MULTI-ANALYTE DETECTION FOR BIOLOGICAL FLUIDS

Towards Continous Monitoring of Glucose, Ionized Calcium and pH using a Viscometric Affinity Biosensor

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Abstract: We present a viscometric affinity biosensor that can potentially allow continuous multi-analyte monitoring in biological fluids like blood or plasma. The sensing principle is based on the detection of viscosity changes of a polymeric solution which has a selective affinity for the analyte of interest. The chemico-mechanical sensor incorporates an actuating piezoelectric diaphragm, a sensing piezoelectric diaphragm and a flow-resisting microchannel for viscosity detection. A free-standing Anodic Alumina Oxide (AAO) porous nano-membrane is used as selective interface. A glucose-sensitive sensor was fabricated and extensively assessed in buffer solution. The sensor reversibility, stability and sensitivity were excellent during at least 65 hours. Results showed also a good degree of stability for a long term measurement (25 days). The sensor behaviour was furthermore tested in fetal bovine serum (FBS). The obtained results for glucose sensing are very promising, indicating that the developed sensor is a candidate for continuous monitoring in biological fluids. Sensitive solutions for ionized calcium and pH are currently under development and should allow multi-analyte sensing in the near future.

1 INTRODUCTION

Continuous detection overtime of analyte levels in complex biological fluids such as blood or plasma is a tricky task since strong interferences from other biomolecules may occur during the measurements. A successful system for continuous monitoring of physiologically relevant parameters would afford great benefits in numerous pathologies such as in the thrombosis with the assessment of blood calcium levels, in diabetes with glucose concentrations or pH in acidosis/alkalosis disorders. In addition, these parameters are also of primary importance for critically ill patients. Today, these controls are performed by hand and continuous monitoring could contribute to reduce the risk of mortality in the intensive care units.

Existing detection methods are currently based on electrochemical principles, which have limitations for *in vivo* monitoring. Electrochemical measurements depend on the analyte diffusion rate. Consequently, biofouling affects the sensitivity and frequent calibrations are required. Furthermore, the presence of other electrochemically active solutes often produces inaccuracies. An alternative approach which could overcome these limitations is affinity sensing. Affinity sensing is more tolerant to biofouling, which results only in an increased stabilization time, and is intrinsically not subjected to electroactive interferences. For these reasons, intensive investigations on affinity binding sensors have been carried out using different technics such as fluorescence (Ballerstadt, 2004) or viscosity measurements (Huang, 2009). Recently, hydrogel-based sensors have emerged as promising materials

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for affinity sensing (Tierney, 2009). Despite many efforts towards the development of continuous biosensors for medical and biological applications, long term reversibility and stability remains a challenge.

In this context, we propose a novel chemico-mechanical method which aims at detecting viscosity changes of a solution which has a selective affinity for the analyte of interest (Fig. 1). A semi-permeable membrane ensures that the analyte concentration in the biosensor is the same as in the patient blood or plasma. The viscosity detection of the sensitive solution is based on a microchannel which exhibits a resistance to the flow circulating through it. The sinusoidal actuation of a piezoelectric diaphragm generates a flow through the microchannel which results in a deflection of the sensing piezoelectric diaphragm, inducing a voltage which can be recorded. The phase shift between the applied voltage and the sensing piezoelectric diaphragm deflection is a measurement of the fluid viscosity.

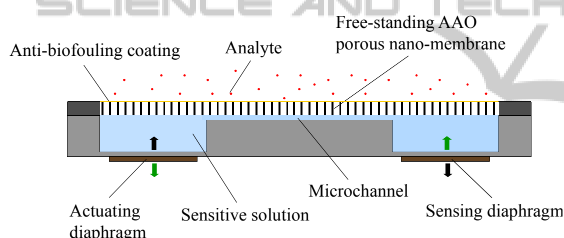

Figure 1: Schematic illustration of the biosensor principle. (Cross view).

A proof of concept of continuous glucose monitoring was previously reported using a macroscopic demonstrator (Boss, 2009). The present paper investigates the sensitivity, reversibility and reproducibility in a buffer solution of a novel glucose sensor fabricated by stereolithography. The sensor behavior in biological fluids was also assessed using fetal bovine serum (FBS). The glucose-sensitive fluid used inside the sensor was based on Concanavalin A (ConA), a protein which specifically binds to glucose, and a high-molecular-weight dextran. When glucose concentration increases, dextran is partially replaced by glucose at the binding sites of ConA. As a result, the network ConA-dextran is weakened, and the viscosity of the sensing fluid decreases.

2 MATERIALS AND METHODS

2.1 Sensor Fabrication

The sensor was fabricated by stereolithography using a biocompatible resin specially dedicated to medical applications (Proform). The actuating diaphragm was made up of a 50 µm thick and 3 mm in diameter lead zirconate titanate (PZT) disc (Audiowell Electronics) glued on a 10 µm thick brass foil (Goodfellow). The sensing diaphragm was made up of a 28 µm thick and 3 mm in diameter Polyvinylidene fluoride (PVDF) disc (Measurement Specialties) glued on a 10 µm thick brass foil. The semi-permeable membrane was a 50 µm thick Anodic Aluminium Oxide (AAO) membrane with 4-6 nm in diameter pores (Synkera Technologies). A $100 \times 100\ \mu m^2$ in section glass capillary was used as microchannel. The sensor assembly was realized using medical adhesive epoxy (Loctite M-21HP).

The sensor was 200 µm thick and the volume of sensitive fluid encapsulated inside was 4 µL. The sensitive fluid was prepared using the protocol described by Kuenzi *et al.* (2000). The sensitive fluid was composed of 2% [w/w] dextran 3200 (PSS) and 0.4% [w/w] ConA (Sigma). The viscosity of the sensitive fluid ranged from 5.9-16.7 mPas (30-2 mM glucose) at 25°C and from 4.2-9.4 mPas (30-2 mM) at 37°C. A low viscosity was selected to keep the glucose diffusion as fast as possible.

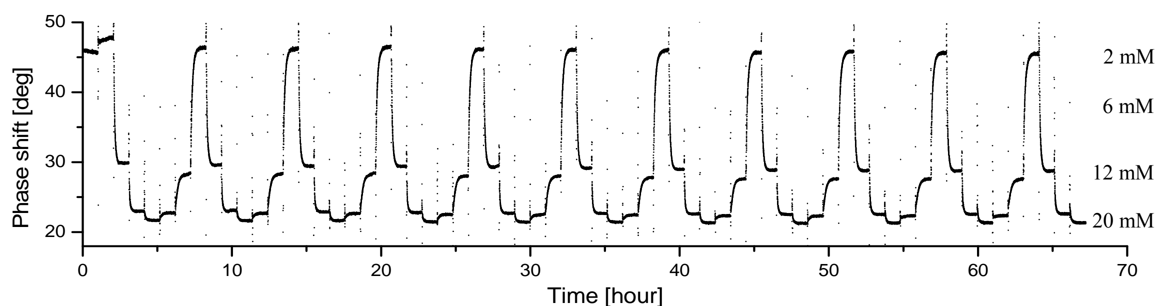

Figure 2: Phase shift response to multiple glucose concentrations (2, 6, 12, 20 mM). Measurement performed in reference solution at 25°C.

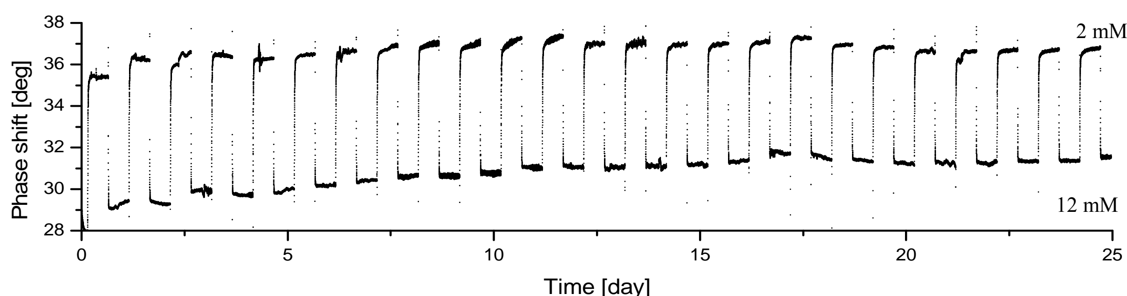

Figure 3: Long term glucose concentration measurement in reference solution at 37°C, between two physiologically relevant glucose concentrations, 2 mM and 12 mM.

2.2 Experimental Setup

Glucose measurements were performed in a reference solution which is an isotonic solution of the sensitive fluid, but without dextran and ConA. Stock solutions with different glucose concentrations were subsequently pumped into the test cell using a computer controlled syringe pump. The whole setup was located in a thermally regulated chamber (±0.01°C), as the viscosity of the sensitive fluid is strongly temperature dependant.

Fetal bovine serum (FBS) was preserved frozen. Before use, 0.1% of sodium azide (NaN_3) was added as preservative. FBS was heated at 56°C during 45 min to inactivate the complement system. FBS was then filtered (Millex HV 0.45 µm syringe filter) to remove aggregates which may clog the semi-permeable membrane. The glucose concentration of FBS was measured using a standard glucose meter (Accu-Chek). Concentrated (2M) D-glucose solution was added to increase the FBS glucose concentration.

3 RESULTS AND DISCUSSION

3.1 Reversibility of Glucose-induced Viscosity Change

In Figure 2, a sensor is exposed successively to increasing and decreasing glucose concentrations (2, 6, 12, 20 mM). Ten full cycles were performed during 65 hours showing an excellent reversibility and stability. The response time of the sensor (time to reach 90% of the final value) was 4.8 min for increasing glucose concentration and 19.3 min for decreasing glucose concentration. The longer time constant for decreasing glucose concentration is explained by the smaller mobility of glucose molecules in the viscous sensitive fluid than in the reference solution. The semi-permeable membrane porosity affects therefore more glucose molecules diffusing out of the sensor than glucose molecules entering inside the sensor. At this stage, miniaturization was not the primary focus and the response time is over the 10 min required from a medical point of view. The response time could be shorted by reducing the sensor thickness or sensitive fluid viscosity. The sensor sensitivity in the physiological and hypoglycemic ranges of glucose concentration (2-6 mM) was 0.1 mM, which is accurate enough for patients monitoring.

3.2 Long Term Stability

The long term stability of the sensor at 37°C was investigated (Fig. 3). Glucose concentrations were changed every 12 hours during 25 days. The sensor showed a remarkable stability over time, but a progressive loss of sensitivity was nevertheless observed. After 25 days, the sensor sensitivity dropped to 73% of the initial sensitivity. The loss of sensitivity was likely due to ConA leakage through the biggest pores and defects of the 4-6 nm pores of the semi-permeable membrane. We are currently assessing new AAO porous nano-membranes with reduced pores size (2-4 nm) which should improve the long term stability of the sensor.

3.3 Determination of Glucose in Fetal Bovine Serum

The sensor behaviour in complex biological fluids was evaluated using fetal bovine serum (FBS). Fig. 4 shows the sensor response to glucose variation (2.6 and 30 mM) in FBS at 37°C. Twelve full cycles were performed during 24 hours, showing a good stability. The response time did not increase with time indicating that biofouling due to protein adsorption on the semi-permeable membrane is not an issue. AAO porous nano-membranes are therefore well-suited as selective interface for biosensors intended to be used in biological fluids.

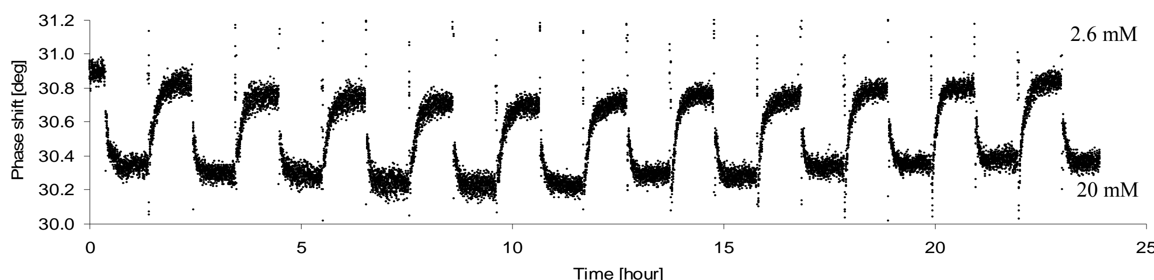

Figure 4: Measurement in fetal bovine serum at 37°C between 2.6 mM and 20 mM.

The sensor sensitivity in FBS dropped to 10% of the sensitivity in buffer solution. After FBS testing, the sensor recovered its initial sensitivity in buffer solution. The loss of sensitivity is therefore reversible, which leads to the following hypothesis. Small glycosylated peptides may enter inside the sensor and competitively interfere with the binding reaction of ConA to glucose. This hypothesis was confirmed by conducting similar experiments with dialysed FBS. A loss of sensitivity of 30% and 40% were observed in 12 kDa and 3.5 kDa dialysed FBS, respectively. The pores size should therefore be significantly reduced to prevent glycosylated peptides from entering the sensor. We are currently assessing 2-4 nm AAO nano-membranes, coated with Al_2O_3 by atomic layer deposition, to minimize the pores size.

4 CONCLUSIONS

A viscosity-based affinity sensor was developed for continuous monitoring in biological fluids. The sensor was extensively tested in buffer solution, showing an excellent reproducibility and stability over 65 hours at 25°C. The sensor sensitivity matched well within the hypoglycemic and physiological ranges (2-6 mM) with a resolution of 0.1 mM. The response time of the sensor was higher for decreasing glucose concentration due to the conjugated effect of both the reduced mobility of glucose molecules in the viscous sensitive solution and the membrane porosity. The sensor showed also remarkable long term stability (25 days) at 37°C. A limited loss of sensitivity was nevertheless observed, which may be explained by ConA leakage through defects of the AAO porous nano-membrane. In FBS, the response time did not increase with time, indicating that biofouling due to protein adsorption is not an issue. The sensitivity in non-dialysed and 12 kDa dialysed FBS were 10% and 60% of the sensitivity in buffer solution, respectively. Glycosylated perptides may enter inside the sensor and interfere with the ConA-glucose reaction. The loss of sensitivity in FBS should be solved by reducing the membrane pores size.

These measurements show good promise for the sensor to be applied as *in vivo* monitoring system. We are currently assessing Al_2O_3 coated 2-4 nm AAO nano-membranes for pores size reduction, which should allow sensitive measurements in FBS. Sensitive fluids for ionized calcium and pH are also under development and should allow multi-analyte sensing in the near future.

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