (MicroChem, USA) was spin coated onto the silicon wafer (Fig. 1(b2)) at a speed of 700 rpm for 30 s and was slowly increased to 3000 rpm for another 30 s. The coated photoresist was soft baked on a hot plate at 65 °C for 5 min and 95 °C for 20 min to evaporate the solvent. A negative film (Fig. 1(b3)) with the microchannel pattern was placed on top and they were exposed to UV light on vacuum contact for 10 min (Fig. 1(b4)). Following exposure, a post expose bake step was performed to selectively cross-link the exposed portions of the film. This was done by putting it on a hot plate at 65 °C for 1 min and 95 °C for 10 min. The wafer was then developed with developer (MicroChem's SU-8 Developer) for 10 min and soaked in distilled water for 2 min to remove the film. The final step was to hard bake the patterned silicon wafer at 175 °C on a hot plate for 20 min to further cross-link the material (Fig. 1(b5)).

To cast the microchannel, PDMS solution with a 10:1 ratio of PDMS to activator was employed (Sylgard® 184 Silicone elastomer, Base & Curing agent, Dow Corning, USA). The solution was degassed in a vacuum chamber for 30 min, poured over the

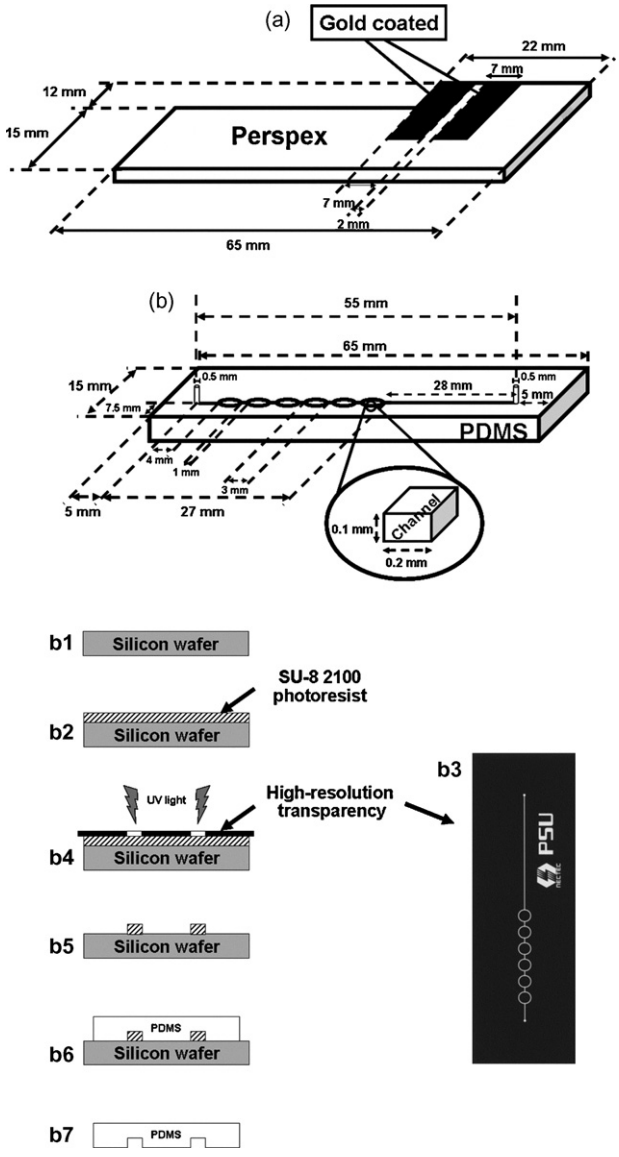


Fig. 1. Schematic diagram showing the microfluidic system: (a) perspex base with gold electrodes; (b) PDMS cast containing microchannel. b1–b7 showing the synthesizing steps of a PDMS cast containing microchannel: (b1) cleaned silicon wafer; (b2) silicon wafer spin-coated with 100 μm layer of SU-8 2100 photoresist; (b3) mask; (b4) UV light through the mask selectively activates the SU-8 2100 photoresist; (b5) photoresist development; (b6) PDMS molding; (b7) PDMS pattern.

patterned silicon wafer, baked at 65 °C on a hot plate for 5 h and left to stand at room temperature overnight (Fig. 1(b6)). The harden PDMS was then peeled from the patterned wafer leaving an impression of microchannels on the surface as shown in Fig. 1(b7).

### 2.3. Immobilization of urease

Urease was immobilized on the surface of the PDMS microchannel using covalent binding method (Fig. 2a). The first step is to prepare the surface of PDMS by chemical treatment to change the chemical properties of the PDMS surface form hydrophobic to hydrophilic (Fig. 2(a1)). This was done by immersing PDMS in acetone and sonicated for 5 min, rinsed with distilled water and dipped into 30% (v/v) hydrogen peroxide ($H_2O_2$) for 1 h. The PDMS was then rinsed thoroughly with distilled water and dried with pure nitrogen gas. The next step is the derivatization of PDMS surface with organosilane by dipping it into 25 ml of 2.0% (v/v) 3-aminopropy-triethoxysilane in 95% acetone for 2 h at room temperature. It was then heated at 50 °C on a hot plate for 10 min. The microchannel was then rinsed with acetone to remove unreacted silane and this produced a layer of amino group on the PDMS microchannel surface (Fig. 2(a2)).

The final step is the coupling of enzyme to the microchannel surface (Fig. 2(a3)). This was done in a flow system (Fig. 2b) by placing the PDMS on top of the perspex base with the microchannel facing the base. This set up was then placed between two perspex blocks and fastened with six screws. On the top block, there were an inlet and an outlet connected to the beginning and the end of the microchannel. The amino group on the PDMS microchannel surface was first activated by glutaraldehyde to yield the carbonyl group by passing 5.0% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer pH 7.0 for 60 min in the flow system with a flow rate of 5 μl $min^{-1}$. During this step the free amino groups (R–$NH_2$) of the PDMS microchannel surface react with carbonyl groups of the glutaraldehyde and the color of the PDMS microchannel changed to orange-red. This color was characteristic of the amount of glutaraldehyde bond to the PDMS microchannel. The channel was then washed by passing distilled water at the same flow rate for 30 min. To immobilize urease, 10 mg (620 units) of urease was dissolved in 5 ml of 50 mM sodium phosphate buffer pH 7.0 and passed through the microchannel at room temperature with a flow rate of 5 μl $min^{-1}$ for 15 h and washed by passing buffer for 1 h. Then, 0.1 M ethanolamine pH 8.0 was passed through at the same flow rate and the reaction was allowed for another 2 h. This step was to occupy all the aldehyde groups which did not couple to the enzyme. When not used, the urease immobilized PDMS microchannel was stored in 50 mM sodium phosphate buffer pH 7.0 + 0.02% sodium azide at 4 °C.

### 2.4. Determination of the amount of protein bound

The amount of protein (enzyme urease) was determined by the silver binding method (Krystal, 1987). In this procedure, protein samples were first treated with glutaraldehyde and then exposed to ammoniacal silver. After 10 min, the reaction was terminated by the addition of sodium thiosulfate and the optical density measured at 420 nm. The quantity of protein bound to the PDMS microchannel was the difference between the concentrations of the protein in the solution before and after immobilization.

### 2.5. Instrumentation

Fig. 2b shows the basic principle of the microfluidic flow injection conductimetric bioreactor system. When the solution containing urea passed through the immobilized enzyme in the microchannel, ions were generated by the hydrolysis reaction

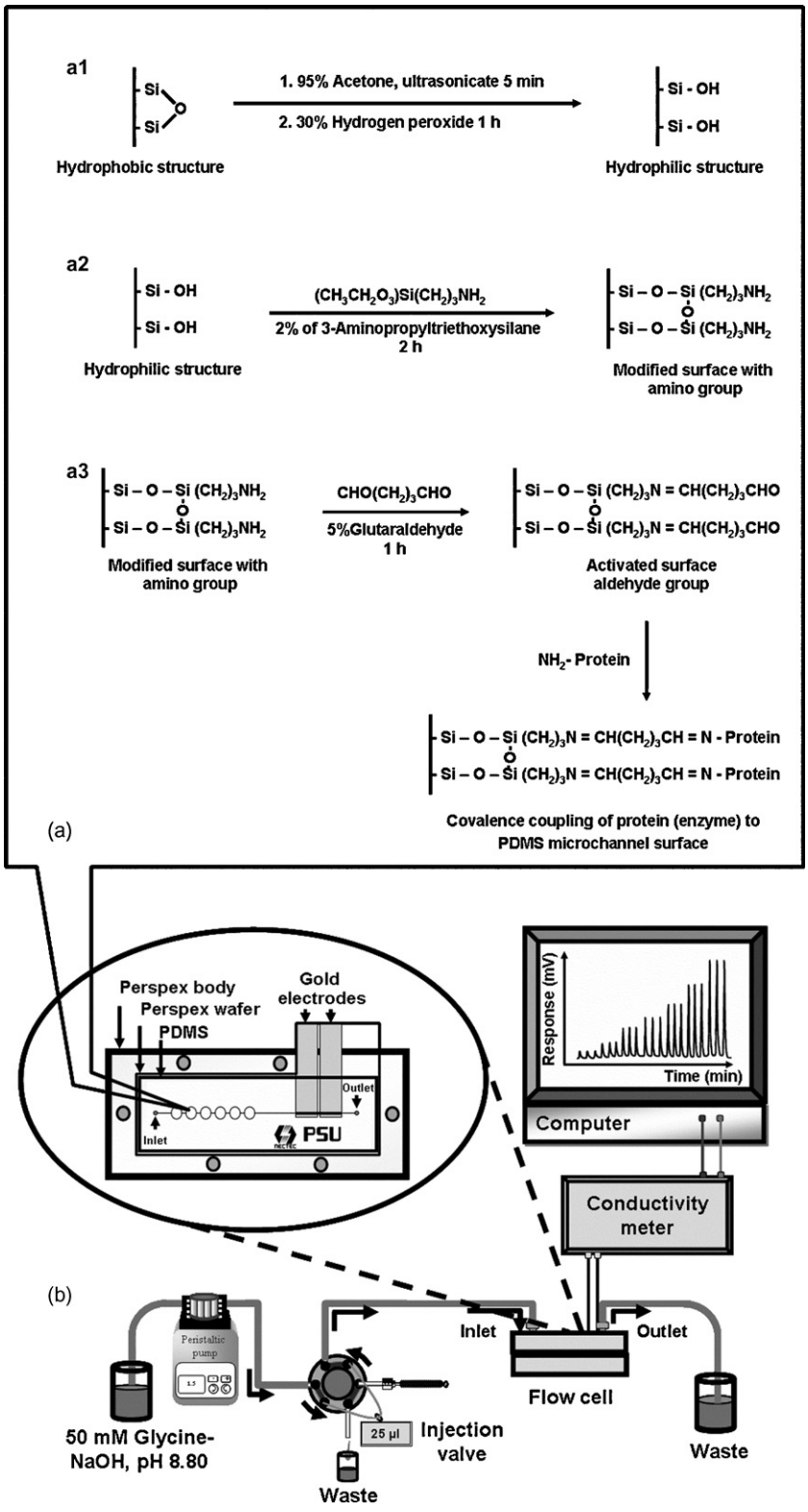


Fig. 2. (a) Immobilization of enzyme on PDMS microchannel surface by covalent binding method: (a1) preparation of PDMS surface by chemical treatment; (a2) derivatization with 3-aminopropy-triethoxysilane; (a3) coupling of enzyme. (b) Schematic diagram showing microfluidic flow injection conductimetric bioreactor system.

(1). The increase of charged products was measured as the change in the conductivity of the solution inside the microchannel section between the two gold electrodes at the outlet of the enzyme microchannel. The conductivity change was measured by a laboratory-built conductivity meter (Wongkittisuksa et al., 2003). In this system, the background conductivity signal of the solution could be adjusted to zero allowing only the change to be detected and amplified. The response, a voltage signal, was

recorded. This voltage signal is linearly related to the solution conductivity.

### 2.6. Optimization of sample volume and flow rate

The sample volume and flow rate of the microfluidic flow injection conductimetric bioreactor were optimized. The buffer used throughout the experiment was 50 mM glycine–NaOH pH 8.80 (Limbut et al., 2004). All standard urea solutions were prepared using this solution. The flow rate used throughout the experiment was 5 μl $min^{-1}$ except when the effect of flow rate was tested. The optimization was performed by changing either the sample volume or flow rate and kept other parameters constant. The optimum value was considered by balancing between response and analysis time.

### 2.7. Determination of urea in serum samples

To demonstrate the use of the microfluidic flow injection conductimetric bioreactor, the system (under optimum conditions) was tested using the serum samples obtained from Songklanagarind Hospital, Prince of Songkla University, Hat Yai, Thailand. The system was first calibrated by injecting standard urea solutions into the system. The calibration curve was prepared by plotting the conductivity change *versus* corresponding urea concentration (mM). The serum samples were diluted using 50 mM glycine–NaOH buffer pH 8.80 at a serum: buffer ratio of 1:99 before injecting into the system. The change in the conductivity of each sample was used to calculate the urea concentration from the calibration done prior to the test. The same samples were analysed by the Berthelot reaction (the results obtained by Songklanagarind Hospital). In this reaction urea was hydrolyzed with enzyme urease to produce ammonia and carbon dioxide. The ammonia products reacted with phenol-nitroferricyanide and hypochlorite to give a blue color and the absorption was measured at 630 nm. The increase of absorbance at 630 mM is proportional to the urea concentration in the sample (Wilcox et al., 1966).

The microfluidic flow injection conductimetric bioreactor was validated by comparing the results to the Berthelot reactions. In making such a comparison, the principle interest will be whether the proposed method gives results that are significantly higher or lower than the established methods. So, the analysis using the Wilcoxon signed rank test (Triola, 1998) was used in this work.

## 3. Results and discussion

### 3.1. Optimization

#### 3.1.1. Sample volume

In enzymatic analysis, the reaction yield depends on the amount of enzyme and target analyte. In this study, the amount of immobilized enzyme in the PDMS microchannel was fixed, only the amount of target analyte had the effect to the conductimetric response. Therefore, sample volume should be optimized. Each concentration of standard urea solution (1, 5, 10, 25, 50, 100 mM) was continuously passed through the system at a flow rate of 5 μl $min^{-1}$ until the constant conductimetric response was obtained and this was after about 5 min. The suitable sample volume of 25 μl was calculated by multiplying the flow rate (5 μl $min^{-1}$) with response time (5 min). This volume was used throughout the rest of the experiments and the peak height was used as the response.

#### 3.1.2. Flow rate

In a flow system, the flow rate of the solution passing through the reactor channel and the detector is the main factor affecting the dispersion of the analyte particles, yield of the reaction and response of the detector. So optimization of flow rate is necessary. The conductivity changes obtained for different flow rates, 3, 4, 5, 7, 9, 10 μl $min^{-1}$ were studied. The conductivity change was found to decrease when the flow rate increased and they differed significantly between each flow rate ($P < 0.05$). Taking into account the size of the response and analysis time for each sample, 5 μl $min^{-1}$ was chosen.

### 3.2. Detection limit and linear range

Under optimum conditions, the detection limit and linear range were investigated. These were performed by injecting standard urea solutions at different concentrations (0.1–80 mM) (Fig. 3a). The fabricated microfluidic conductimetric bioreactor provided good analytical performance with a detection limit of 0.09 mM (based on IUPAC method (Long and Winefordner, 1983)) and a linear range between 0.1 and 10 mM ($r = 0.9944$).

Kinetic parameters of the immobilized urease were calculated from the initial hydrolysis rates at different urea concentrations using the Hanes–Woolf equation (Ho et al., 2000; Shapir et al., 2005):

$$\frac{[S]}{v_0} = \frac{1}{v_{max}}[S] + \frac{K_m}{v_{max}} \quad (2)$$

where $[S]$ is the substrate concentration, $v_0$ is the initial reaction velocity, $v_{max}$ is the maximum velocity catalyzed by a fixed enzyme concentration, $K_m$ (Michaelis constant) is the substrate concentration at which the velocity is half of the maximum

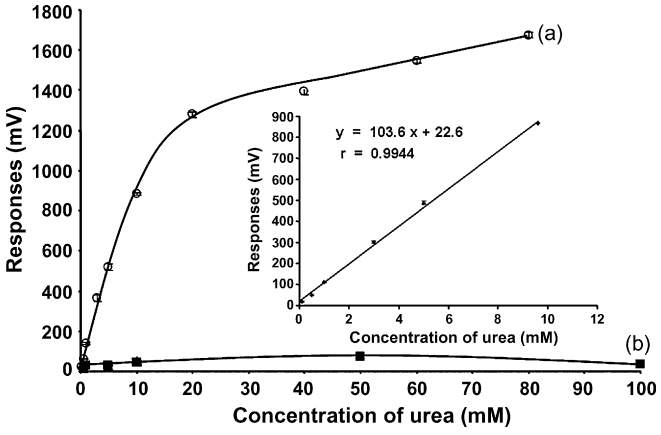


Fig. 3. Responses of the microfluidic flow injection conductimetric system: (a) with immobilized urease; (b) without urease.

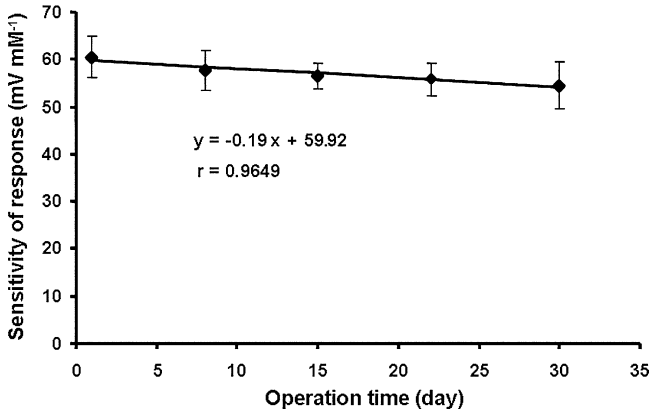


Fig. 4. Stability of the microfluidic flow injection conductimetric bioreactor system.

velocity. $v_{max}$ and $K_m$ were obtained from the slope and intercept of the linear regression of Hanes–Woolf plot of $[S]/v_0$ *versus* $[S]$ (data not show). The $v_{max}$ and $K_m$ values of the immobilized urease on the PDMS microchannel for the hydrolysis of urea were 6 mM $s^{-1}$ and 12 mM, respectively.

Fig. 3b shows the responses when passing urea standard solutions through the microchannel without immobilized urease. The conductivity changes at all concentrations (0.5–100 mM) were relatively small, at 50 and 100 mM the response were approximately the same as 0.5 mM of the microchannel with immobilized urease. So, the response of the system with immobilized urease was due to the hydrolysis of urea.

### 3.3. Stability and repeatability

After prolonged use of the enzyme, denaturation or inhibition of the enzyme may affect the response. The long-term performance of the immobilized urease was evaluated intermittently over a period of 1 month by monitoring the responses to urea standard solutions (0.5–5 mM). Fig. 4 shows the sensitivity (slope of the calibration curve) of the immobilized urease at different operation times. The linear equation being sensitivity (mV $mM^{-1}$) = −0.19(operation time, day) + 59.92. After 30 days of operation time (60 injections, 12 injections/time), the activity of immobilized urease retained about 90% of its original sensitivities. The result indicated that the immobilized urease could provide good stability (the PDMS microchannel chip was stored at 4 °C when it was not being used).

Repeatability was also tested under optimum conditions, 22 injections of 5 mM standard urea solution were done during the same run. The result, 473 ± 11 mV, indicated that the microfluidic flow injection conductimetric bioreactor system gave good repeatability with an R.S.D. lower than 2.3%.

### 3.4. Reusability of the PDMS chip

In the first immobilization, immobilized urease in the microchannel was used for the catalytic hydrolysis reaction of different concentrations of urea for about 120 injections and still gave very good responses. This PDMS chip was then tested to see whether it could be reused. The surface of the PDMS microchannel was regenerated through sonication in 30% hydrogen peroxide followed by acetone for 1 h each, to remove the immobilized urease. It was then washed with an excess amount of distilled water. During this step the orange-red color on the surface of PDMS microchannel disappeared since the urease-glutaraldehyde was removed from the 3-aminopropy-triethoxysilane modified PDMS surface. Then urease was immobilized on the regenerated surface of the PDMS microchannel using the same procedure as indicated in Section 2.3. The reusability of the PDMS chip was evaluated under optimum conditions by considering the sensitivity. The calibration curve was performed by using five different concentrations (0.5, 1, 3, 5, and 10 mM urea standard solution) and each concentration was done in triplicate. In its first use, PDMS microchannel with immobilized urease provides responses with a sensitivity of 87 mV $mM^{-1}$. The amount of urease bound to the surface of the PDMS microchannel was taken as the difference between the amounts of urease in the solution before and after immobilization and this was found to be 333 units. The amount of immobilized urease on the surface of the PDMS microchannel was 9.17 units $mm^{-2}$.

The sensitivity of immobilized urease on the second immobilization of PDMS chip was only slightly lower than when the PDMS was used for the first time (82 mV $mM^{-1}$ *versus* 87 mV $mM^{-1}$). When the amount of immobilized urease was tested the result was of the same trend as the sensitivity, *i.e.*, 283 units *versus* 333 units. This is similar to the results found in our previous works, *i.e.*, more immobilized enzyme provided better sensitivity (Limbut et al., 2004). When the surface of the PDMS microchannel was regenerated two more times the sensitivities of the third and fourth experiments were 92 and 97 mV $mM^{-1}$, respectively. The amount of immobilized urease for these two experiments were not tested, however, it is reasonable to say that these higher sensitivities were most likely due to higher amount of immobilized enzyme.

### 3.5. Comparison between the results obtained from the microfluidic flow injection conductimetric bioreactor system and conventional method

The analysis of urea using the microfluidic flow injection conductimetric bioreactor system and the conventional Berthelot reaction were done on the same serum samples. For the microfluidic flow injection conductimetric bioreactor system, the serum samples were diluted 100 times, to reduce the matrix effect, using 50 mM glycine–NaOH buffer pH 8.80 and injected into the system. The responses from these samples were very high due to the existence of several ions, such as $Na^+$ and $K^+$, in the serum sample. That is, the detected change of conductivity in the solution was from these ions as well as those produced by the hydrolysis of urea. This problem was solved by first passing the serum sample through the microfluidic flow injection conductimetric system without the immobilized urease where the change of conductivity due to various ions in the serum (compare to run-

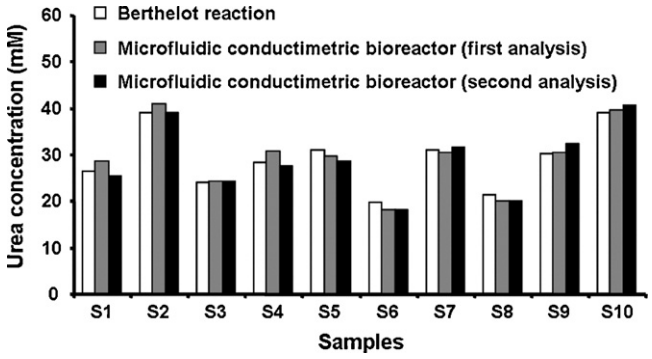


Fig. 5. Comparison of the urea concentration in serum samples obtained from the microfluidic flow injection conductimetric bioreactor system and the Berthelot reaction.

ning buffer) was determined. The same serum sample was then passed through the microfluidic flow injection conductimetric bioreactor system with immobilized urease and the conductivity change (from the running buffer) due to the serum as well as those generated by the hydrolysis process was recorded. The conductivity change of the hydrolysis alone was then obtained by subtracting the former from the latter value and this was used to calculate the urea concentration from the calibration curve done prior to the test.

Ten samples (Fig. 5) were analysed (twice for each sample) and the concentrations were found to be in the range of 19.9–39.2 mM. Comparison between the two analysis techniques was done by the Wilcoxon signed rank test (Triola, 1998). There is no evidence for systematic difference between the results obtained from the microfluidic flow injection conductimetric bioreactor system and the Berthelot reaction ($P < 0.05$). That is, the concentrations determined by the microfluidic flow injection conductimetric bioreactor system are in good agreement to the Berthelot reaction.

## 4. Conclusions

This work presented the fabrication and immobilization of urease on a PDMS microchannel chip and its use in a flow injection conductimetric bioreactor system to determine urea in real serum samples. Good stability and repeatability were obtained. When the enzyme activity was low this immobilized enzyme can be removed from the microchannel surface. PDMS surface can then be reactivated and reused. Good agreement was obtained when urea concentrations of human serum samples determined by the microfluidic flow injection conductimetric bioreactor system were compared to those obtained using the Berthelot reaction ($P < 0.05$).

To overcome the problem of the background conductivity from the ions in real sample future set up may include two sets of gold electrodes, one before and the other after the microchannel. The conductivity change due to the catalytic hydrolysis reaction of urea by enzyme urease can then be measured as the difference between the conductivity signals of the two sets of electrodes. To move the system towards "Lab-on-a-chip" further research may incorporate a micropump to facilitate the flow and a microdialysis membrane to separate unwanted matrix.

## Acknowledgements

This project was supported by The Royal Golden Jubilee PhD-Program supported by Thailand Research Fund; Center for Innovation in Chemistry: Postgraduate Education and Research Program in Chemistry (PERCH-CIC), Thailand; National Electronic and Computer Technology Center (NECTEC), Graduate School, Faculty of Engineering and Faculty of Science, Prince of Songkla University. We thank Sakon Rahong, Supanit Porntheerapat and Tanom Lomas (NECTEC) for their discussion on the preparation of the PDMS chip.

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