Determination of total bile acid levels using a thick-film screen-printed Ir/C sensor for the detection of liver disease

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An electrochemical sensor, based on thick-film screen-printed Ir/C working and counter electrodes, was developed for the detection of total bile acid concentration in a physiological fluid for potential patient management in patients with liver disease. Current electrochemical methods of detecting total bile acid levels involve the use of potentials greater than +0.45 V, *versus* an Ag/AgCl reference electrode, and require a selectively permeable membrane. The proposed detection method did not require any membrane and used a potential of +0.27 V *versus* Ag/AgCl. This biosensor used 3-α-hydroxysteroid dehydrogenase (3α-HSD) (EC 1.1.1.50) immobilized on the thick-film screen-printed working electrode to detect the enzymatically generated NADH. The production of the NADH resulted from the reaction of the enzyme with bile acids such as sodium cholate, taurocholic and taurochenodeoxycholic acid, which could then be used to quantify the total bile acid. Constant potential measurements showed that this biosensor had good linear performance over a 0–200 μM concentration range in the phosphate buffer and the bovine serum. The sensor performance was also examined at different temperatures and pH conditions. This sensor prototype could be used for single use, disposable detection of total bile acids, extending its applicability for simple and early detection of liver disease.

1. Introduction

Rapid and easy detection of liver disease would provide a method of early detection for a growing concern in today's population. Liver disease, including cirrhosis and hepatitis, can be found in more than 170 million people worldwide.[1] Early detection of liver disease can slow, or eliminate, the progression of the disease into the fatal stages. Unfortunately, often those that have the least access to detection are those that need it the most. With impoverished areas such as Tanzania, Rwanda and Mongolia seeing disease levels approaching 10% of the population,[2] it is clear that a simple and effective means of detection of liver disease will be desirable for early patient management. At the onset of the liver disease a series of physiological changes occur in the body, specifically in the liver. It has been found that by monitoring one specific biomarker, total bile acid concentration, the presence and effectiveness of treatment can be evaluated.[3–5] Total bile acid concentration is the term used to describe the concentration of the individual bile acids that can be found in the body, specifically in the enterohepatic system. The acids, produced by the degradation of cholesterol in the liver, are stored in the gall bladder until they are needed for the digestion of fatty foods. The total bile acid principally consists of cholic, taurochenodeoxycholic and taurocholic acids. Accurate identification of these acids can provide proper diagnosis of the condition of the liver.[6–8] Typical levels of total bile acid found in a healthy individual can range from 4–10 μmol L^{-1}, while in a sick individual the level of total bile acid can vary from 67–376 μmol L^{-1}.[9] It appears feasible that a detection method should be able to distinguish between these two levels of the total bile acid as described.

Various methods have been used for the detection of total bile acid. Techniques such as thin layer chromatography,[10] high-pressure liquid chromatography,[11,12] and bioluminescence[13] have all showed their usefulness and uniqueness in their applications towards the detection of total bile acid. While the precision and the selectivity of these techniques are high, for clinical applications they are not particularly well suited due to their technical complexities. The most commonly used method for clinical determination has been the enzymatic colorimetry. In this method, bile acid from the sample is enzymatically oxidized in the presence of NAD^+ using 3-α-hydroxysteroid dehydrogenase (3α-HSD) (EC 1.1.1.50) as shown in eqn (1) and (2).

$$\text{Bile acid} + NAD^+ \xrightarrow{3\alpha\text{-HSD}} NADH + H^+ + \text{Oxidized acid} \quad (1)$$

$$NADH + H^+ + \text{Dye} \xrightarrow{\text{diaphorase}} \text{Reduced dye} + NAD^+ \quad (2)$$

The produced NADH is then combined with a dye such as nitrotetrazolium[14] or resazurin.[15] The changes in the concentration of the spectrometric absorbance of the reduced dye are monitored over time with a spectrophotometer. These changes can then be related to the original bile acid concentration. The downside of these measurements is the need for expensive, non-portable equipment and the total time for testing.

Research into amperometric biosensors based on enzymes has grown recently. Interest in their possible high selectivity, sensitivity and convenience has led to numerous studies.

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Unfortunately, the amount of work focused directly on bile acid detection has been limited.[16,17] In the work by Albery,[16] a conductive organic salt was used as the catalyst for the oxidation of NADH formed by reaction (1). Due to the high oxidizing potential needed, a series of size-selective membranes were needed to prevent oxidation of other species in the test medium. The need for the membrane increases the complexity of the construction of the sensor, and this leads to a greater diffusion resistance and the possibility for quicker fouling of the electrode. Koide[17] approached the problem slightly differently by suggesting the use of a three-enzyme system that ultimately produced hydrogen peroxide. While hydrogen peroxide could be detected using a lower potential, they still used an applied potential of +0.5 V *versus* an Ag/AgCl electrode. They also required a size-selective Nafion membrane to be applied to the sensor. Once again, the membrane acted as a method to prevent the oxidation of the additional species beyond the target hydrogen peroxide. Koide's method provided a means of creating a more easily oxidized species, H_2O_2, but resulted in a sensor with increased complexity and cost.

Both of the proposed sensor designs[16,17] provided a means of amperometrically quantifying the total bile acid levels. However, none of these systems were able to perform at a low potential in order to minimize any interference derived from the oxidation and reduction of other species in the biological fluid. Thus, by reducing the operating potential, other species typically found in blood, such as ascorbic acid and uric acid, would not interfere in the measurement. The previous researchers[16,17] attempted to overcome this problem by using size-exclusion membranes. The use of membranes resulted in an increased diffusion resistance and complexity. Therefore, a sensor that could operate at a lower operating potential without the need for a membrane would provide a considerable advantage over the current systems.

In our study, an Ir/C sensor was prepared using the thick-film screen-printing techniques. Feasibility of NADH measurement with this Ir/C sensor was assessed using cyclic voltammetry and amperometry. The influence of the temperature and the pH value on the detection of the three bile acids, sodium cholate, taurocholic acid and taurochenodeoxycholic acid, was measured. Finally, a determination of the effectiveness of the sensor on detecting the multiple bile acids in both phosphate buffer and bovine serum was made. Our results demonstrated that a sensor would be capable of measuring total bile acid concentrations over the concentration range of 0 to 200 μM, at a potential of less than +0.5 V *versus* an Ag/AgCl reference electrode, without the need for size exclusion membranes.

2. Experimental

2.1 Materials and reagents

Lyophilized 3α-HSD (EC 1.1.1.50) powder was purchased from Diazyme Laboratories (San Diego, CA, USA). Ir/C particles (5% Ir) were purchased from BASF (Somerset, NJ, USA). Potassium chloride, NADH, NAD^+, sodium phosphate monobasic, sodium phosphate dibasic heptahydrate and sodium phosphate tribasic were purchased from Fisher Scientific (Hampton, NH, USA). Sodium cholate, taurocholic acid–sodium salt, and sodium taurochenodeoxycholate were purchased from Sigma-Aldrich (St. Louis, MO, USA). The bovine serum used was from Invitrogen (Carlsbad, CA, USA). Additional chemicals used were of analytical grade. Solutions and buffers made were prepared using de-ionized water.

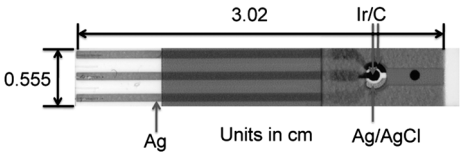

Fig. 1 Typical sensor used during experimentation.

2.2 Thick- film screen-printed prototype

The constructed sensor consisted of three electrodes: a printed Ir/C working and a counter electrode and a printed Ag/AgCl reference electrode. Fig. 1 shows the layout of a typical sensor used for the experimentation in this study. All electrodes were printed onto a polyester surface. Printed silver served as the electrical connections. Surface area of the working electrode was 7.85×10^{-3} cm^2. Preparation of the Ir/C ink and the subsequent manufactured electrode has been described in a previous publication.[18] As mentioned, this sensor prototype was developed as a single use, disposable device which would be invaluable for various bile acids measurements.

2.3 Construction of the 3α-HSD biosensor

The described sensor prototype served as the platform for this total bile acid biosensor. In order to complete the construction of the sensor, 0.05 units of 3α-HSD were deposited onto each sensor. This was accomplished by first dissolving 0.7 mg of lyophilized 3α-HSD into 737 μL of pH 8.9 100 mM phosphate buffer containing 150 mM potassium chloride and 100 mM NAD^+. The solution was mixed to ensure the homogeneity and then stored at 4 °C for 10 minutes. Next, 1 μL of the solution was pipetted onto the working electrode of the sensor prototype. Finally, the sensor with the solution was stored at 4 °C for an additional one hour allowing the chemicals on the sensor to dry. The sensor prototypes were then ready for use.

2.4 Electrochemical testing of the sensors

A CH Instrument 660C electrochemical workstation (CH Instrument, Inc., Austin, TX, USA) was used in this study, and the performance of the sensor was evaluated using cyclic voltammetry and amperometry. Testing was performed using a 0.6 ml micro-centrifuge tube for sample handling, or by directly applying the test sample to the sensor. The experiments performed using the tubes required 200 μL of the test solution and 20 μL of the enzyme solution. This was carried out in order to attain the same 0.05 U/sensor ratio. Experiments using the sensor and immobilized enzyme required a 5 μL test volume. In the case of elevated temperature experiments a water bath (ISOTEMP 202 from Fisher Scientific) was used. Potentials reported throughout this study were in comparison to the Ag/AgCl screen-printed reference electrode.

3. Results and discussion

3.1 Detection of NADH in solution

As shown in eqn (1), bile acid can be detected and quantified if a sensor for the detection of NADH can be accomplished. This will simplify the detection of bile acid by eliminating the processing step shown in eqn (2). Thus, the use of this Ir/C electrode for the detection of NADH was first carried out. The measurement of known concentrations of NADH in a pH 7.0 phosphate buffer with 150 mM potassium chloride was first evaluated, to determine the effectiveness of the Ir/C sensor for detecting NADH. Evaluations were made using both cyclic voltammetry and amperometry.

3.1.1 Detection of NADH using cyclic voltammetry. Cyclic voltammetric curves were generated at ambient temperature (21 °C) using a 200 μM solution of NADH. The potential was scanned from +0.5 V to −0.1 V, starting at the open circuit potential, for eight cycles at a scan rate of 1 mV s^{-1} while monitoring the current. Fig. 2 compares the 3rd and 4th cycles generated for solutions with and without NADH. Oxidation of NADH occurred within the range of +0.18 V to +0.44 V *versus* the Ag/AgCl reference electrode. Based on the experimental results, a potential of +0.27 V *versus* Ag/AgCl, was selected for the detection of NADH in this study. The choice of potential was a balance between a desired low oxidation potential and a relatively high oxidation current. By maintaining a potential lower than +0.3 V *versus* the Ag/AgCl electrode, oxidation of other species, such as ascorbic and uric acid, could be minimized. Consequently, this led to a higher signal to noise ratio in the study.

3.1.2 Quantification of NADH concentrations using chronoamperometry. Amperometric studies were conducted on NADH solutions in pH 7.0 phosphate buffer solutions using the identified potential of +0.27 V *versus* Ag/AgCl. Concentrations of 0 to 200 μM of NADH in phosphate buffer with 15 0mM KCl were studied over a 200 second time period. Fig. 3 shows an example of the experimental results obtained during testing, as well as the repeatability of the measurements. Fig. 3(a) shows that at 200 seconds the system approaches a steady-state. The oxidation current of NADH measured using the sensor prototype can be related to the bulk concentration of NADH using the well-established Cottrell equation. The selection of a recording time was made based on the experimental results over the range of the NADH concentration in this study. In principle, the selected recording time should be relatively short yet still should reach the steady state measurement. As shown in Fig. 3(b), an observed time of 60 seconds was chosen. This time selection allowed any charging effects to be neglected. After 60 seconds, comparison of the linear fit showed only minor improvement. A new sensor was used for each measurement demonstrating the feasibility of this sensor for single use, disposable bile acid detection through the detection of NADH without individual calibration. A good linear response of the sensor current outputs to the NADH concentrations was observed over the concentration range of 0 to 200 μM of NADH. Good repeatability and relatively low standard deviations were also observed through this concentration range as shown in Fig. 3(b).

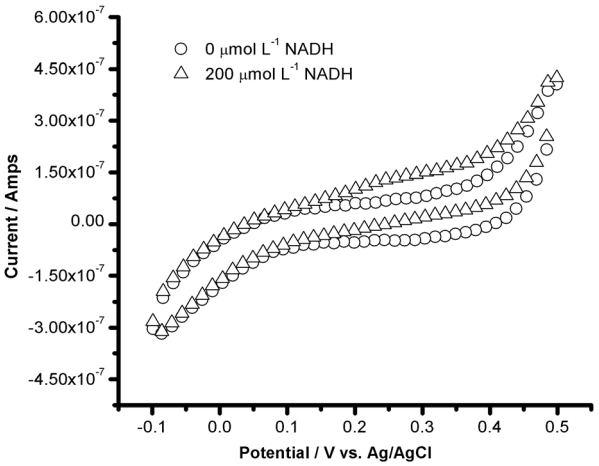

Fig. 2 Cyclic-voltammogram of pH 7.0 phosphate buffer, 150 mM KCl with, and without, NADH.

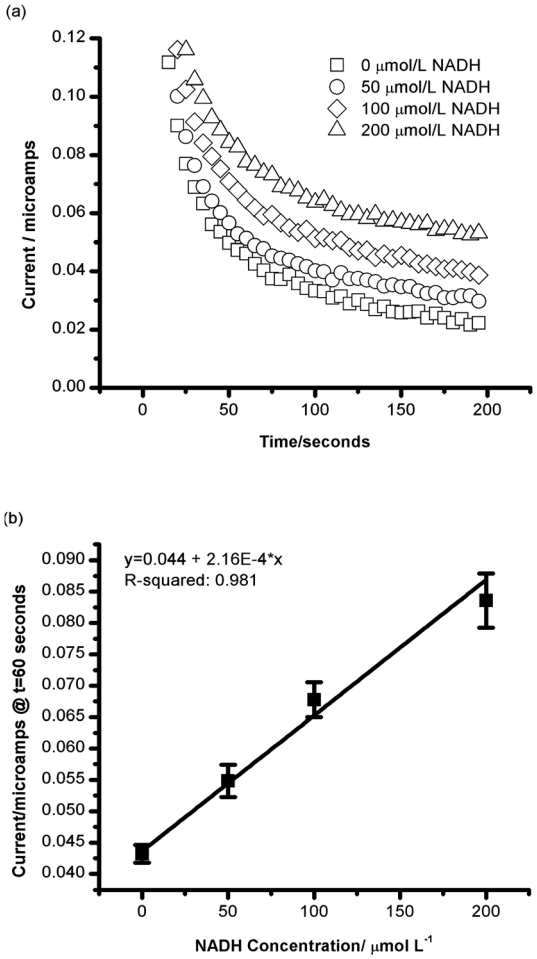

Fig. 3 (a) Examples of amperometric curves obtained at 0.27 V in pH 7.0 phosphate buffer with 150 mM KCl. (b) Repeatability of measurement with a fresh sensor with each measurement $n = 3$.

3.2 Detection of individual bile acids in solution

Based on the detection of NADH described, the measurements of each individual bile acid were then carried out. As mentioned

previously, cholic acid and taurochenodeoxycholic acid comprise the majority of the bile acids and were the focus in this study. Studies were conducted on the acids and their conjugate in order to understand the sensor's performance under various conditions.

3.2.1 Influence of the pH value on the performance of the sensor. While the NADH studies were tested at pH 7.0, it was indicated that a more basic environment would be more favorable to drive the enzymatic reduction of bile acids to completion.[19] Thus, the three individual acids, sodium cholate, taurocholic acid, and sodium taurochenodeoxycholate were experimentally assessed at pH 7.5 and 8.9 in a 0.065 M phosphate buffer with 150 mM KCl supporting electrolyte, over a concentration range of 0 to 500 μM for each acid. Testing was conducted over a time period of 10 minutes with the current being recorded. Fig. 4 compares the performance of the sensor in the presence of the individual bile acids at 37 °C. This increased temperature was selected in order to illustrate the performance of the sensor at a temperature close to the body temperature. Heating was achieved by immersing the sample vial into a water bath set at the desired temperature. An increase in current was observed from pH 7.5 to 8.9 for each acid tested. This increase was anticipated by examining the previously mentioned enzymatic reaction. The presence of the extra base allowed for a more complete oxidation of the bile acid through the increased removal of the chemically produced proton. An additional side effect was the improved linear response observed through 500 μM of bile acid in all cases. It was also noted that the behavior of taurochenodeoxycholic acid at pH 7.5 deviated from the other two at high concentrations. In the case of taurochenodeoxycholic acid the response began to plateau at a lower concentration, namely 200 μM. Similar behavior was observed by Turnberg and Anthony-Mote[20] in their study of bile acids using thin layer chromatography and spectroscopy. It was hypothesized that the presence of the additional hydroxyl group on the cholic acid molecule enhanced in the alignment with the enzyme. This additional alignment allowed for the increased reaction rates between the cholic acid and the 3α-HSD *versus* taurochenodeoxycholic acid.

3.2.2 Assessment of the influence of temperature and the need for the enzyme. An assessment of the influence of temperature was carried out in order to understand the effect of the elevated temperature. While a sensor that incorporated temperature control could be envisioned, design around a sensor that operated at ambient temperature would be more favorable. Thus, studies were conducted comparing the sensor response for cholic acid at 37 °C and 25 °C. The increased current observed in Fig. 5 at 37 °C *versus* the 25 °C could be explained by an electrochemical interpretation of the Arrhenius relationship. Although the 37 °C current was higher, it was encouraging that at 25 °C, over the 0 to 200 μM bile acid concentration range, a good linear response was demonstrated. This finding supported the belief that the sensor could be accurately operated at ambient temperature, and still retain good resolution through the desired operating window.

Studies were also conducted to confirm that the enzymatic byproduct was being detected. Samples were run with and without enzyme over the concentration range. Fig. 6 compares the two conditions for the operating temperatures at 25 °C and 37 °C. At each concentration, excluding zero, the experimental results showed that the presence of the enzyme was required for proper detection. This confirmed that the differences observed in the current were due to the oxidation of the enzymatic byproduct

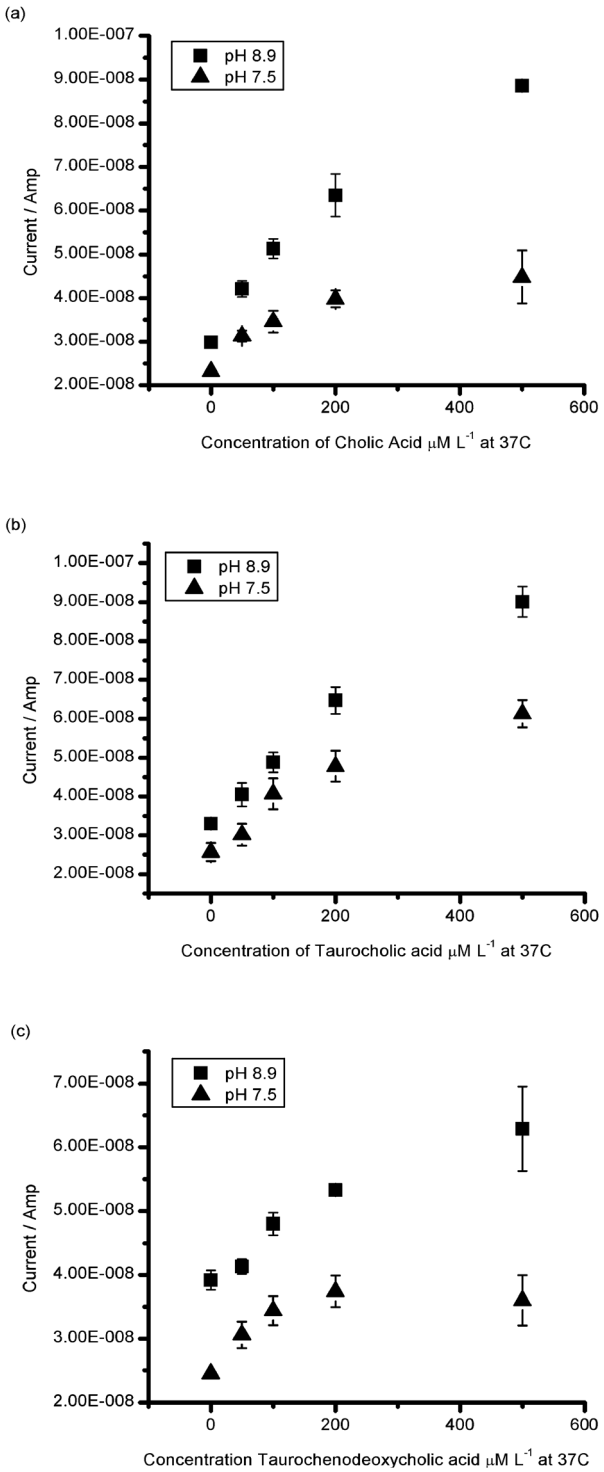

Fig. 4 Performance of the sensor in the presence of individual bile acids at 37 °C in a 0.065 M phosphate buffer. $N = 3$ for all conditions.

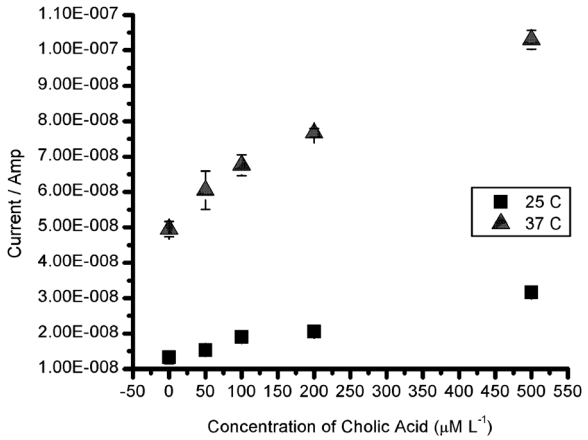

Fig. 5 Sensor response for cholic acid at 25 °C *versus* 37 °C.

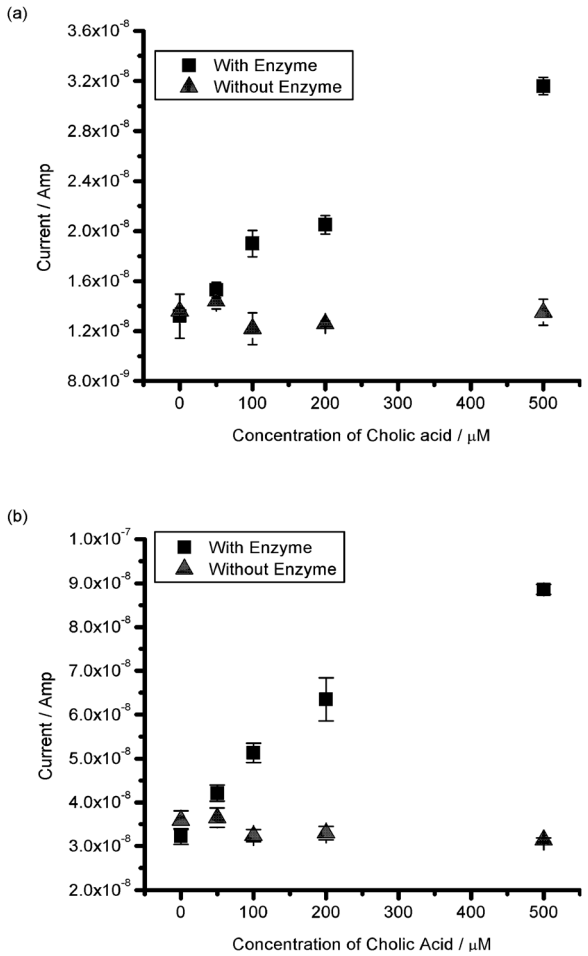

Fig. 6 Sensor response with, and without, enzyme in a 0.065 M pH 8.9 phosphate buffer at (a) 25 °C and (b) 37 °C.

NADH *versus* any other species in solution. The finding supported the belief that the generated NADH was detected and not other chemical compounds. Additionally, it showed that the enzyme was the species that generated the NADH detected.

3.3 Detection of mixture of bile acids in solution

The sensor has demonstrated the ability to accurately detect the concentration differences in the presence of a single bile acid. In the body, the enterohepatic system is a combination of multiple bile acids. In order to achieve a total bile acid measurement, it is important to be able to detect multiple acids in the same sample. Thus, testing was conducted using media that contained multiple bile acids. It was important that the positive performance observed in the single acid detection would also hold with the mixed acids.

3.3.1 Detection of the three mixed bile acids in phosphate buffer. The performance of the sensor was evaluated in the presence of two bile acids using a pH 8.9 0.065 M phosphate buffer. The systems studied were: cholic acid–taurocholic acid, cholic acid–taurochenodeoxycholic acid, and taurocholic acid–taurochenodeoxycholic acid. The enzyme concentration was held constant at 0.05 U μL^{-1}, while the concentrations of each acid were varied as shown in Table 1. The amperometric testing was conducted using a potential of +0.27 V, *versus* an Ag/AgCl reference electrode, for 600 seconds. The concentrations of the individual acids in solution were plotted *versus* the current as shown in Fig. 7. The behavior in the mixed acid system was similar to that observed in the single acid systems. Good linearity was observed in sodium cholate–taurocholic acid containing solutions. Solutions that contained taurochenodeoxycholic acid showed slight deviation from the linearity at concentrations at or above 200 μM. This was expected based on the behavior of the sensors in the taurochenodeoxycholic acid alone in solution. While the deviation from the linearity at high concentrations was less than desirable, the impact on the ability to detect would be minimal, since the concentration of taurochenodeoxycholic acid was lower than the other two acids studied, and thus its influence would be rather marginal. Additionally, the observed deviation

Table 1 Concentration of bile acid used μmol L^{-1} in solution for mixed acid testing

Lot #	SC[a]	TA[b]	TCA[c]
1	0	0	0
2	50	0	0
3	100	0	0
4	200	0	0
5	0	50	0
6	0	100	0
7	0	200	0
8	0	0	50
9	0	0	100
10	0	0	200
11	25	25	0
12	25	0	25
13	0	25	25
14	50	50	0
15	50	0	50
16	0	50	50
17	100	100	0
18	100	0	100
19	0	100	100

[a] Sodium cholate. [b] Taurocholic acid. [c] Taurochenodeoxycholic acid.

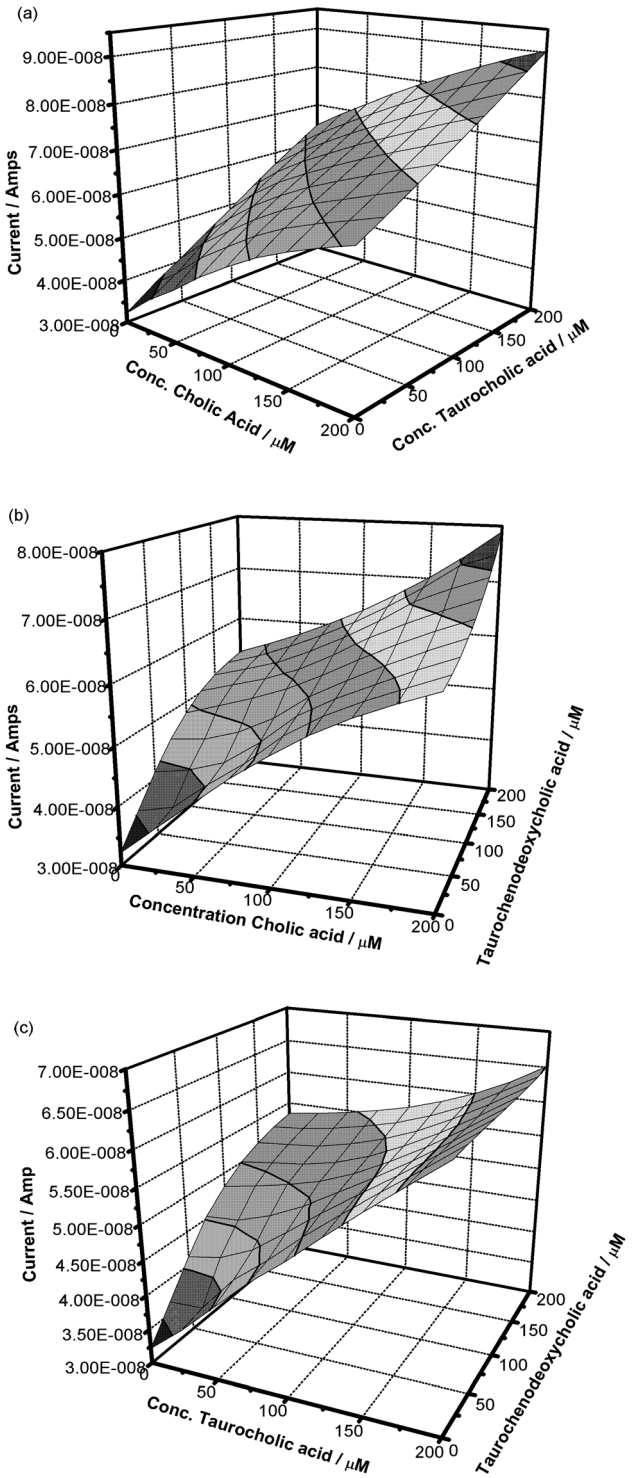

Fig. 7 Sensor response for mixed acid system at 37 °C at 600 seconds (a) cholic and taurocholic acid, (b) cholic and taurochenodeoxycholic acid, (c) taurocholic and taurochenodeoxycholic acid.

occurred at the upper end of the desired testing range, well above the concentration of a normal healthy individual.

3.3.2 Detection of the three mixed bile acids in calf serum. The detection of a combination of the three acids was studied in calf serum. Calf serum, as the testing media, was used to approximate

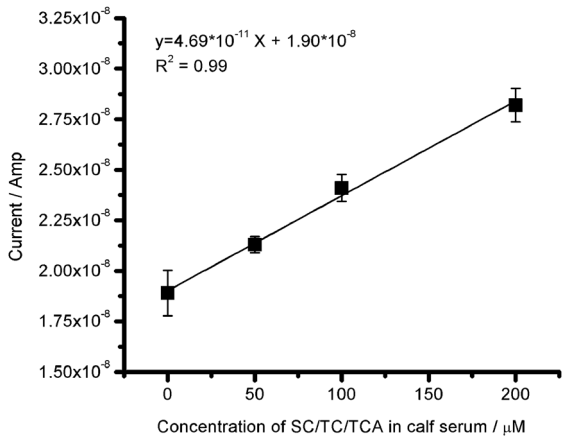

Fig. 8 Sensor response of three-acid system in calf bovine serum at 25 °C.

the physiological solution found in humans. By achieving good results in this media we would have reasonable confidence about the performance of the sensor using human samples. The bile acid concentrations were studied at 0, 50, 100 and 200 μmol L^{-1} of total acid in calf serum at 25 °C. Each acid consisted of one third of the total concentration. Calf bovine serum, and similarly human serum, has a pH of 7.0 to 7.5. It was previously established that higher pH values would be necessary to achieve the complete reaction. Thus, 5 μL of the serum sample containing the acids were mixed with 1 μL of pH 10.97 phosphate buffer. The final pH of the mixed solution was 8.57. The 6 μL sample was pipetted onto the sensor and a potential of +0.27 V *versus* Ag/AgCl was applied. Fig. 8 shows the linear response obtained between the sensor current outputs and the concentrations of the mixed acids. The linear behavior, similar to that observed for phosphate buffer, confirmed that the sensor could be used to accurately detect, within the desired acid concentration range, the multiple acids in calf serum. These results gave high confidence that the sensor would be capable of detecting total bile acids in human samples.

4. Conclusions

In this study, a sensor prototype for the detection of total bile acid was constructed from an Ir/C thick-film screen-printed working electrode, and immobilized 3α-HSD. Detection of the bile acids: sodium cholate, taurocholic acid, and taurochenodeoxycholic acid was based on the amperometric determination of enzymatically formed NADH. Variables including temperature, pH and single and multiple acids in the test medium were explored and experimentally examined. Both phosphate buffer solution and bovine calf serum were used as the test media in this study. It was found that an increased temperature aided in the acid detection, and that the pH of the system played an important role. The sensor had equally good performance when detecting a single acid or a mixture of acids. Detection of sodium cholate consistently showed the most linear response through all variables. Testing the sensor in phosphate buffer and bovine calf sensor showed that the sensor was suitable for both testing conditions. This indicated the sensor's usefulness for a quick

diagnostic testing of human samples. Thus, this sensor prototype was fabricated and tested as a single use, disposable device. It is believed that the application of this sensor to the identification of liver disease worldwide would greatly aid in the treatment and reduction of an often fatal condition.

Acknowledgements

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References

1 B. D. Lindenbach, M. J. Evans, A. J. Syder, B. Wolk, T. L. Tellinghuisen, C. C. Liu, T. Maruyama, R. O. Hynes, D. R. Burton, J. A. McKeating and C. M. Rice, *Science*, 2005, **309**, 623–626.
2 WHO, *Weekly Epidemiological Record*, 2002, **77**, 41–48.
3 P. S. Monroe, A. L. Baker, J. F. Schneider, P. S. Krager, P. D. Klein and D. Schoeller, *Hepatology*, 1982, **2**, 317–322.
4 T. Shima, T. Shima, H. Tada, M. Morimoto, Y. Nakagawa, H. Obata, T. Sasaki, H. Park, S. Nakajo, T. Nakashima, T. Okanoue and K. Kashima, *J. Gastroenterol. Hepatol.*, 2000, **15**, 294–299.
5 G. A. Mannes, C. Thieme, F. Stellaard, T. Wang, T. Sauerbruch and G. Paumgartner, *Hepatology*, 1986, **6**, 50–53.
6 S. Fischer, U. Beuers, U. Spengler, F. M. Zwiebel and H.-G. Koebe, *Clin. Chim. Acta*, 1996, **251**, 173–186.
7 D. Festi, A. M. Morselli Labate, A. Roda, F. Bazzoli, R. Frabboni, P. Rucci, F. Taroni, R. Aldini, E. Roda and L. Barbara, *Hepatology*, 1983, **3**, 707–713.
8 F. Jorquera, M. J. Monte, J. Guerra, S. Sanchez-Campos, J. A. Merayo, J. L. Olcóz, J. González-Gallego and J. J. G. Marin, *J. Gastroenterol. Hepatol.*, 2005, **20**, 547–554.
9 K. Rani, P. Garg and C. S. Pundir, *Anal. Biochem.*, 2004, **332**, 32–37.
10 O. Fausa and B. A. Skalhegg, *Scand. J. Gastroenterol.*, 1974, **9**, 249–254.
11 S. Kamada, M. Maeda, A. Tsuji, Y. Umezawa and T. Kurahashi, *J. Chromatogr.*, 1982, **239**, 773–783.
12 S. Perwaiz, B. Tuchweber, D. Mignault, T. Gilat and I. M. Yousef, *J. Lipid Res.*, 2001, **42**, 114–119.
13 J. Schoelmerich, G. P. van Berge Henegouwen, A. F. Hofmann and M. DeLuca, *Clin. Chim. Acta*, 1984, **137**, 21–32.
14 Diazyme, *Total Bile Acids Test and Clincal Diagnosis*, Diazyme Laboratories, Poway, CA, USA.
15 F. Mashige, T. Osuga, N. Tanaka, K. Imai and M. Yamanaka, *Clin. Chem.*, 1978, **24**, 1150–1154.
16 W. J. Albery, R. B. Lennox, E. Magner, G. Rao, D. Armstrong, R. Hermon Dowling and G. M. Murphy, *Anal. Chim. Acta*, 1993, **281**, 655–661.
17 S. Koide, N. Ito and I. Karube, *Biosens. Bioelectron.*, 2007, **22**, 2079–2085.
18 L. Fang, S.-H. Wang and C.-C. Liu, *Sens. Actuators, B*, 2008, **129**, 818–825.
19 P. Talalay, *Methods Biochem. Anal.*, 1960, **8**, 119–143.
20 L. A. Turnberg and A. Anthony-Mote, *Clin. Chim. Acta*, 1969, **24**, 253–259.