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Array-based titanium dioxide biosensors for ratiometric determination of glucose, glutamate and urea

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ABSTRACT

A novel optical array-based titanium dioxide (TiO_2) biosensor combining with simple vapor deposition method was developed to simultaneously determine multianlaytes including glucose, urea and glutamate in serum samples. Carboxy seminaphthorhodamine-1-dextran (SNARF-1-dextran), serving as a single-molecule fluorescent probe, was co-immobilized with different enzymes including urease, glucose dehydrogenase (GDH), and glutamate dehydrogenase (GLDH) for determination of urea, glucose and glutamate simultaneously. The SNARF-1-dextran was a useful pH sensitive dye and the fluorescence ratio at 585 and 630 nm was used to characterize the change in solution pH. Atomic force microscopic (AFM) and scanning electron microscopy (SEM) images showed that TiO_2 is a crack-free stable sol–gel material, which can effectively trap enzymes in the sol–gel-derived matrices prepared by vapor deposition method. The surface morphology of the sol–gel-derived TiO_2 films changed significantly after the immobilization of macromolecules and enzymes. The array-based TiO_2 biosensors show good analytical performance on the simultaneous determination of multiple samples for multianalytes without obvious cross-interference. The analytical ranges of the three analytes in water samples were between 0.01 and 10 mM and limit of detections (LODs) were in the range 2.4–5.5 μM. In addition, satisfactory dynamic ranges of 2–3 orders of magnitude with LODs of 3.1–7.8 μM in serum samples were obtained. These results demonstrate the first report of combining a sol–gel-derived TiO_2 array biosensor with SNARF-1-dextran for ratiometric determination of multianalytes simultaneously and provide a viable alternative to current silica technology for the fabrication of array-based optical biosensors.

1. Introduction

The demand for high throughput analysis of multianalytes in biological fluids, food and environmental microsamples has recently encouraged the development of miniaturized and inexpensive analytical devices (Cho et al., 2002; Golden et al., 2005; Tsai and Doong, 2005). The fabrication of array-based biosensors each with an individual well has numerous advantages for analysis. One distinct niche is its ability for simultaneous detection of multiple samples in the presence of multiple unrelated analytes (Rowe et al., 1999; Tsai and Doong, 2005). Optical biosensors are powerful tools that can be used to detect a wide variety of analytes because these devices are immune to electromagnetic interferences, capable of remote control and can provide multiplexed detection (Fan et al., 2008). Recently, a variety of optical array-based biosensors have been fabricated for the detection of organic pollutants, microorganisms and mycotoxins (Knecht et al., 2004; Ngundi et al., 2005; Tao et al., 2006; Gonzalez-Martinez et al., 2007). Among the optical methods used for sensing purposes, the single-wavelength fluorescence sensing technique has attracted particular attention because it is sensitive, convenient, and can minimize cross reactivity (Wolfbeis, 2005). However, fluorescence intensity expressed in relative units strongly depends on the environmental and instrumental factors that may vary from system to system and often suffers from photo-bleaching and source fluctuation (Liebsch et al., 2001), thus resulting in the decrease in signal-to-noise ratio and making the measurement inaccurate.

Determination of pH is important for a wide variety of applications in biomedical and environmental fields. The optical biosensors are easy to fabricate, suitable for remote measurement and insensitive to electric interference (Gonçalves et al., 2008). In addition, ratiometric measurement method based on pH change with a single-molecule dye exhibiting two independent fluorescence emission bands that can be calibrated internally is a suitable sensing technique to simultaneously detect multianalytes (Demchenko, 2005). Several ratiometric probes such as carboxy seminaphtharhodafluor-1 (SNARF-1) and xanthene have been used for intracellular imaging and sensing (Gulcev et al., 2002; Kreft et al., 2007; Han et al., 2009). Carboxy SNARF-1 is a long-wavelength pH sensitive fluorescent probe that can produce a change in the ratio

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of fluorescence intensities at two emission wavelengths as a function of pH, and has emerged as an attractive material well suited for optical detection in pH range of 6–9 because of high sensitivity and stability. Carboxy SNARF-1 can be excited with wavelength ranging between 488 and 530 nm, and the emission spectra are pH sensitive, which imparts two inversely related emission signals at two different wavelengths (Gulcev et al., 2002; Kreft et al., 2007). It therefore becomes possible to calculate the change in pH by determining the ratio of peak emission intensities between the acid and base forms (Hunter and Beveridge, 2005; Salerno et al., 2007). In addition, reagentless optical biosensors using carboxy SNARF as the fluorescent probe for the detection of pH and urea has been fabrication (Gulcev et al., 2002; Doong and Shih, 2006). However, the use of carboxy SNARF-1-dextran for array-based biosensors to ratiometrically measure multianalytes has received less attention.

The performance of biosensor incorporating biorecognition probe is directly linked to the method used for immobilization. Sol–gel materials have recently emerged as one of the attractive porous matrixes well suited for the immobilization of proteins (Tsai and Doong, 2007). The silica material derived from sol–gel process under ambient conditions can retain the catalytic activities of enzymes to a certain extent, which has been widely used for the fabrication of biosensing systems. However, several inherent limitations including the poor adhesion of sol–gel onto the solid surface and the cracking of final structure due to shrinkage of the gel are the major challenges that should be overcome before it can be used to fabricate silica biosensors (Gill and Ballesteros, 2000; Jin and Brennan, 2002). Recently, titanium-based biosensing systems have been developed for detection of glucose, hydrogen peroxide and cancer biomarkers because of their superior properties including high uniformity, less cracking and excellent biocompatibility (Yu and Ju, 2002; Yu et al., 2003; Lu et al., 2006). Yu and Ju (2002) first used vapor deposition method to prepare a titania sol–gel film by direct hydrolysis of titanium isopropoxide on the electrode surface to fabricate enzymatic and immunological biosensors. Zhu et al. (2009) fabricated a biosensor for measuring hydrogen peroxide utilizing the sol–gel-derived three-dimensional macroporous TiO_2 structure on which horseradish peroxidase was immobilized into matrices. Recently, an amperometric biosensor based on platinum nanoparticle-decorated TiO_2/carbon nanotubes composite was developed for the detection of glucose (Pang et al., 2009). However, these biosensors only focus on the determination of single analytes electrochemically and the application of array-based TiO_2 biosensor coupled with carboxy SNARF-1-dextran as the fluorescent probe for the optically determination of multianalytes has not been reported.

The objective of this study was to fabricate simple and novel array-based titania biosensors for simultaneous determination of metabolic multianalytes in biological samples. Glucose, urea and glutamate were selected as the model compounds due to their importance in biomedical analysis. Glucose is an important substrate used as an indicator of diabetes and as an additive sweetener of foods. Glutamate is an important energy and nitrogen source in eukaryotic and mammalian cells, and functions as excitatory neurotransmitters in the central nervous system (Castillo et al., 2005). In addition, urea constitutes the major excretory product of protein metabolism and is a dominant non-protein nitrogenous substance in body. Sol–gel-derived TiO_2 was used to immobilize enzymes in the matrices and fluorescence microscopy was used for optical detection. Urease, glucose dehydrogenase (GDH), and glutamate dehydrogenase (GLDH) were co-immobilized with SNARF-1-dextran for ratiometric measurement of urea, glucose, and glutamate at a time. In addition, atomic force microscopy (AFM) and scanning electron microscopy (SEM) were used to characterize the surface morphology of the TiO_2-encapsulated array-based biosensors.

2. Experimental

2.1. Chemicals

Polyvinylacetate (PVAc, MW 113,000) was purchased from Aldrich (Milwaukee, WI). Polyvinylalcohol (PVA, MW 205,000) was obtained from Fluka (Buchs, Switzerland). Urease (EC 3.5.1.5, MW 480,000, type IX, 82.8 units/mg-solid from jack beans), glucose dehydrogenase (EC 1.1.1.47, MW 39,000, 2000 units/mL from recombinant *thermoplasma acidophilum*), glutamate dehydrogenase (EC 1.4.1.4, MW 300,000, 300 units/mg-protein from *Proteus* species) 1,3-bis[tris(hydroxymethyl)methylamino] propane (BTP, 99%), β-nicotinamide adenine dinucleotide phosphate (β-NADP), L-glutamic acid (monosodium salt, 99%), and titanium isopropoxide (TTIP, $Ti(OC_3H_7)_4$) (99.999%) were purchased from Sigma–Aldrich (St. Louis, MO). Carboxy SNARF-1-dextran (MW 70,000) was obtained from Molecular Probes (Eugene, OR). Urea (99.3%) was obtained from J.T. Baker (Phillipsburg, NJ). All other chemicals were of analytical grade and were used as received without further purification.

Enzyme solutions including urease (1090 units/mL), glucose dehydrogenase (100 units/mL), and glutamate dehydrogenase (250 units/mL) were prepared with 5 mM BTP buffer at pH 6.0, 7.0, and 8.0, respectively. The fluorescent dye, carboxy SNARF-1-dextran, was prepared in 5 mM BTP buffer at pH 7.0 to get a concentration of 2.0 mg/mL. All the solutions were stored at 4 °C and were freshly prepared every 2 weeks. All aqueous solutions were prepared by bidistilled deionized water (bdH_2O) (18.3 MΩ cm) unless otherwise mentioned. The room temperature was controlled at 19 ± 1 °C and the relative humidity was in the range 55–75%. The $K_2Cr_2O_7/H_2SO_4$ clean solution was prepared by dissolving 0.84 g of $K_2Cr_2O_7$ in 7 mL of H_2O and then 200 mL of concentrated H_2SO_4 was added slowly.

2.2. Preparation of array-based TiO_2 biosensors

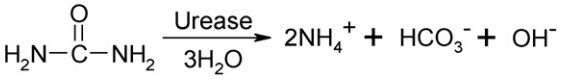
Glass microscope slides (2.5 cm × 7.5 cm) were cleaned with $K_2Cr_2O_7/H_2SO_4$ solution for 2 h to remove any organic contaminants. After washing with copious amount of bdH_2O and drying under a stream of N_2 gas, the glass slides were dipped into PVAc solution (5% in dichloromethane) for at least 1 h to enhance the sol–gel attachment (Tsai and Doong, 2005). The slides were then taken out slowly from the container, purged with N_2 gas, and stored under ambient conditions until used. A 50-well chambered coverslip was affixed on the PVAc-coated slide, and then placed on a thermoelectric cooler stage for contact printing. The printing of sol–gel-derived biosensor array was conducted automatically by a three-dimensional robotic system controlled by a LabVIEW™ program.

To fabricate the sol–gel-derived array-based TiO_2 biosensor, the mixture of solution was prepared by mixing 20 μL of enzyme solution, 5 μL of SNARF-1-dextran solution, 5.5 μL of glycerol, and 7 μL of PVA into a 200 μL vial. After pin-printing enzyme spots on the glass substrate covered with a 50-well coverslip, the glass matrix was placed horizontally into a sealed flask. About 0.4 mL of pure titanium isopropoxide (TTIP) was then injected into the flask from the rubber septum at 27 ± 1 °C (Doong and Shih, 2006). This resulted in absorption of saturated TTIP vapor on the surface of enzyme spots and slow formation of a sol–gel-derived TiO_2 film through the hydrolysis reaction of TTIP on the surface. After vapor deposition of 6 h, the glass matrix was taken out and 10 μL of 5 mM BTP buffer was added to stabilize the titania sol–gel array biosensor for 60 min prior to use.

2.3. Fluorescence detection

The simultaneous analysis of multiple samples in the presence of multianalytes was carried out using the sol–gel-derived array

biosensor. 15 μL of different analytes including BTP buffer (blank control), glutamate, glucose, and urea at various concentrations ranging from 10 μM to 10 mM were added into the array-based biosensor for 10 min. Subsequently, the solution was removed and the fluorescence was measured (Tsai and Doong, 2007). The principles for determination of urea, glucose, and glutamate are as follows:



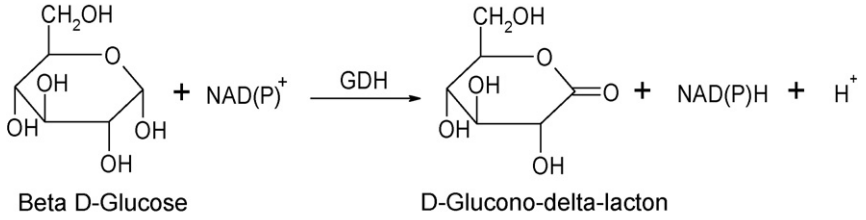


Fig. S1 (see Supplementary Information) shows the schematic illustration of the developed system for the fluorescence detection (Tsai and Doong, 2005). The fluorescence microscope (ECLIPSE E600, Nikon Corp., Tokyo, Japan) equipped with a blue-enhanced silicon detector with a 100 mm^2 sensing area (Edmund Industrial Optic Inc., Barrington, NJ, USA) was used for fluorescence detection. The intensity of the light source (100 W mercury lamp) was attenuated to 1/32 of the original value using neutral density filters in order to prevent the photo-bleaching of the dye molecules. Two filter blocks, G-2A (excitation (EX) 510-560 nm, dichroic mirror (DM) 575 nm, barrier filter (BA) 590 nm, Nikon) and B-2A (EX 450–490 nm, DM 505 nm, BA 520 nm, Nikon), were used for the detection of SNARF-1-dextran, respectively. An optical chopper with 5/6 slot blade (SR540, Stanford Research System, Sunnyvale, CA, USA) was set at the frequency of 392 Hz to minimize the electronic interference. The signal obtained from the silicon detector was integrated with the chopper reference by a signal-board lock-in amplifier (FEMTO Messtechnik GmbH, Berlin, Germany). Usually 50 measurements can be completed automatically within 10 min. Finally, the data was acquired by the data acquisition card (DAQ card, PCI-1200, National Instruments, TX). Fluorescence detection was carried out automatically by scanning over the sol–gel spots by the developed robotic system programmed by the LabVIEW™ software (National Instruments, TX).

2.4. Surface characterization

The topography of sol–gel-derived TiO_2 biosensor was characterized using atomic force microscopy. AFM was performed using a scanning probe microscope (SPM) equipped with a Nanoscope IIIa controller (Digital Instruments, Santa Barbara, CA) and a laser beam deflection to monitor the displacement of a microfabricated Si cantilever having a force constant of 40 N/m. AFM was conducted in the tapping mode at room temperature in air. The scan speed was at 0.5–0.7 Hz with 512 × 512 data acquisitions.

The surface morphology of titania biosensor was determined by SEM (Hitachi S-4700 type II, Tokyo, Japan). All the samples were platinum (Pt)-coated using Ion Sputter ε-1030 (Hitachi, Japan) to increase the conductivity of sample surface. After coating with Pt, samples were placed under high vacuum (10^{-3} to 10^{-7} mbar) conditions. An acceleration electron voltage of 5k was applied to obtain the SEM images.

3. Results and discussion

3.1. Characterization of carboxy SNARF-1-dextran

Carboxy SNARF-1-dextran is typically used by exciting the dye at wavelengths between 488 and 530 nm, while monitoring the fluorescence emission at another wavelength ranging between 580 and 640 nm. The acid tautomer emits peak in the 580–590 nm range, while the emission peak of the base form appears in the range 630–640 nm. Several studies have shown the fluorescence spectra of carboxy SNARF-1-dextran in phosphate buffer (Gulcev et al., 2002; Collinson and Higgins, 2004). However, the characteristics of carboxy SNARF-1-dextran is dependent on the microenvironment of solutions and the relative intensity ratios of acid to base tautomers in BTP buffers remain unclear. Fig. S2 (see Supplementary Information) shows the fluorescence spectra of carboxy SNARF-1-dextran measured in BTP buffer at various pH values. At low pH values, the fluorescence emitted from carboxy SNARF-1-dextran falls predominately in a band centered near 585 nm. A dramatic decrease in the fluorescence intensity near this wavelength occurs when the pH was higher than 7.0. In addition, a new emission peak near 630 nm appeared, and the intensity increased upon increasing pH values. The spectra also show a clear isoemissive point around 610 nm, which is consistent with the spectra prepared in phosphate buffer (Collinson and Higgins, 2004). In this study, both the fluorescence intensity ratio (I_{630}/I_{585}) of the dye molecules excited at 488 and 514 nm increased with the increase in pH values ranging from 6.0 to 9.0. However, a large relative standard deviation (RSD) of 17% was observed at high pH when carboxy SNARF-1-detran was excited at 514 nm, presumably due to the low emission intensity at 585 nm. Therefore, the excitation wavelength at 488 nm and the emission wavelengths at 585 and 630 nm, respectively, were used for further experiments.

3.2. Surface morphology

To further determine the surface morphology of the titania sol–gel films after immobilization of enzymes and dye molecules using vapor deposition method, SEM and AFM were used to

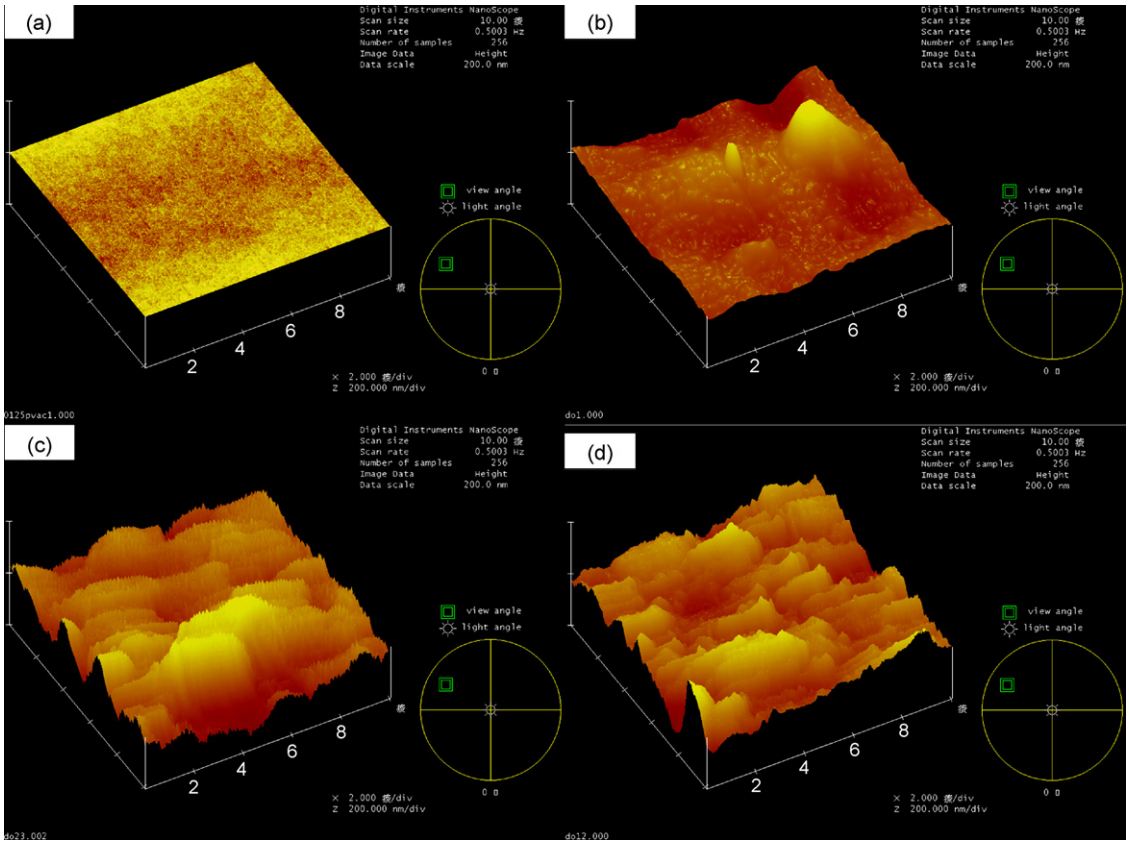


Fig. 1. AFM images of the titania sol–gel films coated with (a) PVA alone, (b) urease, (c) glucose dehydrogenase, and (d) glutamate dehydrogenase.

characterize the surface roughness of the enzyme-encapsulated sol–gel spots. Fig. S3 (see Supplementary Information) shows the SEM images of the titania sol–gel films in the presence and absence of macromolecules. The vapor deposition method can produce a homogeneous and smooth TiO_2 sol–gel film in the absence of biomolecules or organic additives. Addition of macromolecules changes the surface morphology significantly. Surface roughness caused by the doped enzyme and dye molecules into titania sol–gel film could be easily observed from SEM image. In addition, the TiO_2 film is transparent and no crack is found by SEM, showing that TiO_2 is a stable and biocompatible sol–gel material for fabricating optical-based array biosensor.

Fig. 1 shows the AFM images of the surface morphologies of the enzyme-encapsulated array-based TiO_2 biosensors. A smooth surface with roughness of 2.12 nm was observed when the PVAc-coated glass matrix was coated with titania sol–gel. However, the surface roughness increased significantly when TiO_2 was coated onto the surface layer of macromolecules including biomolecules, SNARF-1-dextran and PVA. The surface roughnesses, determined by the distance from peak-to-valley, of the array-based TiO_2 biosensors after immobilization of urease, GDH and GLDH were 36.1, 46.2, and 35.2 nm, respectively, depicting that the biomolecules were successfully immobilized into titania sol–gel matrices using the vapor deposition method. The structure of urease is reported to be a flatted sphere with a diameter of 11 nm and a height of 6 nm (Qin and Cabral, 2002). Zhang and Tan (2001) used AFM to characterize the immobilized GLDH on silica plate surface. The roughness of the biosensor surface increased during the GLDH immobilization processes, and the peak-to-valley distance increased from 20 to over 200 nm after 24 h of immobilization, which is in good agreement with the results obtain in this study by AFM image. It is noted that the immobilized macromolecules including SNARF-1-dextran (MW 70,000), biomolecules (MW 39,000–480,000) and PVAc (MW 113,000) are larger than those used in the previous reports (Qin and Cabral, 2002; Zhang and Tan, 2001), which means that the surface roughness of the TiO_2 thin film would be large. Moreover, macromolecules were probably not adsorbed on the sol–gel surface but entrapped inside sol–gel matrix because the sol–gel networks on the top of the gibbosity could be observed when compared the non-doped and doped sol–gel AFM images.

3.3. *Effect of pH on the array-based TiO_2 biosensors*

The initial pH and buffer concentration of the solutions are crucial parameters influencing the sensitivity and dynamic range of the array-based biosensor. Fig. S4 (see Supplementary Information) shows the responses of pH value and buffer concentration to the array-based TiO_2 biosensors in the presence of various concentrations of urea. The hydrolysis of 1 mole urea by urease produces 2 mole ammonium ions and 1 mole of bicarbonate ion, resulting in the increase in pH values. Therefore, the initial pH values in the range 5.5–6.8 were selected for optimization. At low pH value of 5.5, no significant change in intensity ratio (I_{630}/I_{585}) was observed when urea concentrations increased from 1 μM to 100 mM, presumably due to the fact that the relative fluorescence intensity ratio of carboxy SNARF-1-dextran is low under acidic conditions. In contrast, the intensity ratio of urease-based TiO_2 biosensors increased upon increasing urea concentrations when the initial pH was in the range 6.0–6.5. In addition, the intensity ratios at pH 6.5 were higher than those at pH 6.0. Further increasing the initial pH to 6.8, however, lowered the intensity ratio of the fluorescent probe, clear showing that pH 6.5 is the optimal pH value for urease-encapsulated biosensor when carboxy SNARF-1-dextran was used as the fluorescent probe in the array-based TiO_2 biosensors.

Buffer capacity is another important factor influencing the performance of the TiO_2 biosensors for multianalyte determination.

In general, higher buffer concentration has higher buffer capacity, resulting in decreasing the system sensitivity since it become difficult to change the pH value of the system as buffer capacity increases. However, low buffer capacity cannot maintain the stability of pH which is harmful to enzyme activity. As depicted in Fig. S4(b), Addition of low buffer concentration at 1 mM had insufficient buffer capacity to maintain the stable pH. No obvious change in intensity ratio was observed when 10 mM BTP buffer was used to detect urea (<1 order of magnitude), presumably due to that 10 mM BTP buffer are too strong to change the solution pH. Addition of 2.5 and 5 mM buffer solutions had the similar sensitivity and linear range (10–2000 μM) toward urea determination. Therefore, 5 mM buffer concentration was chosen for further experiments.

Unlike the determination of urea, reactions of glucose and glutamate by dehydrogenase produce proton to decrease the pH of the system. Therefore, relatively high initial pH values ranging from 7.0 to 9.0 were examined. Fig. S5 (see Supplementary Information) shows effects of pH on glucose and glutamate determination. The intensity ratio increased with the increase in pH ranging from 7.0 to 8.5 and then slightly decreased at pH 9.0. The increased response at high pH value is primarily attributed to the fluorescent characteristics of the carboxy SNARF-1-dextran that the emission intensity at 630 nm is strong under alkali conditions. It is noted that the response of the array-based TiO_2 biosensor at pH 8.5 is slightly higher than that at pH 9.0, presumably due to that the optimal pH for glucose dehydrogenase is in the range 7.5–8.0. However, the RSD of the intensity ratio at pH 8.5 (0.3–7.3%) are larger than those at pH 9.0 (0.1–4.1%).

Similar results were observed in GLDH-encapsulated TiO_2 biosensor for the quantification of glutamate. The intensity ratio increased upon increasing glutamate concentration in the pH range 6.5–8.5 and then slightly decreased at 9.0 (Fig. S5b). Several studies depicted that the optimal pH of the NADP-dependent glutamate dehydrogenase from the hyperthermophilic archaeon was in the range 8.0–8.25. Jeffries et al. (1997) reported that both apparent half-saturation velocity (K_m^{app}) and maximum reaction rate (V_{max}) of the immobilized GLDH on a carbon paste electrode increased with increasing pH values, and there was little difference in the rate kinetics between pH 8.2 and 8.9. In addition, addition of 2.5 and 5 mM buffer solutions has similar effect on the response of glutamate determination. These results clearly depict that 5 mM BTP buffer at pH 9.0 could be the optimal condition for the ratiometric determination of glucose and glutamate using the array-based TiO_2 biosensors.

3.4. Cross-interference of multianalytes

In order to understand the chemical cross-interference behaviors of the array-based TiO_2 biosensors, multianalyte experiments were performed. To achieve this objective, urease-, GDH-, and GLDH-doped TiO_2 sol–gels were immobilized on different rows of the array followed by treating each column with corresponding or mixed analytes. Results of multianalyte analyses were compared with those of the single analyte determinations to understand the possible cross reactivity. To address the cross-interference behavior, various concentrations (0–1000 μM) of analytes including urea, glucose, and glutamate were mixed in the 5 mM BTP buffer at pH 6.5 to determine urea concentration, while 5 mM BTP buffer at pH 9 was used to determine glucose and glutamate. Fig. 2 shows the responses of the array-based biosensors in the presence of various concentrations of single or multiple analytes. It is clearly that the intensity ratios of carboxy SNARF-1-dextran in the solutions containing various concentrations of single and mixed analytes were similar. No significant difference in GLD- and GDH-encapsulated biosensors was observed when *t*-test was applied ($p < 0.05$). A slight decrease in intensity ratio was observed when the urease biosensors were used to determine multianalytes in the concentration range of 10–100 μM. However, the difference in intensity ratios between single and mixed analytes was only 1.5–3.7% and was not statistically significant when compared with the precision of the array-based TiO_2 biosensors (RSD = 2.8%). These results clearly

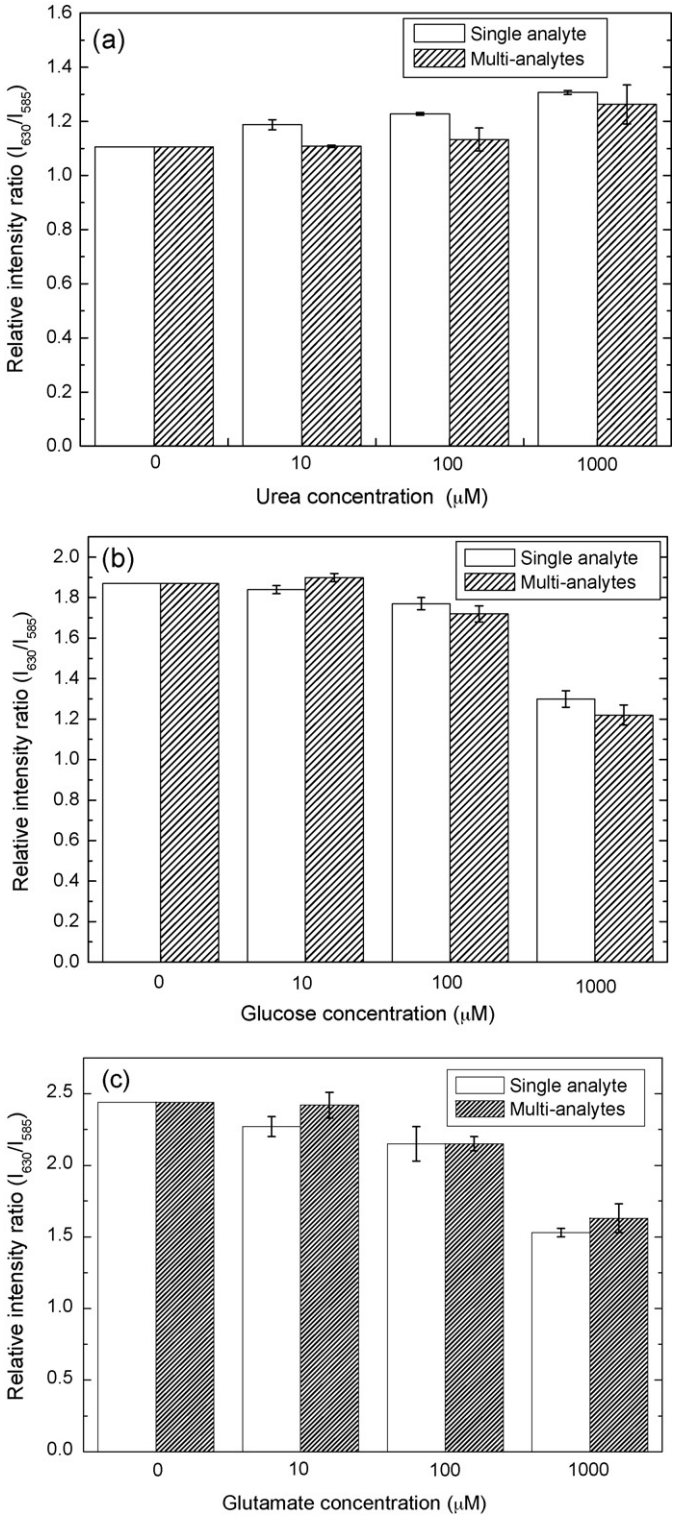


Fig. 2. Responses of the array-based TiO_2 biosensors with respect to various concentrations of (a) urea, (b) glucose, and (c) glutamate. The biosensors consisted of 50 independent wells and each well contained a sol–gel sensing element. The concentrations used in this study were in the range 0–1000 μM. Error bars represent the standard deviation of means ($n = 3$).

Table 1
Analytical performance of array-based TiO_2 biosensors of multianalytes in water and serum samples.

Target analytes	Water samples			Serum samples		
	LOD (μM)	Dynamic range (μM)	Precision (%)	LOD (μM)	Dynamic range (μM)	Precision (%)
Urea	2.4	10–8000	2.8	3.1	2–300	2.4
Glucose	5.5	40–10,000	1.6	5.4	20–10,000	4.6
Glutamate	4.8	10–10,000	4.3	7.8	40–8,000	6.1

show that the array-based TiO_2 biosensors encapsulated with multiple enzymes exhibit good specificities in a mixed analytes and have little cross-interference effect when SNARF-1-dextran was used as the single-molecule fluorescent probe.

3.5. Determination of multianalytes

Fig. 3 shows the response curves of the array-based TiO_2 biosensors in the presence of various concentrations of analytes (urea, glucose, and glutamate). Sigmoidal curves (S-shape) as a function of analyte concentrations were observed. The intensity increased rapidly with increasing amounts of urea, and then leveled off at high concentrations (10–100 mM). In general, sol–gel encapsulated enzymes follow Michaelis–Menten kinetics as is the case for the enzymes in solution. A previous study showed that the apparent Michaelis–Menten constant (K_m) value of urease encapsulated in sol–gel matrix was 2.03 mM (Tsai and Doong, 2007). Since the substrate concentration is much higher than the K_m value in the concentration range 10–100 mM, the urease activity could reach the maximum activity, which is consistent with the response curves obtained in this study.

Table 1 shows the analytical performance of the developed array biosensors in buffer and human serum samples. The array-based TiO_2 biosensor showed an excellent analytical performance on urea determination. A wide dynamic range of 3 orders of magnitude (6–5000 μM) was observed. The limit of detection (value greater than the blank ±3 standard deviations, $p < 0.05$) of urea was 2.4 μM. Similar results were also observed in GDH- and GLDH-encapsulated TiO_2 biosensors. The dynamic range of 2–3 orders of magnitude with the LODs of 5.5 and 4.8 μM for glucose and glutamate, respectively, were observed. In addition, the precision for the determination of urea, glucose and glutamate were 2.8, 1.6 and 4.3% ($n = 3$), respectively. In addition, a good reproducibility with recovery of 102 ± 7% ($n = 5$) was also obtained when the GDH-encapsulated array biosensor was fabricated in the 5 consecutive weeks, showing that the developed protocol for the fabrication of sol–gel-derived array biosensors has good precision and reproducibility for multiple analyte determination.

Several optical and electrochemical biosensors have been developed for determination of glucose, urea or glutamate. A titania sol–gel-derived amperometric biosensor has been developed to immobilize glucose oxidase for the detection of glucose. The dynamic range and detection limit were 0.07–15 mM and 70 μM, respectively (Yu et al., 2003). Singh et al. (2008) summarized the urea biosensors in different fields of application and found that the dynamic range of urea biosensors is generally in the range 2–3 orders of magnitude with detection limits of 3 μM to 5 mM. More recently, several array-based biosensors have been fabricated to detect multianalytes. A multifunctional biosensing chip was designed based on the electrochemiluminescent detection. The detection ranges and limit for the different biosensors were 1–500 μM and 1 μM for glutamate and were 0.02–2 mM and 20 μM for glucose, respectively (Marquette et al., 2003). An array-based optical biosensor based on silica sol–gel immobilization has been reported for simultaneous determination of glucose, urea, uric acid and creatinine. The analytical ranges of the four analytes were between 0.01 and 10 mM with detection limits of 2.5 (urea) to

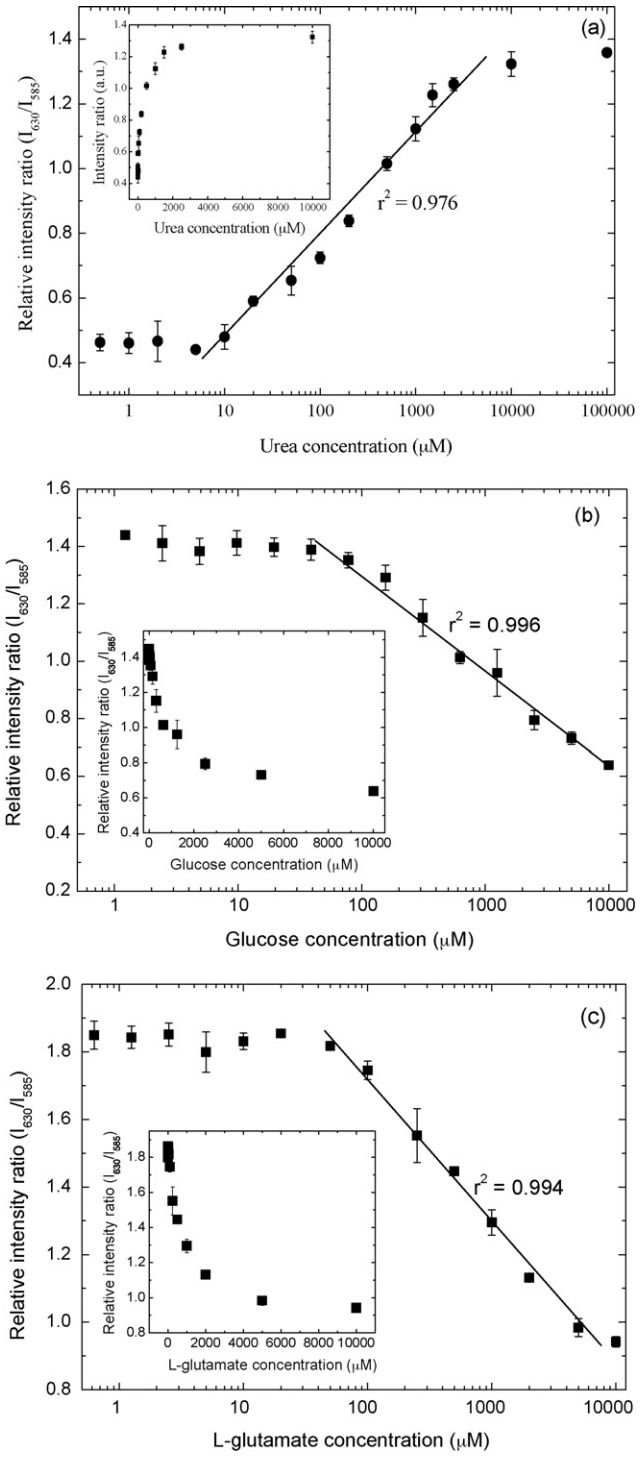


Fig. 3. Responses of array biosensors to (a) urea, (b) glucose, and (c) glutamate in buffer solution. The concentrations used in this study were in the range 0.5 μM to 10 mM. Error bars represent the standard deviation of means ($n = 3$). The insets showed the response of array biosensor to urea, glucose and glutamate using normal–normal plot in buffer solution.

80 μM (glucose) (Tsai and Doong, 2004). These results clearly show that the developed array biosensor has high reproducibility and is comparable or even superior to the reported biosensor systems for multianalyte determination.

The storage stability of the sol–gel-derived array-based biosensor encapsulated with glucose dehydrogenase was also investigated. The biosensor containing 50 spots was pin-printed on a glass slide and stored at 4 °C in buffer solution and the sensitivity was analyzed at a particular time interval. Fig. S6 shows the storage stability of the glutamate-encapsulated biosensor as a function of time. In general, the developed biosensor showed good stability with relative sensitivity >90% within 5 weeks when 100 μM glucose was added into the solution, depicting that the enzymatic activity and fluorescent probe of the developed biosensor can be maintained for at least 1 month and can be used as the disposal biosensor for detection of multianalytes.

3.6. Determination of multianalytes in real samples

The use of array-based TiO_2 biosensors for the simultaneous quantification of small molecules in biological samples such as urine and serum is of great interest in the medical diagnosis and in the therapy of patients suffering from a variety of disorders associated with metabolic diseases such as kidney dysfunction, diabetes mellitus and Alzheimer disease. To evaluate the applicability of the system to real sample analysis, human serum was spiked with various concentrations of analytes. Fig. 4 shows the response curves of the array-based TiO_2 biosensors toward various concentrations of analytes (urea, glucose, and glutamate) in serum samples. The analytical performance of the developed array biosensor in human serum is also shown in Table 1. Urea constitutes the major excretory product of protein metabolism and has now become as a general biomarker for renal function. The array biosensor showed a satisfactory analytical performance on urea determination in serum samples. A dynamic range of 2–300 μM with the correlation coefficient (r) of 0.994 (r^2 = 0.988) for urea determination was observed. The limit of detection (LOD) was found to be 3.1 μM, which is comparable with the existing urea sensors (3 μM to 5 mM) and is sufficiently low to determine urea in serum (2.5–8 mM) (Gaw et al., 1995; Singh et al., 2008).

Glucose biosensors are one of the most well-known amperometric biosensors now developed, and they are commercially available due to their importance to patients suffering from diabetes mellitus. In this study, the optical TiO_2 biosensor also showed good analytical performance on glucose determination in serum samples. The dynamic range of 20–10,000 μM with a detection limit (p < 0.05) of 5.4 μM was obtained. The obtained dynamic range is within the normal range of glucose in serum (3.5–5.8 mM) and the LOD is significantly lower than those of reported data (Yu et al., 2003; Marquette et al., 2003). In addition, glutamate is an amino acid recognized as an excitatory neurotransmitter in the central nervous system, which is believed to be responsible for certain fundamental processes such as learning, memory, and synaptic plasticity. In this study, a dynamic range of 3 orders of magnitude (40–8000 μM) with correlation coefficient of 0.997 (r^2 = 0.994) for GLDH-encapsulated TiO_2 biosensors was obtained. The LOD of glutamate was found to be 7.8 μM, which is comparable with the reported data and is sufficient for glutamate analysis in cerebrospinal fluids (1–48 μM) (Lu et al., 2005). In addition, the precision of the array TiO_2 biosensor was investigated in this study and the relative standard deviations of 2.4–6.1% (n = 45) for different analytes were observed through the entire array, showing that the developed protocol for the fabrication of sol–gel-derived array TiO_2 biosensors has good precision and reproducibility for multiple analyte determination.

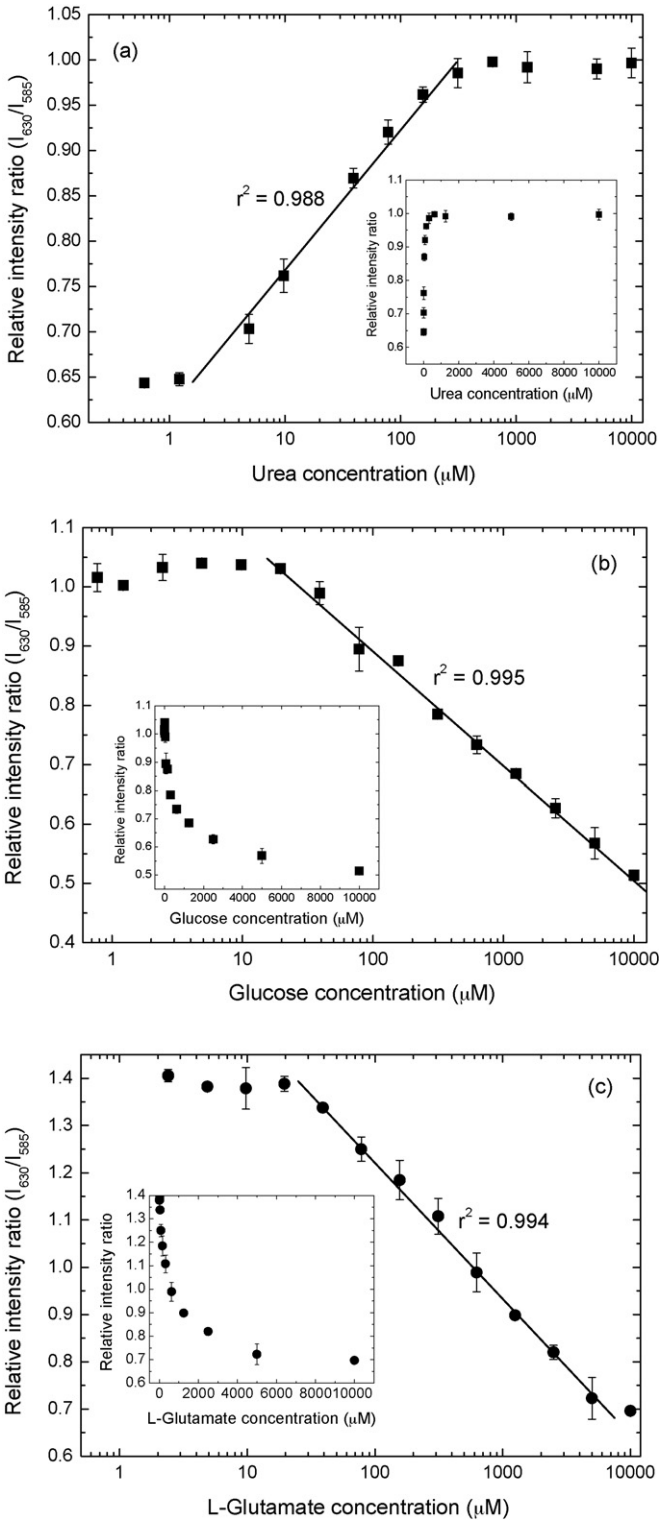


Fig. 4. Responses of array biosensors to (a) urea, (b) glucose, and (c) glutamate in human serum samples. The concentrations used in this study were in the range 0.5 μM to 10 mM. Error bars represent the standard deviation of means (n = 3). The insets showed the response of array biosensor to urea, glucose and glutamate using normal–normal plot in human serum samples.

4. Conclusions

In this study, the combination of the sol–gel-derived array-based TiO_2 biosensors with the carboxy SNARF-1-dextran as the fluorescent probe for the optical determination of multianalytes

in biological samples was first demonstrated. Titanium dioxide is a stable sol–gel material which does not crack with age and vapor deposition method has been shown to effectively immobilize different enzymes into the TiO_2 matrices for multianalyte determination at a time. The applicability of the developed array TiO_2 biosensor using carboxy SNARF-1-dextran as the fluorescent probe for the simultaneously determination of multianalytes in water and serum samples is also demonstrated. The analytical ranges of 2–3 orders of magnitude with detection limits of 2.4–5.5 μM in water samples and 3.1–7.8 μM in serum samples, respectively, were obtained. In addition, the prepared array-based TiO_2 biosensors retained high relative enzyme activity and precise detection even after long-tem storage (1 month) at 4 °C. Results obtained in this study not only provide a viable alternative to current silica technology that low levels of multianalytes can be determined at a time but also most likely find relevant in a variety of biomedical diagnostics, clinic, and environmental applications. It is also noted that the simultaneous determination of multianalytes is based on the change in pH values for optimal detection in the presence of fluorescent dye. Several label-free biosensors such as surface plasmon resonance- and optical waveguide-based biosensors have been developed. These biosensors could be useful analytical tools which can overcome the limitation of pH change for parallel measurement.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.10.044.

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