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Non-invasive mouthguard biosensor for continuous salivary monitoring of metabolites

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The present work describes the first example of a wearable saliva metabolite biosensor based on the integration of a printable enzyme electrode integrated on a mouthguard. The new mouthguard enzymatic biosensor, based on an immobilized lactate oxidase and a low potential detection of the peroxide product, exhibits high sensitivity, selectivity and stability using whole human saliva samples. Such non-invasive mouthguard metabolite biosensors could tender useful real-time information regarding a wearer's health, performance and stress level, and thus hold considerable promise for diverse biomedical and fitness applications.

Introduction

Wearable sensors have recently received considerable interest owing to their promise for real-time monitoring of the wearer's health and fitness in a wide range of biomedical, sport and military scenarios.[1-3] Until recently, most of the activity on wearable sensors has focused on monitoring vital signs from physical signals such as electrocardiography and pulse oximetry, while wearable chemical sensors have received limited attention.[2,4] Yet, non-invasive wearable chemical sensors can yield useful insights into the overall health status and performance of individuals beyond physical parameters alone.[2,4,5,6]

Recent efforts have led to wearable biosensors for detecting chemical biomarkers in human fluids that can be obtained non-invasively, e.g., tears, sweat or saliva.[5,7,8,9] Of these fluids, saliva has been considered extremely attractive for such non-invasive monitoring, in part due to its continuous and convenient availability. Additionally, saliva has good correlation with blood concentrations of numerous analytes.[10,11] Such correlation reflects the permeation of multiple constituents from blood to saliva via transcellular or paracellular paths. Sialochemistry has thus been recognized as a useful non-invasive alternative to blood analysis for monitoring the hormonal, stress and metabolic states of individuals.[10-14] Prior work on wearable in-dwelling salivary sensors have focused primarily on the non-invasive potentiometric monitoring of electrolytes such as fluoride,[15] pH,[16] or sodium.[17] A denture based sensor for monitoring pH and temperature in the oral cavity has also been described.[18] Recently, Mannoor et al.[19] demonstrated a dental tattoo for continuous wireless monitoring of bacteriain saliva. In contrast, there are no reports on non-invasive wearable biosensors for monitoring salivary metabolites despite the established high correlation between the level of salivary metabolites and their corresponding blood concentration.[11-13]

Herein we present the first example of a non-invasive mouthguard biosensor for continuous monitoring of salivary metabolites. To demonstrate the new in-mouth biosensor concept, we integrated a printable amperometric enzymatic biosensor onto an easily removable mouthguard platform toward non-invasive monitoring of lactate. Mouthguards are widely used by athletes in competitive and recreational sports as they offer considerable protection against sports-related dental injuries.[20] Such polymeric devices fit firmly and snuggly over the teeth, and represent an attractive platform with sufficient volume for mounting miniaturized sensors, control/acquisition electronics and wireless transmitters. Unlike the earlier reported permanent in-dwelling saliva sensors, the mouthguard sensor can be easily worn and replaced without any specialized assistance. Importantly, since the device will be always in direct contact with saliva, physiological information can be measured in real-time without interruption, thereby opening a new avenue for continuous assessment of dynamic metabolites changes.

The new concept of mouthgaurd metabolite biosensor is demonstrated here using amperometric monitoring of lactate. Salivary lactate concentrations correspond well with blood lactate levels and have been used *in vitro* for monitoring fitness levels.[8,11,13,14,21,22] Saliva may therefore be suited as a fluid for continuous non-invasive monitoring of lactate levels during sport activities. The presented wearable oral biosensory system is based on a printable Prussian-Blue (PB) transducer and a poly-orthophenylenediamine (PPD)/lactate-oxidase (LOx) reagent layer. Prussian-blue acting as "artificial peroxidase", offers a highly selective detection of the hydrogen peroxide product of oxidase biocatalytic reactions.[23-25] PB has been widely used for oral treatment of poisoning by the heavy metals thallium and cesium, and its use appears to be very safe under

physiological conditions even following high oral doses.[26-28] Poly-orthophenylenediamine (PPD) is commonly employed for the electropolymeric entrapment of oxidases, rejection of potential interferences and protection of the biosensor surface.[29-31] Such coupling of the extremely low-potential detection of the peroxide product afforded by the PB transducer and the exclusion of electroactive constituents of whole saliva leads to high selectivity and stability. In the following sections we will describe the design and *in-vitro* characterization of the new mouthguard-based biosensor toward continuous in-mouth monitoring of lactate.

Materials and methods

Chemicals and reagents

L-Lactate oxidase (LOx) (activity: 101 U/mg) was purchased from Toyobo Crop. (Osaka, Japan). 1,2-phenylenediamine (o-Pd), L-lactic acid, L-ascorbic acid (AA), uric acid (UA), sodium sulfate, potassium phosphate monobasic, potassium phosphate dibasic, and sodium chloride were obtained from Sigma Aldrich (St. Louis, MO) and were used without further purification or modification. Ultrapure water (18.2 MΩ•cm) was employed in all of experiments.

Instrumentation

A CH Instruments (Austin, TX) model 440 analyzer was employed for the electrochemical measurements. Chronoamperometric studies were carried out to evaluate the response of mouthguard sensors; the applied potentials in all experiments were versus the printed screen-printed pseudo Ag/AgCl reference electrode at room temperature (22°C). A MPM SPM semi-automatic screen printer (Speedline Technologies, Franklin, MA) was used for printing electrodes. The sensor patterns were designed using AutoCAD (Autodesk, San Rafael, CA) and stencils were patterned on 75 µm-thick stainless steel stencils (Metal Etch Services, San Marcos, CA).

Fabrication and integration of mouthguard biosensor

Mouthguard biosensors were fabricated by screen-printing three separate layers on a flexible PET substrate. An Ag/AgCl conductive ink (124-36, medical grade, Creative Materials Inc., MA USA) was printed first to provide the reference electrode as well as the contacts for interfacing the three electrodes to the electrochemical analyzer. The second layer, serving as the working and auxiliary electrodes, was printed from a Prussian-Blue-graphite ink (C2070424P2, Gwent Inc, Torfaen, UK). The third (insulator) layer, was printed by using the DuPont 5036 Dielectric paste (Wilmington, DE, USA). After each printing step, the printed layers were cured at 80 ^{O}C for 20 min. Subsequently, the printed electrode system was attached to the PET substrate of the mouthguard body using a double-sided adhesive (**Figure 1A**). The electrochemical analyzer was connected to wires placed through holes inside the mouthguard body that were attached to Ag/AgCl contacts via a silver epoxy. An insulator layer was used for coating the exposed silver-epoxy and Ag/AgCl contacts.

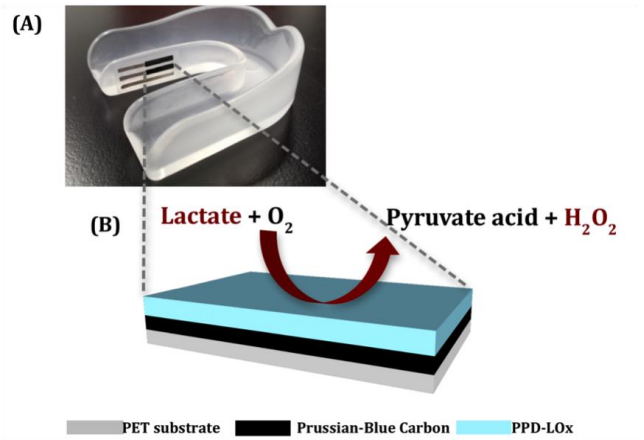

Figure 1. (A) Photograph of the mouthguard biosensor, with the integrated printable 3-electrode system, including the enzyme working electrode. (B) Schematic illustration of the PB working electrode coated with the PPD-LOx layer in the mouthguard biosensor for salivary lactate monitoring.

Working electrode modification (enzyme immobilization)

Lactate oxidase (LOx) was immobilized on the working electrode surface by electropolymeric entrapment in a poly(o-phenylenediamine) (PPD) film. This was accomplished using a 0.1 M phosphate buffer (pH 7.0) solution containing 10 mM o-Pd, 5 mM sodium sulfate, and 800 U/mL LOx, which was purged with nitrogen for 20 minutes. The mouthguard printable transducer was immersed in the polymerization solution; a potential of 0.55 V (*vs* Ag/AgCl) was subsequently applied for 1 min in order to grow the LOx-entrapped PPD film.[29] Following the electropolymerization process, the sensor was rinsed and immersed in a 0.1 M phosphate buffer solution (pH 7.0) for 20 min to remove monomeric residues from the electrode surface as well as any non-bound enzyme. Figure 1B shows the scheme of the modified working- electrode transducer on the mouthguard platform.

Electrochemical characterization in buffer matrix

The electrochemical performance of the mouthguard lactate sensor was evaluated in a 0.1 M phosphate buffer (pH 7.0) solution containing 20 mM NaCl (PBS) (mimicking the Cl concentration in human saliva).[32,33] Chronoamperometric measurements of lactate at the PB-PPD-LOx biosensor were carried out by stepping the potential to 0.042V (vs. Ag/AgCl) for 60s after 2min incubation in the sample solution. The current was sampled after 60sec. The applied potential was chosen based on cyclic voltammetry of the PB-carbon transducer, where the reduction of hydrogen peroxide showed the maximum current (not shown). Stability of the biosensor was examined in 0.5 mM lactate at 10 min intervals over a 2 h operation. The sensor was kept in 0.1 M PBS between such successive measurements.

Human saliva collection and handling

Human saliva samples were collected from healthy volunteers at fasting conditions (at least 8 hrs) using "passive drool method".[34]

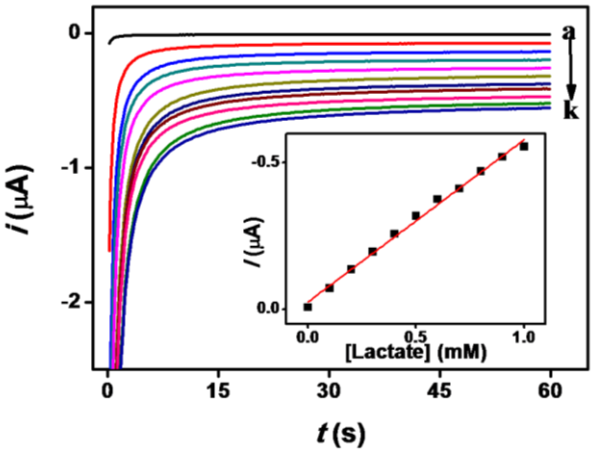

Figure 2. Chronoamperomgrams obtained for increasing lactate concentration in 0.1 mM increments up to 1 mM (a - k) in 0.1 M PBS. All experiments were performed with E_{APP} = 0.042 V (vs Ag/AgCl) and a current sampling time of 60 s. The resulting calibration curve is shown in the inset.

The collected samples were kept at room temperature to allow their sediments to precipitate and the supernatant was used directly (without dilution) for electrochemical measurements. Due to the high viscosity of whole saliva samples, 50µl of undiluted saliva aliquots were mixed with different lactate concentrations and vortexed for one min. The concentration of lactate in the saliva samples was determined via the standard addition method.

Human saliva measurements

Electrochemical measurements of spiked saliva samples were carried out using the same conditions used in buffer matrix (E_{APP} =0.042V for 60 s). Prolonged measurements of such whole saliva samples were performed by changing the sample every 10 min to mimic the replenished in-mouth flow of saliva (unstimulated: 1ml/min, stimulated: 2ml/min).[35] The sensor was kept in saliva between such successive measurements.

Results and discussion

Lactate dynamic range of the mouthguard sensor in buffer media

The lactate concentration of the human saliva varies depending on a person's metabolism and physical performance, with high correlations observed between blood (upto 17.3±1.9mM) and salivary lactate levels (upto 1.6±0.4).[14,21,22]Thus, a wide linear lactate detection range and a fast response time are essential for realizing continuous in-mouth monitoring of lactate in saliva.
To address potential interferences in complex raw saliva samples, the commonly used LOx enzyme has been immobilized onto a printable PB-based transducer by entrapment within a PPD film. The PB-PPD-LOx biosensor, mounted on the mouthguard, was evaluated first in phosphate buffer medium.

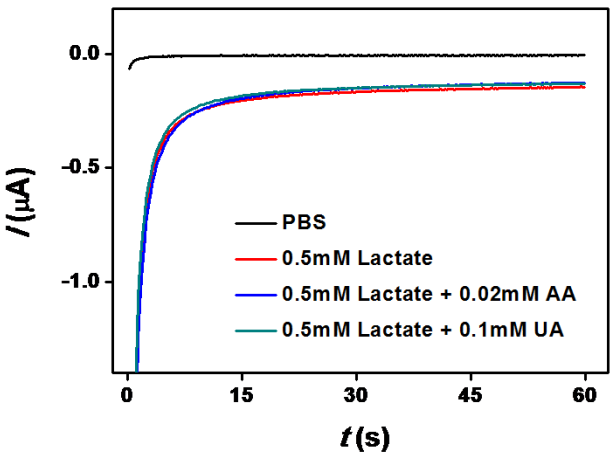

Figure 3. Selectivity of the mouthguard biosensor. Response to 0.5 mM lactate in 0.1 M PBS in the presence of common electroactive physiological interferents. Conditions, as in Figure 2.

The dynamic concentration range was examined in response to increasing levels of lactate over the 0.1-1.0 mM lactate range using a low potential of 0.042V (vs. Ag/AgCl). Figure 2 displays chronoamperograms for increasing concentrations of lactate in 0.1 mM increments (b - k) in PBS medium. These data indicate that the PB-PPD-LOx mouthguard biosensor displays a very high sensitivity toward lactate, with well-defined chronoamperograms and current-signals proportional to the lactate concentration. The resulting calibration plot (shown in the inset) exhibits high linearity (slope, 0.553µA/mM; correlation coefficient, 0.994). Note also the remarkably low background current (a) associated with the extremely low operating potential. A low detection of around 0.050 mM can thus be estimated from the favorable signal-to-noise characteristics of the response for the 0.1 mM lactate (b) (S/N = 3). The PB-PPD-LOx mouthguard sensor could thus detect lactate effectively over the saliva lactate physiological range.[14,21,22]

Selectivity in the presence of physiologically-relevant electroactive compounds

Since the mouthguard biosensor is expected to be exposed to complex raw saliva media, it should offer selective response in the presence of electroactive constituents (e.g., AA and UA) that often interfere with the amperometric detection of lactate. The PB-PPD-LOx transducer-reagent-layer system was designed to minimize potential electroactive interferences by coupling the very low detection potential, offered by the PB surface, with the effective permselective behavior of the PPD layer.[29-31] The selectivity was evaluated in the presence of physiological levels of the relevant electroactive constituents of human saliva, uric acid (100 µM), and ascorbic acid (20 µM).[12,36] Figure 3 displays the chronoamperometric response for 0.5 mM of lactate in the presence and absence of such physiological concentrations of ascorbic acid and uric acid. These data clearly indicate that these potential interferences have a negligible effect upon the lactate response (around 5% for both of AA and UA) and hence that the new mouthguard biosensor system offers high selectivity.

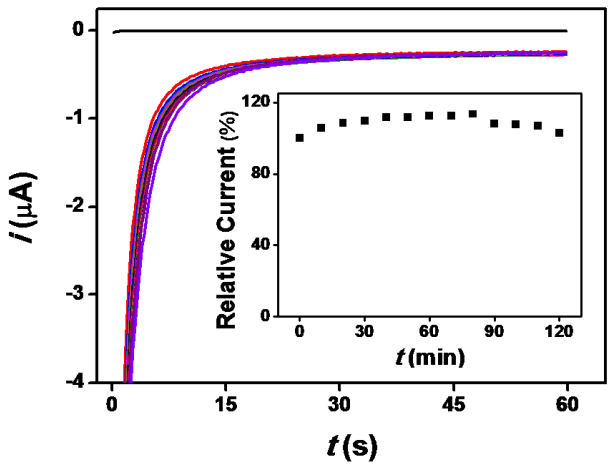

Figure 4. Stability of the electrochemical response of the mouthguard biosensor to 0.5 mM lactate over a 2 h operation. Measurements were carried out at 10 min intervals. The inset shows the relative current, based on original current response (t=0). The sensor kept in 0.1 M PBS between such successive measurements. Other conditions, as in Figure 2.

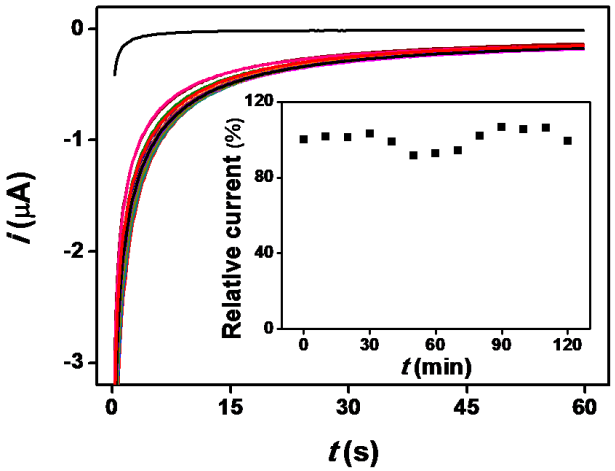

Figure 5. Chronoamperometric response for human saliva sample (a) spiked 0.1-0.5 mM of lactate (b-f). Inset, resulting calibration plot. Other conditions, as in Figure 2.

Continuous monitoring for stability test of mouthguard biosensor

High stability is another important requirement towards continuous in-mouth operation of the new mouthguard lactate biosensor. The stability was initially evaluated over a continuous two-hour operation with repetitive measurements of 0.5 mM carried out every 10 min. **Figure 4** displays the corresponding chronoamperograms along with the time-course profile of the resulting current response (inset; with the initial result at t=0 min normalized to 100%). These data indicate a highly stable current response over the entire 2 hour operation. On-going studies currently evaluate the long-term stability of the sensor.

Biosensing for lactate with mouthguard sensor in human saliva

After the evaluation of the mouthguard biosensor in a synthetic buffer matrix, experiments were carried out by using human saliva samples. The response of the sensor to changing lactate levels was examined using unstimulated human saliva spiked 0.1-0.5 mM of lactate. As indicated from the well-defined chronoamperograms of **Figure 5**, the sensor responds favorably to such changes in the lactate level (b-f). The resulting calibration plot (shown in the inset) exhibits good linearity (slope, 0.202 μA/mM; correlation coefficient, 0.988). The endogeneous lactate level can thus be estimated to 0.010 mM, which is in the normal range in human saliva in rest without stimulation.[21] The small current increments due to the lactate additions (vs those observed in the buffer media) are attributed to the viscosity of the saliva samples that leads to slower diffusion No apparent change in the sensitivity or linear range were observed when testing the sensors at 37° C (body temperature; not shown).

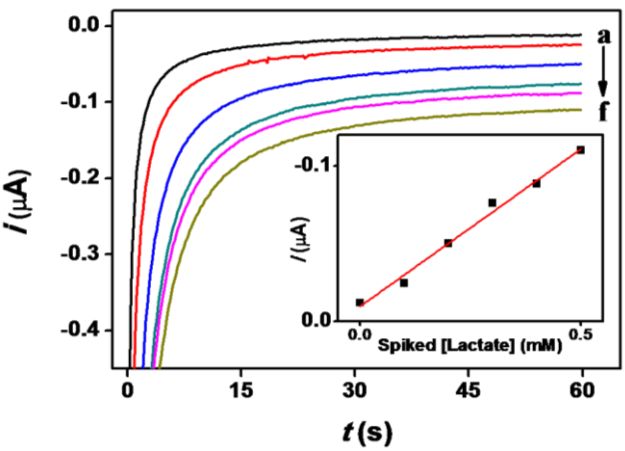

Figure 6. Stability of the response of the mouthguard biosensor to a human saliva sample spiked with 0.5 mM lactate. Repetitive measurements were carried out at 10 min intervals over a 2 h period. The inset is the relative current based on original current response (*t*=0). The sensor kept in saliva between such successive runs. Other conditions, as in Figure 2.

Continuous monitoring of lactate in human saliva

The continuous exposure to complex saliva media and the potential degradation of the sensor response by co-existing proteins requires assessment of the stability of the new oral biosensory system in the presence of such untreated biofuid. **Figure 6** examines the stability of the sensor in an untreated saliva sample over a 2 hour period. Only small variations of the current signal (ranging between 90% and 106% of the original response) are observed. Such good stability reflects the protective action of the PPD coating against co-existing fouling constituents. These data was obtained by repeated measurements every 10 min for 2hr, and replacing the saliva for each measurement to mimic the dynamic oral environment.[35] Whenever needed, the mouthguard sensor system can be readily replaced during actual in-mouth operation to address further degradation of the sensor response by the saliva matrix.

Conclusions

We have demonstrated the first example of a non-invasive mouthguard biosensor towards the continuous in-mouth monitoring of salivary metabolites. The new wearable biosensing platform has been demonstrated for amperometric measurements of lactate in connection to a PB-PPD-LOx system. The system enables highly sensitive, selective, and stable lactate response in saliva samples, reflecting its low-potential signal transduction and rejection of co-existing electroactive and protein constituents. Such attractive performance in undiluted human salivary samples substantiates the potential of the mouthguard-based biosensing platform as a practical wearable device for continuous non-invasive physiological monitoring of the fitness state of individuals. Future efforts towards continuous in-mouth lactate monitoring will focus on miniaturization and integration of the amperometric circuits and electronics for data acquisition, processing, and wireless transmission, as well as critical assessment of all potential toxicity and biocompatibility concerns. The amperometric mouthguard biosensing concept can readily be expanded towards salivary monitoring of other clinically-relevant metabolites and stress markers, hence offering useful insights into the wearer's health and performance and considerable promise for diverse biomedical and fitness applications.

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Notes and references

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