Piezoresistive Cantilever Array Sensor for Consolidated Bioprocess Monitoring

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Summary: Cellulolytic microbes occur in diverse natural niches and are being screened for industrial modification and utility. A microbe for consolidated bioprocessing (CBP) development can rapidly degrade pure cellulose and then ferment the resulting sugars into fuels. To identify and screen for novel microbes for CBP, we have developed a piezoresistive cantilever array sensor which is capable of simultaneous monitoring of glucose and ethanol concentration changes in a phosphate buffer solution. 4-mercaptophenylboronic acid and polyethyleneglycol-thiol are employed to functionalize each piezoresistive cantilever for glucose and ethanol sensing, respectively. Successful concentration measurements of glucose and ethanol with minimal interferences are obtained with our cantilever array sensor. SCANNING 31: 204–210, 2009. © 2009 Wiley Periodicals, Inc.

Introduction

Consolidated bioprocessing (CBP), a one-step microbe-mediated process for directly converting plant biomass into ethanol, has recently attracted much attention as a game-changing strategy for cellulosic biofuel production. Current efforts are examining isolates, consortia, and mutants to search for improved microbial characteristics and enzymes. The goal is to find cellulolytic fermentative microorganisms that have the potential for industrial utility. Key traits are rapid degradation or conversion of native biomass and good fermentative potential (Mielenz 2009). In order to screen many organisms, a low-cost flexible multi-well format is required. The measurement of degradation is challenging for current sensors due to the presence of the solid lignocellulosic substrate combined with the growing microbial biomass. There is also the challenge that while ethanol may be the current desired fermentative product, the production of any fermentative products (alcohols or organic acids) is desirable. This presumes that genetic manipulation will be able to adjust the fermentative products. Therefore, we sought to use microcantilevers to detect the presence of soluble sugars and cellulose degradation intermediates or the change in fermentation products.

The microcantilever, an essential part of atomic force microscopy (AFM), has been widely utilized as a physical, chemical, and biological sensor platform for a decade (Hansen and Thundat 2005; Lavrik et al. 2004; Thundat et al. 1997; Weeks et al. 2003). Microcantilever sensors have two operational modes: the static mode and dynamic mode. The static mode measures the variation in the deflection of a cantilever due to adsorption-induced surface stress changes, whereas the dynamic mode measures the variation in the resonance frequency of a cantilever due to adsorption-induced mass or stiffness changes (Chen et al. 1995; Thundat et al. 1994). Although dynamic mode is well suited for measuring adsorption-induced mass in vacuum and air, its mass resolution is very poor due to hydrodynamic loading when microcantilever sensors are operated in liquid environments (Oden et al. 1996; Tamayo et al. 2001). Furthermore, the viscosity and density changes of a mixture solution complicate the measurement of an analyte concentration. The dynamic mode, therefore, is generally not used for the highly

sensitive and selective detection of analytes in liquid. On the other hand, the static mode has been widely used for highly sensitive and selective measurements of analytes in liquid. A thin gold film is typically coated on one side of the microcantilever in order to selectively functionalize the surface of the cantilever with thiol compounds. Specific interactions between the analytes and thiol compounds induce an apparent surface stress change and micromechanical bending of the cantilever (Berger *et al.* 1997; Kohale *et al.* 2007; Raiteri *et al.* 2001).

The deflection of a cantilever is conventionally measured by an optical beam deflection technique, which is employed in a standard AFM. However, optical beam measurements require bulky optical components which make the device miniaturization difficult. Moreover, refractive index change of a mixture solution and liquid turbidity make the quantitative measurements more complicated. Therefore, recently, piezoresistive microcantilevers have attracted much interest in the development of compact and simple device operation sensors (Boisen *et al.* 2000; Mukhopadhyay *et al.* 2005; Rasmussen *et al.* 2003; Yu *et al.* 2002). Piezoresistive cantilever arrays have been exploited as a platform for "electronic nose" applications in gaseous environments and have shown some successful results (Pinnaduwage *et al.* 2004; Seo *et al.* 2007). However, very limited studies have been reported on multiple analytes detection with piezoresistive cantilever arrays in liquid environments. Here, we present a piezoresistive cantilever array sensor which is capable of simultaneously monitoring glucose and ethanol concentration change in a phosphate buffer solution. This sensor shows great potential for the fermentation process monitoring in consolidated bioprocessing (CBP), a one-step microbe-mediated process for directly converting plant biomass into ethanol.

Materials and Methods

Materials

1-dodecane thiol and 4-mercaptophenylboronic acid (4-MPBA) were used as received from Sigma-Aldrich (St. Louis, MO). Polyethyleneglycol (PEG)-thiol (HS-$[CH_2]_{11}$-$[OCH_2]_6$-OH) was used as received from Prochimia (Sopot, Poland). Phosphate buffer solution was prepared by dissolving 8.5 g of NaH_2PO_4 and 11 g of Na_3PO_4 in 1.0 L of Millipore water. Dilute HCl and NaOH ($\sim$0.1 M) were used to adjust the pH of the phosphate buffer solution ($\sim$pH 7.0). All chemicals used in the experiments were of analytical grade or higher. High-purity de-ionized water was obtained with a Millipore water

system. Two types of piezoresistive cantilever were employed. One is a Canti-4^{TM} chip from Cantion (Aalborg, Denmark) which has four piezoresistive cantilevers in an array and the dimensions of each cantilever are 120 μm long, 35 μm wide, and 500 nm thick. The other is a Cantimer chip from Cantimer Inc. (Menlo Park, CA) which has a 300 μm long, 50 μm wide, and 3 μm thick piezoresistive cantilever in the middle of silicon protection hole. One side of each cantilever was coated with 2 nm of chromium and 20 nm of gold by using an e-beam evaporator.

Surface Functionalization

In all cases, before surface functionalization, the microcantilevers were carefully cleaned with acetone, isopropyl alcohol, methanol, and absolute ethanol and then immersed in piranha solution for 30 s (*Caution: Piranha solution reacts violently with organic matter and is highly corrosive. It should be handled with the utmost care while wearing the appropriate personal protective equipment*). They were then rinsed with de-ionized water and ethanol. At this stage, the cleaned cantilevers were ready for self-assembled monolayer (SAM) formation on the gold surface. Formation of 1-dodecane thiol SAM, 4-MPBA SAM, and PEG-thiol SAM were achieved by immersion of the cleaned, gold-coated cantilever portion of the chips into a 5 mM of 1-dodecane thiol, a 3.25 mM of 4-MPBA, and a 2 mM of PEG-thiol, respectively, in ethanol for overnight. Following the SAM formation, the functionalized cantilevers were rinsed with ethanol to remove any loosely bound thiol compound and dried with argon gas.

Piezoresistive Cantilever Array System

The piezoresistive cantilever array system used in this study consists of a reference piezoresistive cantilever coated with a 1-dodecane thiol SAM which permits reliable subtraction of background noise signal due to temperature fluctuation and fluid exchange dynamics (i.e. differential measurement), and two functionalized piezoresistive cantilevers, one with a 4-MPBA SAM, and the other with a PEG-thiol SAM. Figure 1 shows the basic concept of differential measurements for glucose and ethanol detection with functionalized cantilevers. 1-dodecane thiol SAM was selected as an inert surface coating layer for glucose and ethanol concentration change. 4-MPBA SAM was chosen for selective detection of sugar in solution. The sensing mechanism of sugar in solution with a 4-MPBA

SAM was described in detail in our previous publication (Baker *et al.* 2008). PEG-thiol SAM is usually employed to passivate the surface to prevent nonspecific adsorption of biomolecules in solution (Yue *et al.* 2008). Especially, PEG has been exploited to resist the nonspecific adsorption of proteins as well as the adhesion of bacterial and mammalian cells (Ostuni *et al.* 2001). In addition to this functionality, PEG swells in aqueous solution and ethanol molecules partition into PEG. This makes the PEG-thiol SAM-coated cantilever bend when it is exposed to the low concentrations of ethanol. A two-channel Wheatstone bridge circuit and all associated electronics as shown in Figure 2 were constructed on a single circuit board. A commercially available universal serial bus (USB) data acquisition box (NI-USB 6008) allowed the system to be connected to a desktop computer using a USB cable. A LabVIEW™ program was written to collect and save data.

C1: Ethanol sensing cantilever (PEG-thiol SAM)

○ Glucose
* Ethanol

C2: Reference cantilever (1-dodecane thiol SAM)
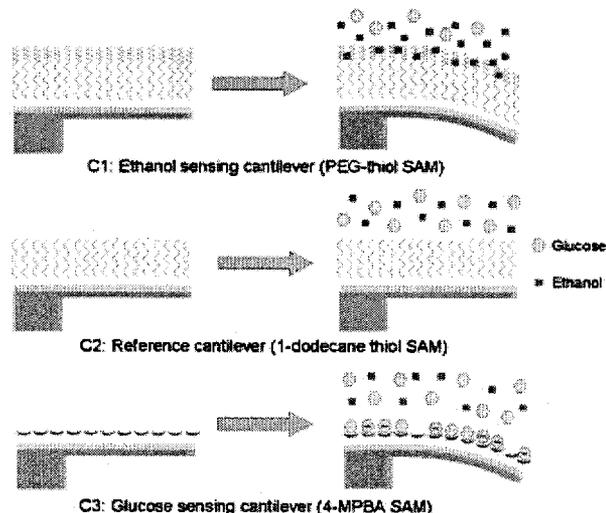
C3: Glucose sensing cantilever (4-MPBA SAM)

Fig 1. Schematic drawing illustrating specific interactions between analytes and functionalized surface interfaces for ethanol (C1) and glucose (C3) sensing. A reference cantilever (C2) permits reliable subtraction of background noise signal due to temperature fluctuation and fluid exchange dynamics.
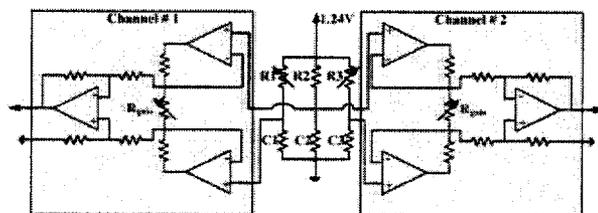

Fig 2. Schematic diagram of a two-channel Wheatstone bridge circuit and all associated electronics. Two differential signals (C1-C2 and C3-C2) are amplified, and sent to USB data acquisition box which is connected with a computer for data storage and analysis.

Stationary and Dynamic Flow Experiment Setup

Initially, we tested the functionalized cantilevers using two sandwiched Canti-4™ chips (one is a reference cantilever and the other is a sensing cantilever) in a standard 24 well plate with a 3 mL solution per well (as shown in Figure 3) to verify the sensitivity and selectivity of each functionalized cantilever and investigate the interference effects on glucose and ethanol sensing. Previously, we have shown that 4-MPBA SAM functionalized cantilever can detect and quantify the concentration of fructose ranging from 2 to 25 mM in a phosphate buffer solution (Baker *et al.* 2008). However, CBP does not involve fructose in the biomass conversion and fermentation process. Cellulose is generally converted to cellobiose and then to glucose which is fermented to ethanol by microbes and enzymes. Therefore, in this study, we chose glucose as a representative analyte. After developing a steady baseline, the cantilever array sensor was carefully moved to another well filled with a phosphate buffer solution containing glucose or ethanol at different concentrations. For the dynamic flow experiment, the plastic flow chamber was machined and integrated with the three Cantimer chips as shown in Figure 4. After getting a steady baseline with a phosphate buffer solution at a constant flow rate of 50 μL/min, the sample solution was loaded into a 2 mL sample loop and introduced into the flow chamber via a HPLC six-way switch valve system with a syringe pump. All experiments were performed in laboratory room temperature (~24°C). The differential bending signals were recorded as a function of time for both stationary and dynamic experiments.
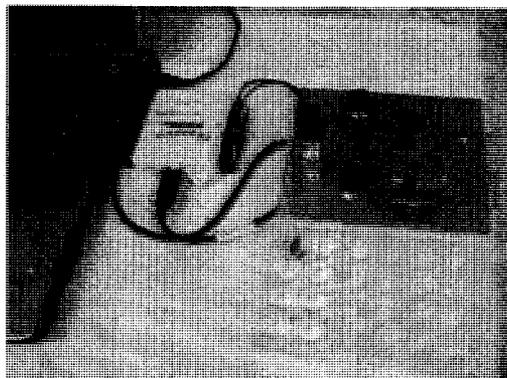

Fig 3. A photograph of experimental setup with two sandwiched Canti-4™ chips (one is a reference cantilever (C2) and the other is a sensing cantilever (C1 or C3)) in a standard 24 well plate. Each well is filled with a 3 mL phosphate buffer solution containing glucose or ethanol at different concentrations.

Fig 4. A photograph of experimental setup with three Cantimer chips (one is a reference cantilever (C2) and the other two are sensing cantilevers (C1 and C3)) in the plastic flow chamber for the dynamic flow experiment. Sample solution is loaded into a sample loop and introduced into the flow chamber via a HPLC six-way switch valve system with a syringe pump.

Results

Glucose Detection

The relationship between cantilever bending response and glucose concentration was tested for various concentration values up to 20 mM of glucose. The 4-MPBA SAM functionalized cantilever responded well to the concentration change of glucose. A control experiment was also performed to investigate ethanol interference effect on the glucose sensing cantilever. Figure 5A shows the representative result of glucose detection with two sandwiched Canti-4^{TM} chips (one is a 1-dodecane thiol SAM-coated reference cantilever and the other is a 4-MPBA SAM-coated sensing cantilever) in a stationary well. After dipping the cantilever array in a pure phosphate buffer solution, the bridge circuit was initially balanced by adjusting the potentiometer. After developing a steady baseline, the cantilever array sensor was carefully moved to another well filled with a phosphate buffer solution containing glucose at different concentrations (10 and 20 mM of glucose). Although a small drift was observed in the initial stage, the differential cantilever sensor signal became stable after dipping in a sample solution. Although we could observe the differential signal of 2 mM of glucose in a very careful experiment, there was chip-to-chip variation which limits the determination of minimum detectable concentration. However, we still anticipate that a sub-millimolar level of sensitivity will be attainable on the basis of noncommercial thiolated boronic acid interfaces with an optimized setup.

Figure 5B shows the result of a control experiment with ethanol concentration changes up to 5% and no glucose for the glucose sensing cantilever
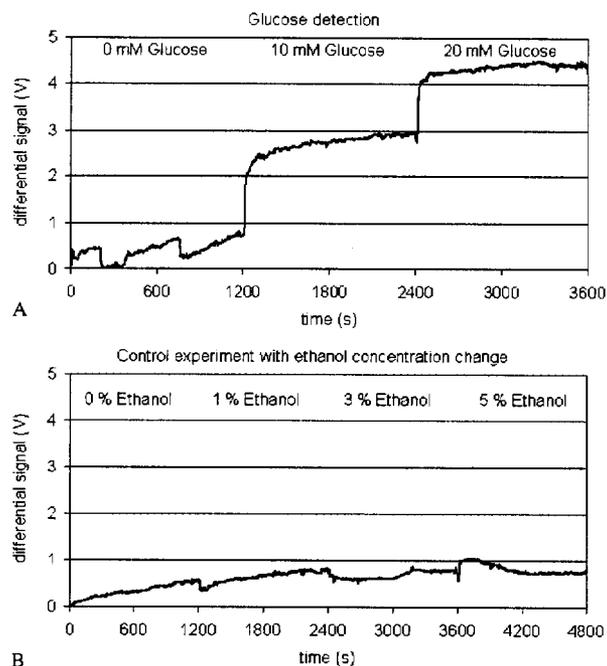

Fig 5. The response of a 4-MPBA SAM-coated piezoresistive cantilever for glucose and ethanol concentration change: (A) the representative result of glucose concentration detection with two sandwiched Canti-4^{TM} chips (one is a 1-dodecane thiol SAM-coated reference cantilever and the other is a 4-MPBA SAM-coated sensing cantilever) in a stationary well. (B) The representative result of a control experiment with ethanol concentration change up to 5% for the glucose sensing cantilever array in a stationary well. Minimal interference signal is observed in this concentration range.

array in a stationary well. The glucose sensing cantilever array was sequentially dipped in a phosphate buffer solution containing ethanol at different concentrations (0, 1, 3, and 5 % of ethanol) for 20 min. Minimal interference was observed with this ethanol concentration level which is acceptable for the fermentation process in CBP. Figure 6 shows the result of 20 mM glucose detection with a 4-MPBA SAM-coated cantilever (Cantimer chip) and the minimal interference effect of glucose on a PEG-thiol SAM-coated cantilever (Cantimer chip) in the plastic flow chamber shown in Figure 4. Three cantilever chips were initially exposed to a constant flow of a phosphate buffer solution at 50 μL/min and the cantilevers were equilibrated until stable baselines were obtained and then each bridge circuit for channel 1 and 2 was balanced by adjusting the corresponding potentiometer. After 20 min, 20 mM glucose was introduced and passed into the flow chamber via a HPLC six-way switch valve system with no change in flow rate. We chose a maximum glucose concentration of 20 mM as a reasonable maximum for soluble sugar intermediates. Tracking consumption of glucose and production of ethanol is of practical interests in this case. Therefore, the
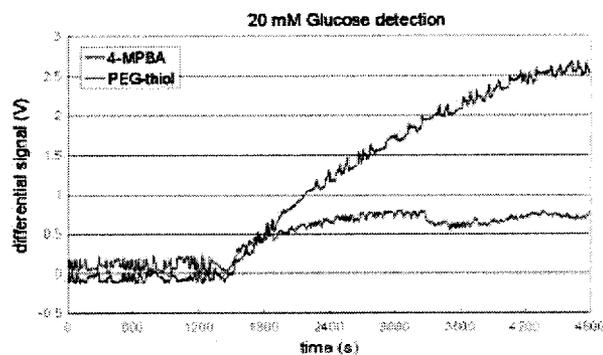
Fig 6. The simultaneous responses of a 4-MPBA SAM coated piezoresistive cantilever and a PEG-thiol SAM-coated piezoresistive cantilever (Cantimer Chips) for 20 mM glucose sample in the plastic flow chamber. The sensor signal for a PEG-thiol SAM-coated cantilever reflects approximately maximum interference signal from glucose in our fermentation process of interest.

differential signal for a PEG-thiol SAM-coated cantilever in Figure 6 reflects approximately the maximum interference signal from glucose.

This glucose sensor will also respond to other small soluble sugars as discussed in our previous publication (Baker et al. 2008). In many industrial fermentation applications, this partial selectivity can either be ignored (if glucose is the only substrate) or is a weakness. However, the degradation of lignocellulose may result in the release of a number of intermediates (e.g. cellobiose, xylose, and arabinose). Therefore, in a preliminary screen, the detection of any sugar release is a positive aspect and the details of sugar release of the positive samples can be examined by HPLC in a subsequent experiment.

Ethanol Detection

The relationship between cantilever bending response and ethanol concentration was also tested for various concentration values up to 5% ethanol by mass. The PEG-thiol SAM functionalized cantilever responded well to the concentration change of ethanol in this concentration range. A control experiment was also performed to investigate the glucose interference effect on the ethanol sensing cantilever. Figure 7A shows the representative result of ethanol detection with two sandwiched Canti-4^{TM} chips (one is a 1-dodecane thiol SAM-coated reference cantilever and the other is a PEG-thiol SAM-coated sensing cantilever) in a stationary well. After dipping the cantilever array in a pure phosphate buffer solution, the bridge circuit was initially balanced by adjusting the potentiometer. After developing a steady baseline, the cantilever array sensor was carefully moved to another well filled with a
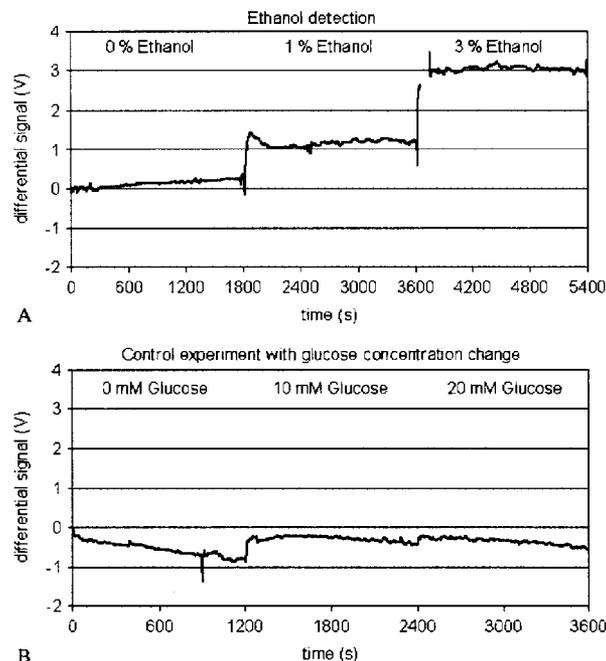

Fig 7. The response of a PEG-thiol SAM-coated piezoresistive cantilever for glucose and ethanol concentration change: (A) the representative result of ethanol concentration detection with two sandwiched Canti-4^{TM} chips (one is a 1-dodecane thiol SAM-coated reference cantilever and the other is a PEG-thiol SAM-coated sensing cantilever) in a stationary well. (B) The representative result of a control experiment with glucose concentration change up to 20 mM for the ethanol sensing cantilever array in a stationary well. Minimal interference signal is observed in this concentration range.

phosphate buffer solution containing ethanol at different concentrations (1 and 3% of ethanol). Figure 7B shows the result of a control experiment with glucose concentration changes up to 20 mM and no ethanol for the ethanol sensing cantilever array in a stationary well. The ethanol sensing cantilever array was sequentially dipped in a phosphate buffer solution containing glucose at different concentrations (0, 10, and 20 mM of glucose) for 20 min. Minimal interference was observed with this glucose concentration level which is acceptable for the fermentation process. Although a small drift was observed in the initial stage, the differential cantilever sensor signal became stable after dipping in a sample solution. Figure 8 shows the result of 5% ethanol detection with a PEG-thiol SAM-coated cantilever (Cantimer chip) and the minimal interference effect of ethanol on a 4-MPBA SAM-coated cantilever (Cantimer chip) in the plastic flow chamber shown in Figure 4. Three cantilever chips were initially exposed to a constant flow of a phosphate buffer solution at 50 μL/min and the cantilevers were equilibrated until stable baselines were obtained and then each bridge circuit for channel 1 and 2 was balanced by adjusting the corresponding
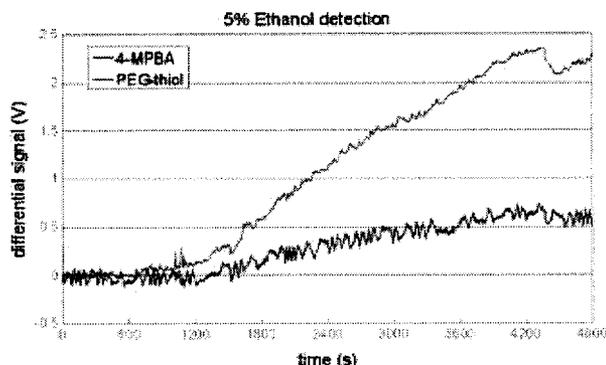
Fig 8. The simultaneous responses of a 4-MPBA SAM-coated piezoresistive cantilever and a PEG-thiol SAM-coated piezoresistive cantilever (Cantimer Chips) for 5% ethanol sample in the plastic flow chamber. The sensor signal for a 4-MPBA SAM-coated cantilever reflects approximately maximum interference signal from ethanol in our fermentation process of interest.

potentiometer. After 20 min, 5% ethanol were introduced and passed into the flow chamber via a HPLC six-way switch valve system with no change in flow rate. We chose a maximum ethanol concentration of 5% as most native microbes cannot tolerate over 5% ethanol in medium and this is often considered the minimum titer for an industrial process. Therefore, the differential signal for a 4-MPBA SAM-coated cantilever in Figure 8 reflects approximately the maximum interference signal from ethanol.

This PEG-thiol SAM-coated cantilever can also respond to the presence of other alcohols. In this circumstance of a preliminary screen, this partial selectivity is a positive and the fermentative production of measurable amounts of any alcohols indicates a potentially good candidate CBP microorganism for further study and manipulation.

Discussion

The deflection of microcantilevers has been used for highly sensitive and selective measurements of analytes in liquid for a decade (Fritz *et al.* 2000). A thin gold film is typically coated on one side of the microcantilever in order to selectively functionalize the surface of the cantilever with thiol compounds. However, the gold coating produces significant residual stress that affects the sensitivity and reproducibility of the cantilever's response to molecular adsorption (Lee *et al.* 2009; Mertens *et al.* 2007). Godin et al. investigated the origin of variation in the surface stress of fully gold-coated cantilevers and demonstrated that the deflection of a cantilever strongly depends on the surface roughness of the gold film (Godin *et al.* 2004). The sensitivity and

reproducibility of a cantilever's static response also depends on the uniformity and the molecular size of the immobilized functional layer and cleanliness of the sensing surface (Desikan *et al.* 2007; Kim *et al.* 2007; Tabard-Cossa *et al.* 2007). Therefore, great care should be taken to prepare the microcantilever array sensor. In addition, selectivity of detection in complex medium still remains as a challenging task and the stability of functional layer is an issue that potentially limits shelf life and long-term reliability of the sensor.

Conclusion

We have developed a piezoresistive cantilever array sensor which is capable of simultaneously monitoring sugar and alcohol concentration changes in a phosphate buffer solution. Successful concentration change measurements of glucose and ethanol with minimal interference were obtained with a 4-MPBA and PEG-thiol SAM-coated cantilever array sensor. This sensor shows great potential for the fermentation process monitoring by tracking consumption of glucose and production of ethanol in liquid buffer medium. These two sensors will be combined with existing sensors for temperature and pH. The partial selectivity of the two cantilever coatings is a positive for a preliminary screen as the actual soluble sugar intermediates or fermentative products are not known *a priori*. The detection of high levels of any soluble sugar intermediates and alcohol product will indicate a microbial sample worthy of further more detailed study. This microcantilever array sensor appears to be useful for screening the efficiency of enzymatic or microbial biomass hydrolysis based on a multi-well plate format. Thus, currently, we are working on developing an integrated sensor system for high-throughput screening with a multi-well format. Finally, improving the robustness of this cantilever array sensor for use in more complex and challenging media is an aspect deserving further attention.

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