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Short communication

Micromechanical oscillators as rapid biosensor for the detection of active growth of *Escherichia coli*

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Abstract

A rapid biosensor for the detection of bacterial growth was developed using micromechanical oscillators coated by common nutritive layers. The change in resonance frequency as a function of the increasing mass on a cantilever array forms the basis of the detection scheme. The sensor is able to detect active growth of *Escherichia coli* cells within 1 h which is significantly faster than any conventional plating method which requires at least 24 h. The growth of *E. coli* was confirmed by scanning electron microscopy. This new sensing method for the detection of active bacterial growth allows future applications in, e.g., rapid antibiotic susceptibility testing by adding antibiotics to the nutritive layer.

Keywords: *E. coli*; Biosensor; Micromechanical oscillators; Active growth

1. Introduction

Food safety is an increasingly important public health issue. In 2000 alone, 2.1 million people died from diarrhoeal diseases. A great portion of these cases can be attributed to contamination of food and drinking water. In USA, for example, around 76 million cases of foodborne diseases, resulting in 325,000 hospitalizations and 5000 deaths, are estimated to occur per year (WHO, 2002). Food contaminations create an enormous economic burden on communities and their health systems. In UK, a single case of laboratory-confirmed salmonellosis was estimated to cost nearly £800 in 1990. This figure includes the hospitalization costs of some patients as well as their loss of earnings and losses in economics output (Sockett and Roberts, 1991). Foodborne pathogens pose a risk to food safety and concerns have arisen due to the globalization of the food trade (WHO, 2004).

Escherichia coli naturally occur in the intestinal tract of humans and warm-blooded animals. Outside the intestine, *E. coli* is normally used as marker organism to detect fecal pollution of water and food products. *E. coli* is a facultative pathogenic or opportunistic bacterium and can cause, for example, urinary tract infections, inflammations and peritonitis in immunosupressed patients as children and elderly people. Besides this, several strains as the enteropathogenic *E. coli* (EPEC), the enterotoxic *E. coli* (ETEC), the enterohemorragic *E. coli* (EHEC) and the enteroinvasive *E. coli* (EIEC) are able to cause diarrhea. These infections are often correlated with direct or indirect pollution of drinking water and food products (Bettelheim, 1991).

For routine proof of the innocuousness of food products, usually only the presence of indicator organisms (e.g., *E. coli*, coliform *Enterobacteriaceae*) is determined as the analysis for multiple pathogens is time- and cost-consuming. These tests continue to rely on conventional culturing techniques, as in many countries the food safety legislation is based on the counting of viable cells. These methods take at least 24 h until an interpretation is feasible (SLMB, 2000). In regard to a future application as a fast antibiotic susceptibility sensor,

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the fast detection of active bacterial growth is very important since the tested antibiotics only act on the metabolism of viable bacteria.

In recent years, nanomechanical bending and oscillating cantilevers have been used as a new class of biological and chemical sensors (Lang et al., 1998). Such cantilever sensors are successfully applied in the field of genomics (Fritz et al., 2000; Hansen et al., 2001; McKendry et al., 2002), gas-sensing (Baller et al., 2000), and proteomics (Arntz et al., 2003; Liu et al., 2003; Moulin et al., 2000). The elegance of these sensing methods is that the detection of an analyte requires no labeling as well as that the various application fields only differ in the functional layers on the cantilever interface. The detection scheme remains common for all the different applications. In principle, any detection method, which is based on molecular recognition, is able to be implemented (Battiston et al., 2001). With the help of micromechanical mass sensors single bacterial cells and virus particles could be detected in a dry environment (Gupta et al., 2004; Ilic et al., 2001). A first attempt for the application of micromechanical oscillators as growing cell sensors was made by Prescesky et al., 1992.

In this work, detection of active bacterial growth using a resonance-frequency-based mass sensor has been accomplished.

2. Materials and methods

2.1. Reagents

All reagents were purchased from Fluka (Buchs, Switzerland), unless otherwise stated, and were of analytical grade.

2.2. Bacteria and culture method

E. coli XL1-Blue was obtained from Stratagene Inc. (USA). *E. coli* was grown in Luria Bertani (LB) broth (Sambrook and Russel, 2001) overnight at 37 °C before use. Cells from 1 ml were harvested by centrifugation (Genofuge 16M, Techne, 14,000 rpm, 20 min, 4 °C) and the resulting pellet was resuspended in 500 μl LB broth.

2.3. Cantilever functionalization

Eight cantilevers are linearly arranged in an array at a pitch of 250 μm, each of them 500 μm long, 100 μm wide and 7 μm thick. The cantilever arrays were obtained from the micro-fabrication group of IBM Zurich Research Laboratory. To remove contaminations from the surfaces, the cantilever arrays were cleaned for 20 min in Piranha solution (95–97% H_2SO_4 concentration in 30% H_2O_2, 1:1) and subsequently rinsed twice with sterile-filtrated water and once with deionized water. The cleaning procedure was performed twice. The cantilever arrays were then immediately immersed in silylating solution (1% (3-glycidyloxypropyl)-trimethoxysilane, 0.5% *N*-ethyldiisopropylamine in water free toluene) for about 4 h at room temperature to form a self-assembled monolayer, which provided a hydroxyl-reactive surface towards primary hydroxyl groups (e.g., agarose). After the silylanization, the cantilevers were rinsed twice with toluene for 20 min and dried under argon.

Agarose was dissolved in deionized water at a concentration of 1% (w/v), melted and stored at 60 °C. Just prior to use, the pH of the agarose solution was adjusted to pH ∼ 11 with 5M NaOH. The agarose coating of each cantilever was accomplished in quartz micro-capillaries (outer-diameter 250 μm, inner diameter 150 μm, Garner Glass Inc., Clarmont, CA). The cantilevers were immersed into the micro-capillaries using micro-manipulators, as described previously (Bietsch et al., 2004) and exposed to the agarose solution for 10 s. Longer incubation of the cantilever led to agarose collar formation at the base of the oscillators. The agarose layer thickness was determined by scanning force microscopy (MultiMode™, Digital Instrument, Santa Barbara, CA, USA). After the agarose coating the arrays can be stored in a dry environment for up to 1 month.

The bacterial inoculation of a single agarose coated cantilever was performed in the above mentioned quartz capillaries. The sensor cantilevers were exposed to an *E. coli* suspension (see bacteria and culture method) for 10 min. The agarose coated reference cantilevers were immersed for 10 min in pure LB broth without *E. coli* cells. The functionalized cantilevers had to be used immediately for the measurement.

2.4. Experimental setup

Experiments were performed in a scanning probe MultiMode™ head with NanoScope® control software (Digital Instruments, Santa Barbara, CA, USA). The measurement principle is as follows: a laser is focused onto the tip of an oscillating cantilever. The reflected laser beam is then deflected onto a position-sensitive detector (PSD), where changes in the frequency can be measured.

To obtain suitable conditions for bacterial growth, the MultiMode™ head was placed inside a temperature and humidity controlled box. Humidity and temperature was measured using a HygroClip-SC05 sensor (Rotronic, Bassersdorf, Switzerland). LabView software and a data acquisition board (6036E, National Instruments, Austin, TX) were used to control the humidity handling system of the setup, i.e., massflow meters and controllers (EL-Flow and FlowDDE, Bronkhorst HI-TEC, Reinach, Switzerland), as well as the temperature setting of the cooling/heating box (Intertronic, Interdiscount, Switzerland). For ideal measurement it was important to keep the temperature and rel. humidity constant ($\Delta T \sim 0.2$ °C; Δrel. humidity ∼ 2%).

In order to determine a frequency change due to bacterial growth, frequency spectra were taken of the cantilever every 30 min. For differential measurements the laser had to

be switched manually between the sensor and reference cantilever. The time difference in probing of two cantilevers was 15 min.

3. Results and discussion

Fig. 1(A) shows a typical frequency response curve obtained by incubating a sensor- and a reference-cantilever in a humid and warm environment (37 °C, 93% rel. humidity). For this experiment, both cantilevers were coated by a nutritive layer, but only the sensor-cantilever was exposed to *E. coli* XL1-Blue cells. No decrease in the resonance frequency of the reference-cantilever, which was not exposed to bacterial cells, was detected over a period of 8 h. In contrast to this, the resonance frequency of the cantilever exposed to bacteria dropped exponentially during the first 5 h and then slowly leveled out to a constant value. To exclude any undesired environmental changes (e.g., temperature- and rel. humidity-shifts in the humidity box) the frequency curves of the reference-cantilevers were always subtracted from the curves of the sensor-cantilevers in the following experiments. After the completed measurement the cantilevers were examined with scanning electron microscopy (SEM) in order to confirm bacterial growth. Fig. 1(B) shows the SEM of the sensor cantilever after exposure to a humid and warm environment. It can clearly be seen that bacterial cells were attached to the sensor-cantilever, as well as that they started to form small colonies. On the other hand, no bacterial cells can be detected on the reference-cantilever (data not shown). As the cantilever had to be completely dry for SEM micrograph, some salt crystallization can occur as a result of the drying procedure. The blisters visible were caused by exposure of the cantilever to the e-beam.

Additional mass loading onto a cantilever causes a shift in its resonance frequency (see Fig. 1A). Assuming that the added mass is much less than the mass of the cantilever, shifts in resonance frequency depend linearly on the additional mass loaded onto a cantilever (Ilic et al., 2000):

$$\Delta f \propto m_{\text{load}} \tag{1}$$

For a deeper insight in the mass/frequency dependence we would like to refer to the work of Chen et al. (1994). In addition to Eq. (1), the resonance frequency depends also on the damping of the oscillating system (Unbehauen, 2002). As we observed no change in the quality factor Q, which is equal to the reciprocal of the damping, no change in the damping of our system was detected during the measurements (data not shown). Steric hindrance due to bacterial cells attached to the cantilever surface could lead to a change of the spring constant. We neglect a change of the spring constant during our experiment due to bacterial adhesion, as the *E. coli* cells do not cover the cantilever surface with a closed film (see Fig. 1). Thus, the measured frequency shifts were caused by additional mass load onto the cantilevers. The calculated mass sensitivity of our cantilever sensors is ∼140 pg/Hz, This allows theoretically the detection of 200 *E. coli* cells at the best. Since, the nutrition which the bacteria assimilate out of the layer stays inside the bacterial cell, the mass balance between the bacteria and the thin nutrition layer (∼200 nm) on the cantilever is zero. Consequently, no decrease in frequency due to bacterial growth should be observed. Furthermore, the loss of carbon dioxide during the aerobic metabolism would lead to an increase in the resonance frequency as the cantilevers get lighter. In spite of these facts, we still detect a resonance frequency decrease caused by active bacterial growth. We interpret the detected frequency shifts due to mass load as follows (Fig. 2). The absorbed water in the nutritive layer stays in equilibrium with the humid environment (A). The bacterial cell growth implies an assimilation of, e.g., water, salts, carbohydrates and proteins, and therefore, these compounds will be diminished in the nutritive layer (B). This leads to

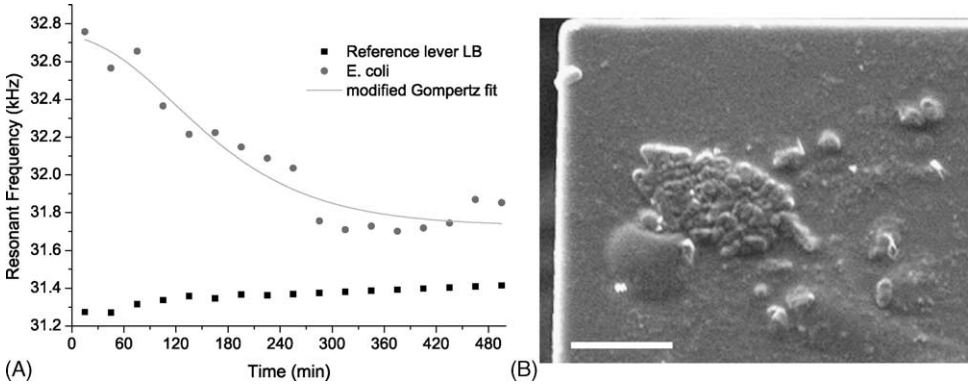

Fig. 1. Detection of bacterial growth. (A) Typical experiment showing frequency decrease due to bacterial growth, whereas the resonance frequency of the reference lever (no bacteria) remains stable. The shown curve corresponds to a modified Gompertz model fit (Baty and Delignette-Muller, 2004). (B) Scanning electron micrograph (SEM) of sensor-cantilever coated with *E. coli* XL1-Blue and a nutritive layer (LB) after 24 h exposure to a warm, humid environment (93% rel. humidity; 37 °C). Accumulations of *E. coli* XL1-Blue cells were observed. In order to reduce the charging effects during SEM imaging, samples were prepared by evaporating a thin layer of Au. The scale bar corresponds to 10 μm.

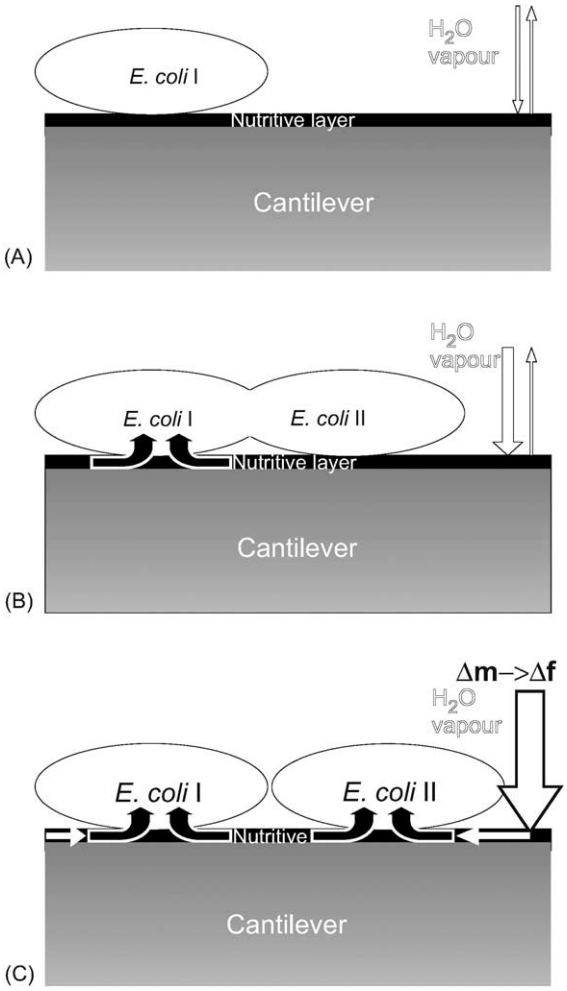

Fig. 2. Scheme of the increasing mass due to bacterial growth on agarose coated cantilever. (A) Freshly deposited *E. coli* cells on top of the coated cantilever. The thin nutritive layer (∼200 nm) stays in equilibrium with the humid environment. (B) The bacterial cells start to grow and assimilate water, protein, salts and carbohydrates out of the nutritive layer. (C) To regain the equilibrium with the humid environment, the nutritive layer absorbs water. This compensation leads to additional mass load onto the cantilever.

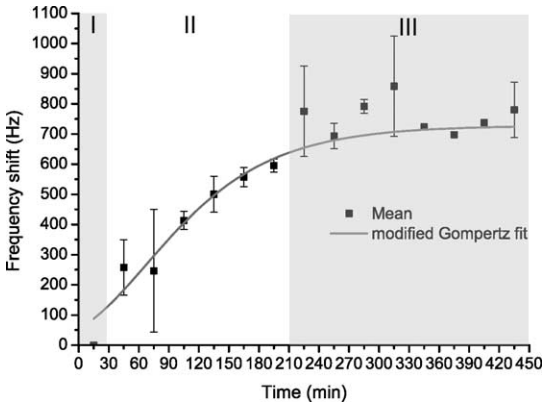

Fig. 3. Mean values of resonance frequency shifts due to bacterial growth of the sensor-cantilever of multiple measurements as a function of time. The shown curve corresponds to a modified Gompertz model fit (Baty and Delignette-Muller, 2004). Bacterial growth phases: (I) lag-phase; (II) exponential-phase; (III) stationary-phase. Bars correspond to the standard deviation.

a compensating absorption of water back into the nutritive layer on top of the cantilever to regain the equilibrium with the environment (C).

For the following experiment, the cantilevers were all coated with agarose within the same batch in order to obtain similar layer thicknesses and furthermore, comparable mechanical properties. In Fig. 3, the mean values of the resonance frequency shifts of multiple measurements are plotted as a function of time. The obtained data were fitted using the modified Gompertz model (Baty and Delignette-Muller, 2004) which describes the bacterial growth. According to Eq. (1), different researchers observed a linear dependence of the frequency shift to cell number (*E. coli*, vaccinia virus particle) bound to the cantilever (Gupta et al., 2004; Ilic et al., 2001). As an outcome of this, we compare the fitted curve with a conventional bacterial growth curve. The growth curve is usually divided into four phases. The "adaptation period", where newly inoculated cells in a fresh medium typically show a lag phase, followed by an exponential growing phase, at which the bacteria cells duplicate at each generation time. The third phase, the stationary phase, starts when the cell density reaches its maximum. The growth is partly inhibited as a fact of the limitation of nutrition. And the last phase, the cell death, where the bacteria start to die due to accumulation of acids (e.g., *Lactobacillus*) or the action of cell's own enzymes (e.g., autolysis). The cell death phase cannot always be observed. In the curve plotted in Fig. 3, the first three mentioned growing phases can be detected. During the exponential growth phase the frequency shifted at a rate of ∼200 Hz/h. The stationary phase was already reached after 5 h of measurement. The short exponential growing phase can be explained by the shortage of nutrition, as the nutritive layer on our sensors had a thickness of approximately 200 nm only. The very short lag phase as well as the quickly starting exponential growth resulted in *E. coli* growth which could be detected easily after 1 h of measurement as a differential shift in the resonance frequency. This is significantly faster than the conventional plating/culturing method where the detection takes at least 24 h (SLMB, 2000). In recent years, different detection methods have been employed to rapidly detect microorganisms such as immunomagnetic separation of the organism followed by PCR identification (Chapman et al., 2001), techniques which are based on antibody–antigen recognition such as surface plasmon resonance (Fratamico et al., 1998), electrochemical impedance sensors (Yang et al., 2004) and quartz crystal microbalance (Su and Li, 2004) as well as bioluminescence measurements (Fujinami et al., 2004; Squirrell et al., 2002). Each of these methods has its own set of drawbacks. Immunological techniques cannot differentiate viable and non-viable cells and often require enrichment. PCR methods as well cannot be used to determine viability. Therefore, we

demonstrate for the first time the use of a micromechanical sensor for the detection of active bacterial growth in less than 1 h.

4. Conclusions

We have demonstrated the use of a nanomechanical sensing device for the detection of active bacterial growth in less than one hour. This new detection system provides numerous advantages such as rapid real-time detection, label-free and small analyte volume, compared to conventional culturing methods. Furthermore, by altering the nutritive layer, as well as the gas-phase in which the measurement take place, the growth of a wide range of different bacterial species can be detected. Moreover, the mass sensitivity of future sensors will be increased by minimizing the dimensions of cantilevers. In addition, the system can easily be adapted to an antibiotic susceptibility testing device by adding antibiotics to the nutritive layer. Therefore, this detection method is a candidate for fast susceptibility measurements in clinical environments. In the future, surface coupled antibodies can be implemented as an additional feature to identify clinical relevant *E. coli* serotypes (e.g., *E. coli* O157:H7). After selective fishing of *E. coli* cells, the measurement can proceed in a humid and temperature controlled environment as reported in this paper to investigate the capability of the attached cells to duplicate.

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