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Real-time *in vivo* uric acid biosensor system for biophysical monitoring of birds

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Research on birds has long played an important role in ecological investigations, as birds are relatively easily observed, and their high metabolic rates and diurnal habits make them quite evidently responsive to changes in their environments. A mechanistic understanding of such avian responses requires a better understanding of how variation in physiological state conditions avian behavior and integrates the effects of recent environmental changes. There is a great need for sensor systems that will allow free-flying birds to interact with their environment and make unconstrained decisions about their spatial location at the same time that their physiological state is being monitored in real time. We have developed a miniature needle-based enzymatic sensor system suitable for continuous real-time amperometric monitoring of uric acid levels in unconstrained live birds. The sensor system was constructed with Pt/Ir wire and Ag/AgCl paste. Uricase enzyme was immobilized on a 0.7 mm sensing cavity of Nafion/cellulose inner membrane to minimize the influences of background interferents. The sensor response was linear from 0.05 to 0.6 mM uric acid, which spans the normal physiological range for most avian species. We developed a two-electrode potentiostat system that drives the biosensor, reads the output current, and wirelessly transmits the data. In addition to extensive characterization of the sensor and system, we also demonstrate autonomous operation of the system by collecting *in vivo* extracellular uric acid measurements on a domestic chicken. The results confirm our needle-type sensor system's potential for real-time monitoring of birds' physiological state. Successful application of the sensor in migratory birds could open up a new era of studying both the physiological preparation for migration and the consequences of sustained avian flight.

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Introduction

Research on birds plays a large role in investigations related to ecology, evolution and behavior. Birds are diurnal and visual, and they are readily observable and trackable for a great variety of research in the field. Their high metabolic rates make them highly responsive to their environments, and they therefore tend to be good indicators of environmental change.[1] The current state-of-the-art for avian physiological monitoring in the field is to take blood samples upon capture, yielding a "snap shot" of information on the covariation of metabolites and hormones with the phases of migration and breeding in wild birds.[2–6] The metabolism of individual birds over the course of their annual migrations or for their once in-a-lifetime dispersal event is still poorly understood because of the difficulties of studying birds that travel tens to thousands of kilometers in their movements.[3] The ability to track and study migratory birds has increased in recent years with the development of new generations of radio-tags and data-loggers capable of increasingly precise monitoring of bird's dynamic locations.[7–9] A system that can combine this positional tracking data with a real-time dynamic measurement of the physiological state of the birds could open up an entirely new way of studying avian movement biology and behavior. For example, being able to track physiological changes and location in a single moving bird could yield priceless information on its internal state and location-associated environmental conditions to enable an unprecedented understanding of the movement-decision-making of individual birds.

Metabolite sensing and quantification in capillary blood is a well-established technology. The vast majority of existing technologies are based around an enzymatic reduction of the target metabolite and detection of the products such as glucose,[10–12] cholesterol,[13] or lactic acid.[14–16] In general, the importance of these measurements to human health monitoring (in particular glucose monitoring for type 1 diabetes) has led to the development of a large diversity of robust, commercially available devices.[17] Despite the wealth of data on blood chemistry, placing sensors in blood vessels presents difficult challenges for sensor

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placement and attachment as well as for the life-time of the sensor, which tends to get fouled by defensive cells in the blood. A major recent advancement that has enabled the deployment of real-time continuous versions of these systems has the ability to accurately determine glucose levels from measurements made in the interstitial space between capillaries, rather than sampling the blood directly.[18,19] This has led to the development of implantable biochips, which enable continuous monitoring of glucose and lactate levels to influence patient outcomes following trauma-induced hemorrhage.[14] Implantable electrochemical sensors have also been used for *in vivo* biosensing applications in animals such as glucose monitoring in fish[11] and continuous alcohol monitoring in a Wistar rat.[20]

Because birds' energy pathways are dominated by lipid rather than carbohydrate metabolism, variation in somatic glucose levels are not as informative as they are in mammalian systems. Glucose levels do not change, for example, over the full range of physiological states shown by arriving and refueling godwits on a migratory stop-over in the Wadden Sea.[21] By contrast, uric acid is a good indicator of protein catabolism in birds since its presence in the body is a consequence of the breakdown of protein, either from recently ingested proteinaceous food or from catabolism of body protein.[22] There are various approaches to be able to detect uric acid levels: chemiluminescence,[23] spectrophotometry,[24] fluorescence[25] and electrochemistry.[26] In general, electrochemical methods for detecting uric acid can be classified as either non-enzymatic or enzymatic methods. Enzyme-based electrochemical methods are preferred due to their high selectivity and sensitivity.[27,28] In most of these methods, determination of uric acid is performed by oxidation of enzymatically generated H_2O_2 at the sensing electrode.[29] Uricase enzymes are immobilized onto various modified electrodes such as polyelectrode multilayer films,[30] iridium-modified carbon electrode,[31] polypyrrole and polyaniline film,[32,33] eggshell membrane,[34] polyaniline-multiwalled carbon nanotube composite film,[35] zinc oxide nanowires[27] and o nano-particle/multiwalled carbon nanotube layer deposited on gold electrode.[36] Although these methods have focused on parameters such as simplicity, sensitivity, low detection limit and low cost, none of them has been able to implement uric acid sensing for *in vivo* applications.

Here for the first time, we have developed a miniature needle-type enzyme sensor system suitable for real-time amperometric monitoring of uric acid in birds. The biosensor uses uricase enzyme to catalyze the uric acid reaction, and reduces the produced hydrogen peroxide at the sensing cavity, where it is detected amperometrically with good sensitivity and stability characteristics. We have also designed and integrated our sensor with a two-electrode wireless potentiostat system. Finally, we demonstrate the *in vivo* monitoring of uric acid in a domestic chicken.

Experimental

Chemicals and reagents

Uricase (from *Candida* sp.; E.C. 1.7.3.3), bovine serum albumin (BSA, lyophilized powder, RIA grade, ≥96%), Nafion (~5% in a mixture of lower aliphatic alcohols and water), cellulose acetate (CA, 39.8% acetyl content), 0.1 M pH 7.4 phosphate buffered saline (PBS) were purchased from Sigma Aldrich (St. Louis, MO). Uric acid (UA) stock solution was prepared freshly by dissolving UA in 1 M NaOH, then diluted using pH 7.4 PBS and the pH level of the prepared solution adjusted to pH 7.4 by HCl. A 5% CA solution was prepared by dissolving CA in 2 : 1 acetone–ethanol. Uric acid (≥99%, crystalline), L-ascorbic acid, glucose, polyurethane and glutaraldehyde (grade II, %25 aqueous solution) were also obtained from Sigma-Aldrich (St. Louis, MO). All other reagents used for the experiments were analytical grade.

Structure of uric acid sensor

The uric acid sensor, shown in Fig. 1, was designed similar to previous needle type *in vivo* glucose sensors.[10–12] The sensor was made using Teflon coated platinum–iridium (Pt/Ir; 10%) wire (0.125 mm o.d.; Advent Research Materials Ltd, Oxford, England). It was stripped at one end to create a sensing cavity of 1 mm. The tip of the wire was sealed with epoxy resin. Silver wire (0.1 mm o.d., from Sigma Aldrich, St. Louis, MO) was wrapped around the Teflon coated surface. Ag/AgCl paste (CH Instruments Inc, USA) was applied onto the sensor body to create a reference/counter electrode. Following that, the sensing cavity was degreased with acetone, isopropyl alcohol, and deionized water to strip any coating residues before inner membrane deposition.

To prevent changes in other species (*e.g.*, ascorbic acid and glucose) from influencing the sensor output, the Pt/Ir electrode

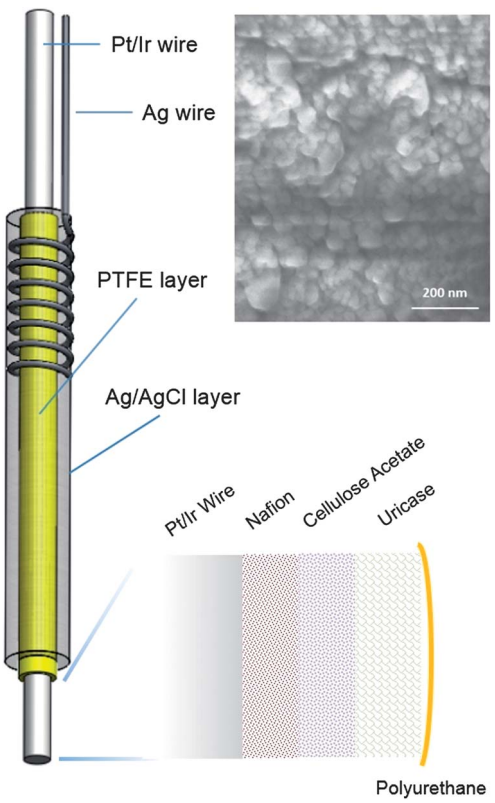

Fig. 1 View of needle type *in vivo* enzymatic uric acid biosensor, inset shows the SEM image of the sensing cavity of the biosensor containing immobilized uricase enzymes.

was coated with an inner membrane composed of Nafion and CA. Twelve alternating coatings of Nafion and CA were found to be sufficient to eliminate the interfering species effectively. Uricase enzyme was immobilized on the coated electrode and crosslinked with glutaraldehyde to protect the enzyme layer from heat degradation, proteolytic enzymes and hydrolysis.[37] The sensing cavity was first coated with two thin layers of Nafion by dipping it into a well containing 5% Nafion and air-drying for 10 min between each coating. After Nafion coating, the sensor was coated with two thin layers of CA by dipping it into the 5% CA solution in 2 : 1 acetone–ethanol for 5 s followed by drying at room temperature for 10 min between coat. This Nafion and CA deposition cycle was repeated 2 additional times. Uricase enzyme was immobilized on the working electrode by absorption on inner membranes. 0.7 μL of freshly prepared enzyme solution containing 1% uricase, 0.6% bovine serum albumin and 0.4% glutaraldehyde was transferred to the sensing cavity while the sensor was held in a horizontal position. The sensor was then allowed to dry for 1 h. A polyurethane (PU) outer layer was deposited around the entire sensor to prevent degradation of the sensor and to make the output current independent of external mass transfer by serving as a rate-limiting barrier for diffusion.[15] Using the wire loop technique, 5% polyurethane (PU) solution was prepared in 98% tetrahydrofuran (THF)-2% dimethylformamide (DMF). Three turns of wire loop (3 mm inner diameter) were constructed from copper wire (0.5 mm outer diameter) and the inside of the loop was filled with 17 μL PU solution. The whole sensor passed through this loop horizontally which enabled uniform deposition of the outer layer. The sensor was then cured in air for 12 hours and then stored in 0.01 M phosphate buffered saline (pH 7.4) for 7 days to permit outer layer conditioning.

In vitro sensor characterization

In vitro evaluation of the sensor characteristics was carried out in a 1.5 mL cell at room temperature (25 °C). All *in vitro* measurements were conducted in 0.1 M PBS (pH 7.4) and dissolved oxygen was saturated with stirring. A potential of +650 mV was applied between the working and the reference/counter electrodes by using a Keithley 2400 SourceMeter (Keithley Instruments Inc., OH, USA) for amperometric detection. The background output current was stabilized for 30 minutes before first measurement. The resulting current data were collected by a digital acquisition card installed in a PC controlled with LabView software (National Instruments Corp., Austin, TX). To carry out the calibration of the sensor, increasing amounts of uric acid (or interferent) solutions were injected into the cell with a pipettor every 3 minutes, which was enough time to get steady-state response. The sensors were stored in 0.01 M phosphate buffer, pH 7.4, at 4 °C when not in use.

Two-electrode potentiostat system for *in vivo* measurements

By combining a low power microcontroller (MCU) MSP430F2274 (Texas Instruments, TX), amplifiers (Maxim Integrated, CA), and filter blocks, we formed a two-electrode potentiostat system that drives the biosensor at 650 mV.[38] It

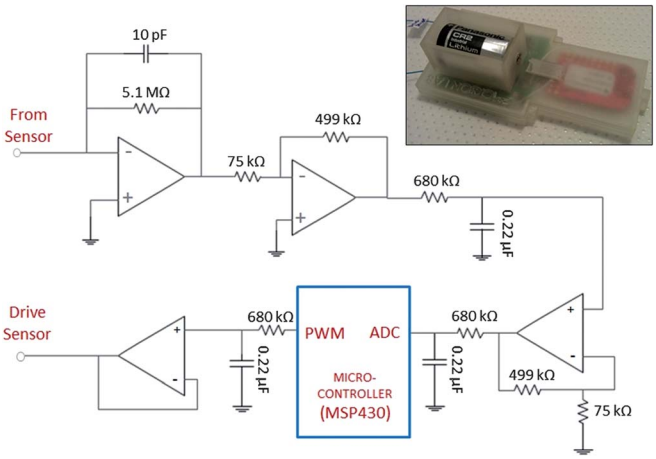

Fig. 2 Two electrode potentiostat system used to drive the uric acid biosensor at 650 mV and read the output current (1–20 nA) in real time.

reads the ultra-low output current of the sensor (1 to 20 nA) and transmits the corresponding voltage data to the base microcontroller connected to a remote computer (Fig. 2). We chose low power, low noise MAX407 op-amps (Maxim Integrated, CA) to build amplifiers and filter blocks. Since MAX407 op-amp has a very high input impedance and low input bias current, it was suitable for a two-electrode potentiostat system application. We build the circuit with a minimum number of components to keep the circuit board area small. A Pulse amplitude modulated (PWM) signal from the MCU was converted to DC voltage by low-pass filtering for driving the sensor after buffering. Since the sensor output current is very small, we amplified and converted it to voltage using a transimpedance amplifier, so that it could be measured by the microcontroller's analog to digital converter (ADC). Data was transferred to a base station using an integrated CC2500 2.4 GHz wireless transceiver and recorded to a computer for further analysis.

In vivo experiments for monitoring interstitial uric acid levels in a domestic chicken

To evaluate the *in vivo* performance of the sensor, experiments were done on an adult chicken. Feathers in the dorsal feather tract, anterior of the uropygial gland, were clipped at their bases and the skin disinfected using Betadine antiseptic solution (Purdue Pharma L.P., NJ). A 20-gauge catheter consisting of an outer polyurethane layer and inner puncture needle (Terumo Medical Corporation, NJ) was inserted slightly over 1 cm immediately beneath the skin without anesthesia. After removing the inner puncture needle, the excess catheter was cut just above the skin. The uric acid sensor was then inserted into the flexible polyurethane outer layer and secured with sterile Tegaderm® film (3M Corporate, MN). Then the wireless two-electrode potentiostat tag was attached directly over the inserted sensor and fastened to the back with a Rappole harness[39] made of dental floss and tightened around the bird's femurs through holes at the sides of the tag case.

Prior to the experiment, the chicken was fasted for 6 hours, lowering the baseline uric acid levels. A blood sample was taken

from the chicken before installing the biosensor tag. After installing the tag, we waited 10 minutes for sensor current to stabilize. Then the bird was allowed to eat in order to increase its uric acid levels. The system collected data for a total of one hour. Before removing the sensor tag, a second blood sample was taken. We determined the uric acid levels of two blood samples taken before and after feeding the bird by using a uric acid kit reagent set (Teco Diagnostics, CA) and spectrophotometer (Molecular Devices LLC, CA). We compared the biosensor output to the uric acid level data from the uric acid kit. We then used a two-point calibration method for uric acid level estimation.[18] For the change in uric acid levels (G) from G_1 to G_2 (in mM), and the corresponding sensor output currents (I_1 and I_2 in nA), sensor sensitivity S will be $(I_2 - I_1)/(G_2 - G_1)$ and the theoretical sensor output I_0 (which would be observed in absence of uric acid) will be $I_1 - (S(G_1))$.

Results and discussion

In vitro sensor characterization

The needle-type UA biosensor is shown in Fig. 1. Uricase catalyzes the oxidation of uric acid to allantoin in the presence of oxygen, producing CO_2 and H_2O_2 simultaneously. A 650 mV potential is applied to the Pt/Ir working electrode with respect to the Ag/AgCl reference electrode. Hydrogen peroxide is reduced on the working electrode surface when potential is applied. This generates a current which is then measured amperometrically. Characterization of the sensor uses 0.1 M phosphate buffer solutions doped with various concentrations of uric acid in the range of expected physiological state.

$$\text{Uric acid} + O_2 \xrightarrow{\text{uricase}} \text{allantoin} + CO_2 + H_2O_2 \quad (1)$$

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (2)$$

The sensor has a fast response time, and output current reaches steady state levels in less than 60 seconds. This is an important property for real-time *in vivo* measurements. The sensor has good sensitivity for UA and the sensor output is linear from 0.05 mM to 0.6 mM UA (Fig. 3). UA levels in avian blood vary from around 0.1 mM to 0.65 mM,[40] and our biosensor thus has the ability to detect the complete range of normal uric acid levels in birds. The polyurethane membrane served as a rate-limiting barrier for diffusion of uric acid to the enzyme layer and helped to get a linear dynamic output current range independent of external mass transfer.[15]

Electrochemical interference is one of the major problems in biological determination of uric acid since it has a similar oxidation potential to hydrogen peroxide. To prevent the effects of potentially interfering species, the Pt/Ir electrode was coated with inner membranes of alternating Nafion and cellulose acetate (CA). These polymers are negatively charged and inhibit the diffusion of anionic species.[10,41] In order to test the selectivity achieved, the performance of the UA sensor was tested against interference from ascorbic acid and glucose. As shown in Fig. 4, addition of 0.1 mM ascorbic acid and 5 mM glucose did not result in detectable changes in sensor response. We have also

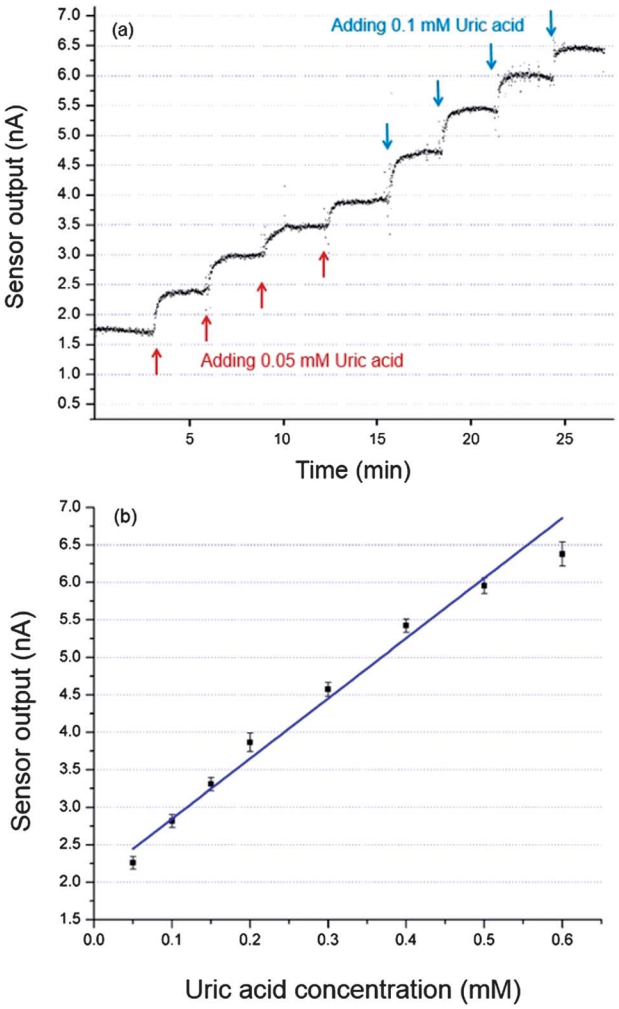

Fig. 3 (a) *In vitro* calibration of the uric acid biosensor by adding various concentrations of uric acid over the expected physiological range. (b) Linear fit of the *in vitro* uric acid calibration data. Error bars are the standard deviation of the sensor output after stabilization.

tested our biosensor against 0.05 mM L-cysteine and 0.2 mM fructose, which also didn't cause any changes in the sensor response. We observed slight decreases in current output from the dilution of the uric acid solution with ascorbic acid and glucose standard solutions, but the sensor still was able to detect UA reliably. Clearly, the uricase enzyme retains its activity and a good turn-over time after immobilization, so it has the ability to deliver fast response to small changes in UA concentrations. This results show that our biosensor system gives a sensitive response to uric acid by rejecting the interferences.

We also tested the UA biosensor at various values of pH and temperature to evaluate their effects on the activity of the uricase enzyme. The pH-dependence of our sensor response was investigated in 0.2 mM uric acid solutions over a pH range of 5.8 to 8.3. The experimental results indicate that the sensor's output was dependent on pH of the solution and got the best response between pH 6.0 and 7.5 (Fig. 5a). Despite the fact that plasma pH does not change that much, our biosensor has the ability to keep its activity against pH decrements and increments. Since the pH values of a bird's blood and interstitial

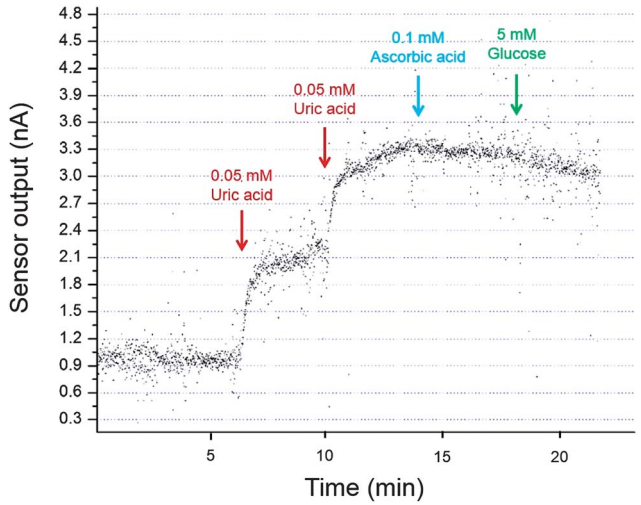

Fig. 4 Interference effect on the response of uric acid biosensor, addition of 0.1 mM ascorbic acid and 5 mM glucose did not result in detectable changes in the sensor response.

fluids are around 7.2 to 7.5, we can conclude that our sensor is likely to have good performance during *in vivo* measurements.[42] The sensor's response in 0.2 mM uric acid solution was also tested between 22 °C and 45 °C. Since higher temperatures increase the activity of the enzyme and mass transport in surrounding solutions, higher sensor responses were generally obtained at higher temperatures (Fig. 5b). Temperature changes in bird's body will not cause notable changes in the enzyme activity. Even though temperature rises to high values, it will still be able to response effectively. Since the body temperatures of birds are generally around 40 °C,[43] these results once again promise good prospects for *in vivo* applications of this UA biosensor.

To test the sensor stability in storage, we measured sensor responses to 0.1 mM UA over a period of 3 weeks. Fully conditioned sensors were kept in 0.01 mM PBS (pH 7.4) at 4 °C while not in use. The UA sensor was able to keep 75% of its activity after three weeks of storage, and the loss of activity was gradual over this period, with no indication of any threshold decays in performance (Fig. 6). After preparing the sensor, we can store the biosensor for 3 weeks. The reason for this response decrease could be deactivation of the uricase (UOX) enzyme or releasing of the UOX from the sensing layer over time.

To test operational stability, the sensor was stored in a 0.1 M PBS solution at 37 °C, which contained 0.1 mM UA without any applied electrical potential. After a week of storage, not significant change was observed in the sensor response. This indicates that this UA sensor could be used for continuous measurements of uric acid over a period of several days.

In vivo measurements of extracellular uric acid levels in a domestic chicken

Although technological advances have been made in recent years for increasingly precise methods to measure uric acid levels in clinical and biological samples,[26,36,44] this is apparently the first development of an *in vivo* UA biosensor. Miniature

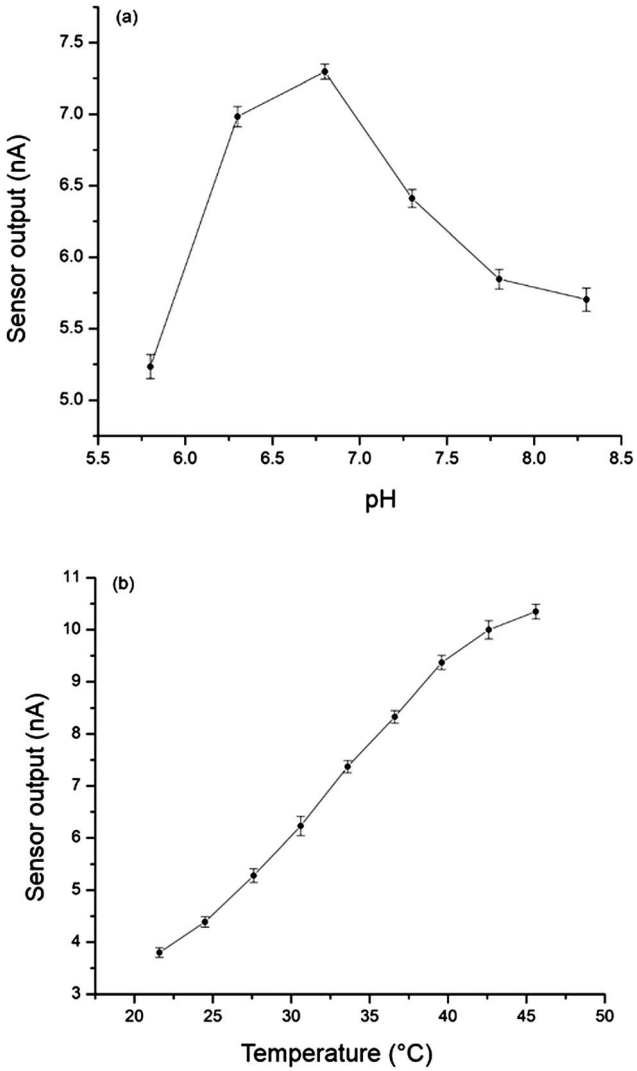

Fig. 5 (a) Effect of pH on the response of the uric acid biosensor in presence of 0.2 mM uric acid at 25 °C. (b) Effect of temperature on the response of uric acid biosensor in presence of 0.2 mM uric acid in 0.1 M PBS at pH 7. Error bar = ±S.D. and $n = 3$.

needle-based sensors, with simple and robust fabrication procedures,[10,12] are good candidates for *in vivo* sensing applications, and their structure makes them suitable for subcutaneous monitoring.[18] The needle-type *in vivo* uric acid biosensor system developed here is really important to fulfill the need to understand the avian physiological states in real-time.

The sensing approach is based on real-time measurements in the interstitial fluid rather than a blood directly which helped the probe to be more free to move in response to movement of the bird without the worry of bleeding. The chicken fasted for 6 hours prior to the experiment to be able to decrease the uric acid levels.[21] After inserting the biosensor system under the skin, sensor current stabilized within 10 minutes and the bird was then allowed to eat. No behavioral changes was observed in bird. The blood UA level was 0.1535 mM at the beginning of the experiment, and it reached 0.2511 mM (Fig. 7) at the end of the experiment. Not only the timing and size of the climb in uric

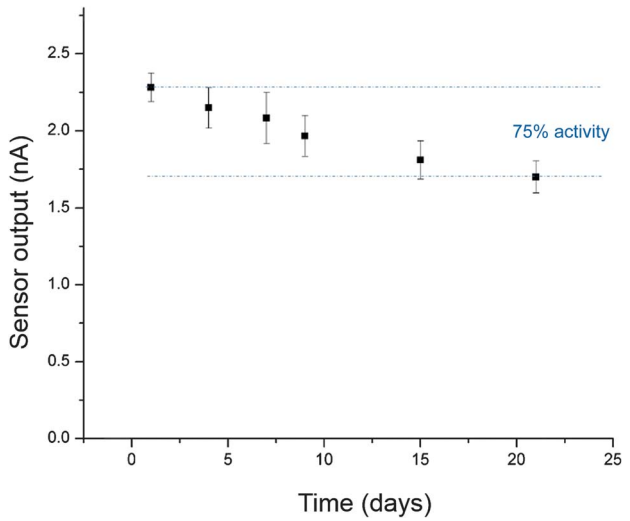

Fig. 6 Uric acid sensor storage stability. Sensor responses were measured for 0.1 mM uric acid. The sensor was kept in 0.01 mM PBS (pH 7.4) at 4 °C while not in use. Error bar = ±S.D. and $n = 3$.

acid biosensor output, but also having an inner membrane which prevents the effects of potentially interfering species, indicates that this climb was a consequence of feeding after fasting which effects the UA levels since it is the end product of protein catabolism in birds.[22] Our biosensor system was able to sense this UA level change in real-time successfully. The rapidly increasing uric acid levels demonstrate both that the sensor is sensitive and rapid-sensing and that uric acid levels in the interstitial fluid track those in blood closely. Through the experiment, it has been shown that the needle type UA biosensor developed here can be used for *in vivo* measurement of interstitial uric acid levels in birds. This is the first real time measurement of a blood chemical of a bird, which gives us an information about its physiological state. This study has a potential to open an innovative way to study of the physiological state of the avians.

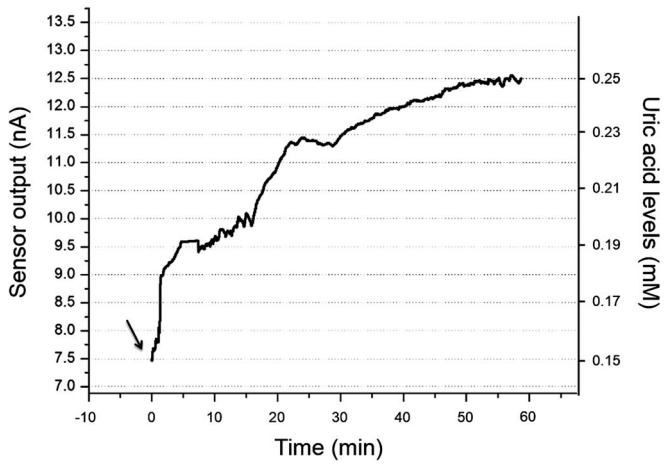

Fig. 7 *In vivo* uric acid sensing. Sensor was placed subcutaneously under the skin and successfully detected uric acid level changes in domestic chicken. The arrow indicates where the bird was given access to feed.

Conclusion

In this work, we have developed a biosensor system that can continuously monitor *in vivo* subcutaneous uric acid levels of birds in real-time. The uricase-based amperometric needle type biosensor developed here exhibits good performance in detecting uric acid at physiological levels with fast response time, good sensitivity, long-term stability and good resistivity against interfering chemical species. The results confirm a promising application of biosensors for *in vivo* monitoring purposes of uric acid in birds. The impact of the study goes beyond the state-of-the-art uric acid sensors giving uric acid measurements in real-time. The system developed here could open up a new way of studying avian behavior which would lead to a better understanding of avian biology by enabling the integration of precise positional tracking data with a real-time dynamic measurement of the physiological state of the birds.

Acknowledgements

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