

Micromechanical cantilever array sensors for selective fungal immobilization and fast growth detection

Natalia Nugaeva, Karin Y. Gfeller, Natalija Backmann, Hans Peter Lang, Marcel Düggelin, Martin Hegner*

Institute of Physics, University of Basel, Klingelbergstrasse 82, CH-4056 Basel, Switzerland

Received 1 November 2004; received in revised form 4 February 2005; accepted 4 February 2005
Available online 2 March 2005

Abstract

We demonstrate the use of micromechanical cantilever arrays for selective immobilization and fast quantitative detection of vital fungal spores. Micro-fabricated uncoated as well as gold-coated silicon cantilevers were functionalized with concanavalin A, fibronectin or immunoglobulin G. In our experiments two major morphological fungal forms were used—the mycelial form *Aspergillus niger* and the unicellular yeast form *Saccharomyces cerevisiae*, as models to explore a new method for growth detection of eukaryotic organisms using cantilever arrays. We exploited the specific biomolecular interactions of surface grafted proteins with the molecular structures on the fungal cell surface. It was found that these proteins have different affinities and efficiencies to bind the spores. Maximum spore immobilization, germination and mycelium growth was observed on the immunoglobulin G functionalized cantilever surfaces. We show that spore immobilization and germination of the mycelial fungus *A. niger* and yeast *S. cerevisiae* led to shifts in resonance frequency within a few hours as measured by dynamically operated cantilever arrays, whereas conventional techniques would require several days. The biosensor could detect the target fungi in a range of 10^3 – 10^6 CFU ml⁻¹. The measured shift is proportional to the mass of single fungal spores and can be used to evaluate spore contamination levels. Applications lie in the field of medical and agricultural diagnostics, food- and water-quality monitoring. © 2005 Elsevier B.V. All rights reserved.

Keywords: Micro-fabricated cantilever array; Biosensor; Fungal spore detection; Microfungal selective functionalization; Fungal growth measurement

1. Introduction

Microscopic fungi are human pathogens and serious contaminants in industry and food production. Fast detection of microfungal species is of great importance in medical diagnostics, food quality control, as well as fungal protection for pure material production. Conventional fungal detection and identification methods usually include sampling, cultivation of fungi on artificial media under a variety of conditions, isolation, morphological evaluation of microscopic fungi and microscopy analysis. Although standard microbiological techniques allow detection of single fungal spores, amplification of the signal through growth of a single spore into a colony is required. This process is time-consuming and

requires at least 5–14 days. Moreover, the majority of fungal species are still identified by morphological criteria that require broad taxonomic skill and experience (Hoog de et al., 2000). Current modern tests using immunological and molecular biology methods can identify fungal species without culturing the organism. These methods include radioimmunoassay (RIA) (Talbot et al., 1987), enzyme-linked immunosorbent assay (ELISA), fluorescent antibody methods (Stygen et al., 1995), Western blot technique, nucleic acid hybridization and polymerase chain reaction (PCR) (Hermanson, 1996; Madigan et al., 2003). Because of their high sensitivity, RIA and ELISA are two widely used immunological techniques. They employ radioisotopes and enzymes, respectively, to label antibodies used for microbial antigen detection (Menning et al., 2004). Nucleic acid hybridization is another very sensitive and selective analytical method for detection of specific DNA sequences associated with specific organisms (Meng

* Corresponding author. Tel.: +41 61 367 37 61; fax: +41 61 267 37 84.
E-mail address: martin.hegner@unibas.ch (M. Hegner).

et al., 1996; Sperveslage et al., 1996). However, these procedures are complex and often demand special labelling and require hours of processing.

The development of highly sensitive, selective, label-free, real-time and in situ detection methods for specific fungi will enhance our ability to effectively track microorganisms in different environments. Micro-fabricated cantilever sensors with selective coatings for target immobilization are ideal candidates for biosensing applications. The mass resolution obtained with cantilevers in air is in the pico and the femtogram range (Lang et al., 1999; Baller et al., 2000; Bhalerao et al., 2004; Gupta et al., 2004). Mass changes on the cantilever can accurately be determined by running the instrument in a “dynamic mode”, where the cantilevers are actuated at their resonance frequency. If additional mass is adsorbed onto the cantilever surface, the resonance frequency will be shifted to lower frequencies. These shifts allow calculation of the mass change (Lang et al., 2002). There are few published reports about microcantilever biosensors operating in dynamic mode capable of the detection of microorganisms (Ilic et al., 2000, 2001; Gupta et al., 2004). These authors immobilized bacterial cells and virus particles on antibody activated silicon interfaces and operated their instruments in vacuum or under dry conditions. The obtained results showed high microcantilever sensitivity in dynamic mode in the range of 1.1–7.1 Hz pg⁻¹ (Ilic et al., 2000; Ilic et al., 2001) and 6.3 Hz ag⁻¹ (Gupta et al., 2004) depending on cantilever dimension. However, these experiments were done with inactivated microbes. There is an obvious need for further development of online detection of vital microorganisms where microfungi can serve as an experimental model for eukaryotic cells.

Reproducible coating of individual silicon cantilevers by proteins with high affinity towards specific fungal spores allows selective detection on its interface. In our approach we exploited the affinity of concanavalin A (Con A), fibronectin (Fn) and an anti-*Aspergillus niger* polyclonal antibody (IgG) to provide specific binding sites for fungal membrane surfaces. Con A contains multiple binding sites with high affinities to specific α -D-mannosyl and α -D-glucosyl residues of fungal cell surface carbohydrates (Becker et al., 1976; Bitiger and Scneble, 1976; Mislovicova et al., 2002). Fn is a protein which shows binding affinities to several fungal mannoproteins and negatively charged carbohydrates on the fungal spore surface (Wasylnka and Moore, 2000). Pathogenic yeast and mycelial fungi adhere to Fn molecules at different sites: the 40-kDa GAG-binding domain, the 120-kDa domain and the RGD sequence (Guar and Klotz, 1997; Lima et al., 2001). The ability of Con A and Fn to interact specifically with microbial glycoconjugates made it possible to employ these proteins as surface bound receptors for cells. For a more selective fungal detection fungi type specific antibodies can be used (Muramatsu et al., 1986). We performed *Aspergillus* spore immobilization via highly specific anti-*Aspergillus* polyclonal antibodies for qualitative, specific detection. The antibody molecules can be attached to

the interface in an oriented manner using protein A. This enhances the binding efficiency of the functional layer on the cantilever interface. Thus, applications of different protein coated cantilever allow to reveal microorganisms with glycoproteins on the outer membrane surface (in case of Con A and Fn application), or to extract single species using specific antigen/antibody interactions (IgG case).

Microfungi are gaining importance with the increasing incident of mycoses and production of toxins in contaminated food. More than 100 species can cause human and animal diseases (Hoog de et al., 2000). In our experiments we used two major morphological fungal forms: the mycelial form *Aspergillus niger* and the unicellular yeast form *Saccharomyces cerevisiae*, as models to explore a new method for growth detection of eukaryotic organisms on cantilevers. *A. niger* is the main air contaminants in industry and food production. Furthermore, it is able to cause human and animal aspergilloses (Yeldandi et al., 1995; Severo et al., 1997; Vivas, 1998). *Aspergillus* infections are clearly associated with environmental hygiene and have a high mortality rate in immunocompromised patients (London et al., 1996). Yeast is used for bread, beer production and can cause food spoilage. *S. cerevisiae* and *A. niger* are used as a model to simulate the behavior of the opportunistic human pathogens *Candida albicans* and *A. fumigatus*. Both strains are relatively easy to handle experimentally and are so far the most studied fungi.

In this paper we present a microcantilever based biosensor for fast qualitative detection of fungal species. We propose reproducible Con A, Fn and IgG protein coating procedures of cantilever surfaces for selective immobilization of fungal spores. We demonstrate that cantilever arrays operated in dynamic mode enable both detection of single fungal *A. niger* spores or *S. cerevisiae* yeast cells and monitoring of their germination *in situ*. A major advantage of such a method is a direct growth measurement in humid air (without pre-enrichment and labeling) and the use of reference cantilever to exclude false positive detection.

2. Materials and methods

2.1. Instrumentation

A MultiMode™ AFM head (Digital Instruments, Santa Barbara, USA) was used to measure the frequency response of the microcantilever during excitation. The light of the laser diode was focused on the apex of the cantilever, from where it reflected onto a position-sensitive detector. The resulting resonance spectrum of the excited cantilever was analyzed with the AFM control software (NanoScope® control software).

2.2. Humidity and temperature control

The MultiMode™ head was placed in a temperature controlled box (Intertronic, Switzerland). Temperature and hu-

midity were kept constant during the measurements in a flow chamber. Humidity was generated by flowing pressurized air through water. The temperature and the relative humidity (RH) in the flow chamber were measured with a HygroClip-SC05 sensor (Rotronic, Bassersdorf, Switzerland). The parameters were monitored with a data acquisition board (Type: 6036E, National Instruments, Austin, TX). LabView fuzzy logic control software allowed the regulation of the flow controllers (EL-Flow, Bronkhorst HI-TEC, Reinach, Switzerland), as well as the temperature feedback of the cooling/heating box.

2.3. Cantilever arrays

Two types of microarrays were used: Firstly, silicon cantilever arrays coated with 2 nm Cr/20 nm Au (MikroMasch, Schaeffer-Tec AG, Burgdorf, Switzerland), secondly bare silicon and silicon gold-coated (2 nm TiO₂/30 nm Au) cantilever arrays (Micro-/Nanomechanics Department, IBM Zurich Research Laboratory). The typical dimensions of the MikroMasch cantilevers are: 250 μm/300 μm/350 μm long, 35 μm wide and 2 μm thick; IBM cantilevers are 500 μm long, 100 μm wide and 1 or 4 μm thick. To remove residual traces from micro-fabricated cantilevers a standard cleaning procedure in H₂SO₄ (conc.) in H₂O₂ (30%) (1:1, Piranha solution, caution!) was used. Thereafter TiO₂/Au layers were deposited by e-beam deposition and the interface activated with proteins (Arntz et al., 2003).

2.4. Protein coating procedure

Three different proteins were used for cantilever coating: concanavalin A, fibronectin and anti-*Aspergillus niger* polyclonal antibodies (Fig. 1). Con A and Fn protein coatings were performed directly on gold-coated cantilevers.

IgG was bound specifically onto a protein A layer which was covalently anchored on bare silicon surface as described

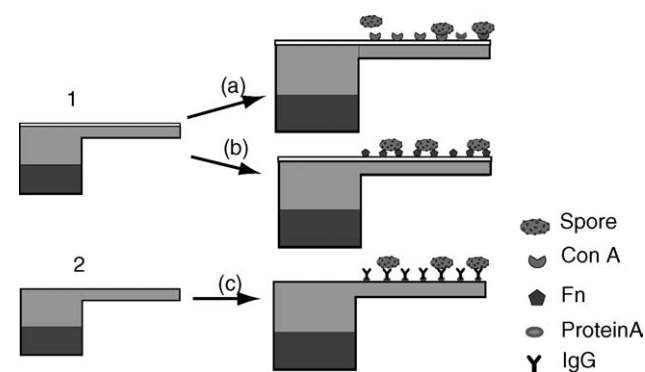


Fig. 1. Schematic of protein coatings and spore immobilization on cantilevers. (1) Gold-coated silicon cantilever: (a) concanavalin A (Con A) binds to sugar residues of glycoproteins of the fungal membrane surface; (b) fibronectin (Fn) interacts with cell-surface receptors of fungi. (2) Silicon cantilever: (c) oriented immunoglobulin G (IgG) anchored on cantilever via protein A selectively reacts with the fungal spore surface.

below. All chemicals and buffer components were purchased from Sigma–Aldrich Chemie GmbH (Buchs, Switzerland).

Con A was used at a concentration of 25 μg ml⁻¹ in 0.5 M 2-(*N*-morpholino)ethanesulfuric acid buffer (MES, pH 6.4) containing 1 mM CaCl₂ and 1 mM MnCl₂. Freshly prepared gold-coated cantilevers were incubated in Con A solution for 1 h at 24 °C. Then the cantilevers were rinsed three times in MES buffer.

Fn was used at a concentration of 25 μg ml⁻¹ in 0.15 M phosphate buffered saline (PBS, pH 7.4). Gold-coated cantilevers were incubated in Fn solution for 1 h at 24 °C, afterwards the cantilevers were rinsed three times in PBS. Fn was provided by Dr. M. Chiquet (University of Bern, ITI Institute).

IgG and protein A were adjusted to 100 μg ml⁻¹ in 0.15 M PBS (pH 7.4). Before the cantilevers could be activated with IgG a protein A coating had to be performed. The silicon arrays were silanized using a 1% (3-aminopropyl)-triethoxysilan solution in toluene for 1 h at 24 °C. The amino-groups of the final silanized layer were activated by 2.5% glutaraldehyde (in PBS buffer) for 1 h at 24 °C and then washed in PBS (in our experiments all samples were washed three times in buffer). Afterwards, the pre-activated cantilever arrays were incubated in a protein A solution over night at 4 °C and washed in PBS. To functionalize the surface with IgG the cantilevers were individually placed in a capillary device containing IgG solution for 2 h at room temperature. After incubation the arrays were washed in PBS. IgG was purchased from Virostat (Portland, ME, USA).

Two experiments were also performed on silicon squares activated with proteins (“control surface”, square size was 5 mm × 5 mm) and placed in a humidity chamber under constant temperature and humidity (24 °C and 96% RH). The first experiment was done to study efficiencies of different protein coatings (as spore immobilization, germination and growth). The second experiment was dedicated to clarify optically the events which took place on the IgG coated surface with *A. niger* immobilized spores. In this experiment we placed silicon samples coated in the same way as sensor cantilever with spores in the microscopy humid chamber under identical conditions (“control surface”) in parallel and collected three samples each 30 min for visualization.

2.5. Fungi and spore immobilization

Both strains, mycelial fungus *A. niger* (#1988) and yeast *S. cerevisiae* (#70449), were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). Strains were revitalized with PBS solution and grown on solid malt extract media. Three week old fungal strains were diluted with 10 ml sterile 0.15 M PBS (pH 7.4) or 0.5 M MES (pH 6.4), mixed for 30 sec and filtered through nitrocellulose filters (Sartorius, Switzerland, pore diameter 8 μm) to separate spores from mycelia. The spore concentrations in the incubation suspension were: for

A. niger 7×10^6 colony-forming units per ml (CFU ml⁻¹), and for *S. cerevisiae* 2×10^3 CFU ml⁻¹. Functionalized cantilevers were incubated in a spore suspension for 45 min using microcapillaries. Microarrays were rinsed first in PBS buffer and then in a nutritive solution (buffer/malt extract mixture, 9:1). Malt extract was used as a soluble nutritive nitrogenous and carbohydrate source for fungal growth. Cantilevers with immobilized spores were intermediately dried and then mounted into the MultiMode™ device for resonance frequency measurements.

2.6. Resonance frequency measurement

The resonance frequency of each cantilever was measured after cleaning and silanization, first in dry and then in humid conditions to obtain the resonance frequency of the “unloaded” sensors in the state prior to exposure to spores. After the immobilization of the receptor proteins and the target spores, the frequency of each cantilever was measured three times in one point in dry conditions (30–31% RH), and afterwards at high humidity (96–97% RH) and constant temperature (27 °C) to obtain frequency of the “loaded” sensor. Every experiment was performed 3–5 times to archive reasonable reproducibility.

2.7. Scanning electron microscopy

Philips XL 30 ESEM was used to visualize the protein-spore-loaded cantilevers.

3. Results and discussion

3.1. Fungal spore detection

Our experiments have demonstrated for the first time that an AFM based technique enables the detection of single fungal spores immobilized on cantilever surfaces functionalized with various proteins.

In all the following experiments, irrespective of cantilever type, fungal species or protein coating, we observed a resonance frequency shift ($\Delta f_1 = f_1 - f_2$) in air after exposure of the functionalized cantilever to a suspension of spores due to absorption of spores onto the cantilevers (Fig. 2). Variations in the peak amplitude reflect minor changes in the position of the laser beam which leads different angular motion read-out. These variations do not affect the resonant frequency, which depends on the spring constant k and the mass m . The spring constant of cantilevers was monitored during the ex-

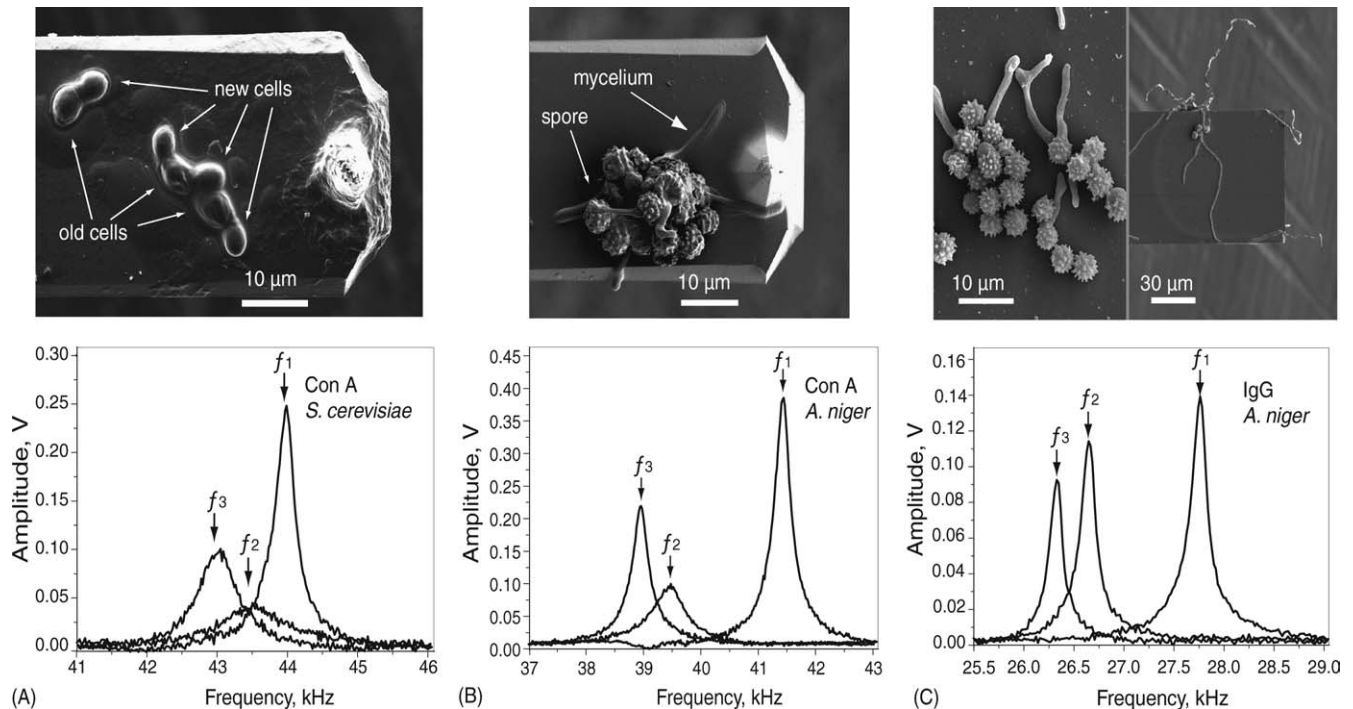


Fig. 2. SEM images of immobilized fungal spores and corresponding frequency spectra of the cantilevers after spore immobilization and spore growth on the protein functionalized surface. (A) yeast *Saccharomyces cerevisiae* on Con A coating: old (three wrinkled cells) and new (four smooth cells) generations (MicroMasch cantilever of 250 μ m long, 35 μ m wide and 2 μ m thick, fundamental mode), (B) mycelial fungus *Aspergillus niger* on Con A coating, mycelia growing from spores (MicroMasch cantilever of 250 μ m long, 35 μ m wide and 2 μ m thick, fundamental mode), (C) *A. niger* on IgG coating, active mycelial growth from spores on IBM cantilever (500 μ m long, 100 μ m wide and 4 μ m thick, fundamental mode; left image—growth time 4 h, right image—growth time 9 h). Resonance frequencies: f_1 —unloaded cantilever, f_2 —cantilever with immobilized spores, f_3 —cantilever with growing immobilized spores after exposure (14, 12 and 4 h for A, B and C, respectively). The first resonance frequency shift $\Delta f_1 = f_1 - f_2$ corresponds to fungal spore mass loaded on the cantilever; second shift $\Delta f_2 = f_2 - f_3$ corresponds to *S. cerevisiae* cell reproduction (A) and to *A. niger* mycelial growth (B, C).

periments. We did not observe changes in the spring constant during the experiment. Thus, we assume that the additional mass of microfungi decreased the resonance frequency of the cantilever.

3.2. Mycelium growth detection

Our results showed that it was possible to distinguish active functionalized fungal spores from dormant/inviable spores within a few hours using cantilever arrays.

One requirement for microbial biosensors is the discrimination between live and dead cells (“viable cell count”) (Ivnitski et al., 1999). It is important to detect not only spores in a sample, but also their ability to germinate and grow. The germination of spores and fungal mycelium growth is clear indication of fungal vitality to produce the next generation of spores, and thus, to be a source of further contamination and infection. In medical diagnostics and food control it is crucial to assess the vitality of microbe spores to use correct treatment.

During subsequent experiments we measured additional reduction of the resonance frequency (a second resonance frequency shift, $\Delta f_2 = f_2 - f_3$), when the activated cantilevers including immobilized spores were placed in the humidity chamber providing favorable conditions for spore germination (27 °C at 97% RH) (Fig. 2). This shift was due to spores starting to germinate on the cantilever surface. In all experiments where a second shift was registered, we visualized that the spores were germinating and mycelia were growing. The minimum time for spore detection was ~1 h. Generally, this time was required to reach stable environmental conditions and a steady measurement of the cantilever frequency. The minimal detection time for spore germination and growth on the protein coated cantilever surface was ~4 h. Conventional microbiological detection and identification methods are considerably more time consuming and require 5–14 days (Davet and Rouxel, 2000; Hoog de et al., 2000).

3.3. Visualization of mycelium growth

We observed spore germination in our experiments by SEM visualization as shown in Fig. 2. The pictures in Fig. 2 correspond to the respective graphs in Fig. 2. The SEM (Fig. 2A) shows that *S. cerevisiae* cells immobilized on Con A are growing by budding. We recognized seven yeast cells on top of the cantilever: three wrinkled “mother” cells producing four new smooth “daughter” cells. This budding, which adds mass to the cantilever, can be measured as resonance frequency shift ($\Delta f_2 = f_2 - f_3$) as shown in Fig. 2A. To discriminate old from new generations of budding yeast cells one needs SEM visualization. The two generations differ slightly only in the topography of the cells (wrinkled versus smooth). To be able to clearly distinguish two well-defined morphological phases (spores stage, and germinating/growing stage) by optical microscopy or SEM we utilized mycelial fungus *A. niger* (Fig. 2B and C). Spores of *A. niger* were immo-

bilized on Con A (Fig. 2B) and on IgG (Fig. 2C) layers, and fungal mycelia germinated from spores grew in different directions. The mycelia were visually spreading as a net of hyphae, which expanded over the cantilever surface over large distances (i.e. several tens of microns in few hours). Germination and active mycelial growth of *A. niger* spores on Fn coated cantilever surfaces were also confirmed by SEM images.

3.4. Differential measurement

To eliminate external parameters (undesired environmental changes in the chamber) which could affect the resonance frequency measurement of the sensor cantilever, we used two reference cantilevers. Fig. 3 shows the resonance frequency measurements of a sensor cantilever with spores immobilized on a IgG layer and two reference cantilevers: one uncoated (bare, reference cantilever 1) and another coated

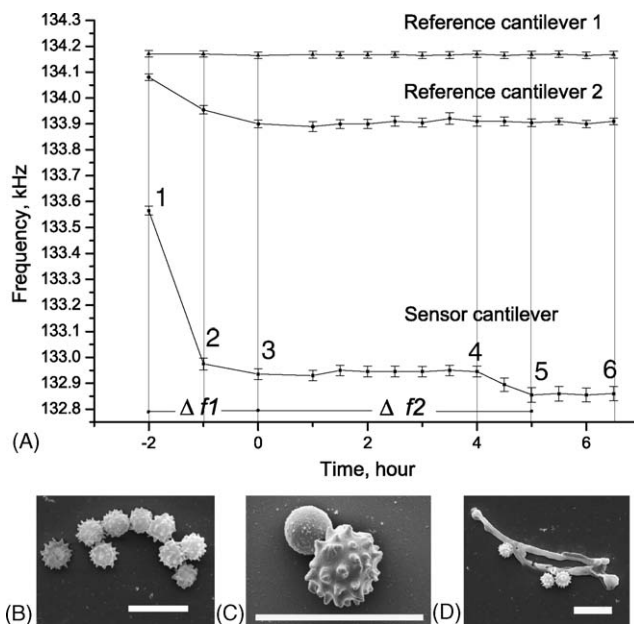


Fig. 3. (A) Resonance frequency changes of two reference cantilevers with-out spores (1—uncoated cantilever, 2—coated with IgG cantilever) and a sensor cantilever (activated with IgG and immobilized spores of *A. niger*). Points: 1–2—dry conditions (30% RH), 2–3—intermediate period (relative humidity was increasing from 30% to 97% RH) and 3–6—humid environment (96% RH). The resonance frequency of uncoated reference cantilever 1 did not change significantly during the complete experiment. The reference cantilever 2 coated with corresponding proteins reacted on protein immobilization and water vapor adsorption on the surface. Immobilization and growth of fungal spores caused decreases in resonance frequency of the sensor cantilever with respect to the reference cantilevers. The Δf_1 corresponded to spore, protein layer immobilizations (1–2) and water absorption (2–3) (the relative humidity was increased from 30% to 97%), Δf_2 included different spore growth stages—lag phase (3–4), log phase (4–5), deceleration and stationary phases (5–6). SEM images of the events that occurred simultaneously on control silicon surfaces with IgG and immobilized *A. niger* spores: (B) dormant spores (lag phase, 3–4); (C) spores broke their dormancy and started to germinate and grow (log phase, 4–5); (D) the mycelial growth from spores (deceleration and stationary phases, 5–6). Scale bar is 10 μ m.

with IgG only (reference cantilever 2 without spores). Measurements were performed under constant temperature in dry conditions (points 1–2, 30% RH) and under humid condition (points 3–6, 97% RH), when an equilibrium between adsorption from the vapor phase and desorption from the cantilever was reached and the cantilever frequency reached a steady state. The resonance frequency of the bare reference cantilever did not change significantly during the experiment ($134,170 \pm 20$ Hz). The reference cantilever 2 coated only with protein IgG reacted on protein deposition and increased humidity due protein immobilization and water adsorption from the surroundings. The resonance frequency decreased during this period by 180 ± 5.7 Hz. Afterwards the cantilever frequency stabilized at constant level $\sim 133,900$ Hz.

A quite different mechanical response of the sensor cantilever with immobilized spores was observed. Two clear steps in resonance frequency measurement were recorded. The first was measured after spore immobilization, their swelling and water adsorption on the protein layer ($\Delta f_1 = -635$ Hz, points 1–3). The second step was observed after 4 h exposure of the spore laden sensor cantilever to a humid environment ($\Delta f_2 = -95$ Hz, points 4–5). After 5 h the resonance frequency of sensor cantilever settled at $\sim 132,860$ Hz. We simulated the same conditions on the IgG activated silicon surfaces (“control surface” see Section 2) and visualized the changes that occurred on the interface by SEM. It was found that spores were dormant for approximately 3 h and then started to germinate (to form germinative tubes) and 1 h later active mycelial growth was registered. We interpreted the last shift $-\Delta f_2$ as the start of the germination of immobilized spores and the mycelium spreading across the cantilever surface. Since about 92–94% of the living functional fungus consists of water, this is the substance which is taken up in the large quantity from the environment in humid chamber. During growth, the extended size of the microfungus (increasing of membrane surface and intracellular volume) demands water absorption from coating layer and humid air which leads to more water bound to the interface in a physical manner, water accumulation inside cell/mycelium, and, therefore, a mass increase.

Generally, to grow on the cantilever the fungus consumes nutrition from coating layer and metabolizes it, and as a result, the mass of cantilever should decrease. However, at the same time, the growing fungus takes up water (the content of water of metabolizing fungi is more than 90% to total fungal mass). The process of water absorption dominates the metabolic process, which leads to additional mass on the cantilever.

The growth of mycelia continued for ~ 1 h until an essential nutrient became limiting and/or metabolic products accumulated to growth inhibitory levels and as the result the resonance frequency leveled off (points 5–6). There was no measurable decrease in resonance frequency when the spores were dormant or inviable under humid conditions (data not shown). The fungal spore development on the protein coated cantilever is comparable to the mycelial fungal growth curve

in culture (Melitiadis et al., 2001). The phases 3–4, 4–5, 5–6 correspond to lag phase (spore stage), exponential (log phase), deceleration and stationary (mycelial stage) phases respectively.

In our experiments, triggering factors for fungal growth on the thin protein layers (thickness of 20–30 nm) were high environmental humidity and the presence of easily degrading nutrition.

3.5. Determination of the mass of an individual spore

Minuscule mass changes on a cantilever can be detected by oscillating the cantilever at its resonance frequency using an external piezoelectric crystal for actuation. The resonance frequency shift of the oscillating cantilever depends on the mass change, Δm . For a rectangular cantilever oscillating in air, Δm can be calculated as follows (Chen et al., 1995):

$$\Delta m = k_{CL}(4n\pi^2)^{-1}(f_1^{-2} - f_0^{-2}), \quad (1)$$

where k_{CL} is the spring constant of the cantilever, n is a geometry-dependent correction factor ($n=0.24$ for rectangular cantilevers), f_0 is the fundamental resonance frequency in air, f_1 is the resonance frequency after mass loading.

We measured the resonance frequencies of the oscillating cantilever f_0 and f_1 during the experiments. The normal spring constant, k_{CL} , was determined using the dimensions of the cantilever visualized by SEM, its fundamental resonant frequency and quality factor in air (Sader et al., 1999):

$$k_{CL} = 0.1906\rho b^2 L Q f^2 \Gamma_i^f(f), \quad (2)$$

where ρ is the density of the medium in which the cantilever oscillates, b and L are the width and length, Q and f are quality factor and resonance frequency in fundamental mode, respectively, and Γ_i^f is the imaginary part of the function Γ (refer to Eq. (20) for analytical expression for Γ , Sader, 1998). The equation for the calculation of mass loaded on the cantilever is valid under the following conditions: the cantilever consists of a rectangular beam with one fixed and one free end; length l_{CL} significantly larger than thickness t_{CL} ; the flexural rigidity of the bare cantilever is assumed to be constant; the mass loaded on the cantilever should be much less than the mass of the cantilever; the mass is considered to be distributed uniformly or at the apex of the cantilever (Chen et al., 1995; Lang et al., 1999; Sader et al., 1999; Davis et al., 2000; Abadal et al., 2001; Battiston et al., 2001; Pinnaduwege et al., 2003; Ilic and Craighead, 2004; Ziegler, 2004). These considerations led to a calculated k_{CL} of 0.85 N/m and 0.73 N/m for the cantilevers used (Fig. 2A and B, respectively).

We estimate the mass changes after the immobilization of fungal *S. cerevisiae* cells and *A. niger* spores on Con A functionalized cantilevers for the experiments shown in Fig. 2. The resonance frequencies before and after *S. cerevisiae* immobilization were 43,940 Hz and 43,020 Hz, respectively, with a Q factor of ~ 116 (see Fig. 2A). The resonance fre-

Table 1
Efficiencies of concanavalin A, fibronectin, immunoglobulin G coatings for *A. niger* spore immobilization, germination and growth

Type of coating	Density of spores (CFU mm ⁻¹)	Number of germinated spores, % to total number	Mycelial rate, μm h ⁻¹
Concanavalin A	320 ± 28	14 ± 3	1.8 ± 0.6
Fibronectin	599 ± 58	23 ± 5	1.0 ± 0.3
Immunoglobulin G	642 ± 34	32 ± 7	3.8 ± 0.5

quencies before and after *A. niger* spore immobilization were 40,900 Hz and 39,480 Hz, respectively, giving a Q factor of ~ 108 (Fig. 2B). Thus, the resonance frequency shifts were ~ 920 Hz due to the mass of *S. cerevisiae* cells and ~ 1420 Hz due to the mass of *A. niger* spores. Using Eqs. (1) and (2) and counting the cells respectively the spores as visible by SEM, we calculated that the mass of a single *S. cerevisiae* cell was ~ 69 pg, and *A. niger* spore was ~ 47 pg. The sensitivity of the cantilevers used (MicroMasch cantilevers of 250 μm length, 35 μm width, 2 μm thick) were 1.9 pg Hz⁻¹ (fundamental mode) and 0.2 pg Hz⁻¹ (second mode). Experimentally obtained data can be used for the evaluation of the spore contamination level in samples to be investigated using the mass detection technique.

In conclusion, online observation and monitoring of microscopic spores using the high sensitivity of cantilever arrays in humid air is presented as a new method to detect fungal contamination at the level of individual spores.

3.6. Protein coatings

In our experiments we exploited the affinity of Con A, Fn and IgG proteins to target different structures of the fungal membrane surface. While less specific than IgG, protein coatings with Con A and Fn present a possibility to immobilize a wide range of microorganisms which have glycoproteins embedded into their outer membrane surface. However, the selective immobilization of *Aspergillus* spores is possible using the highly specific anti-*Aspergillus* polyclonal antibodies. In our preliminary test experiments the selectivity of surface-bound IgG towards *A. niger* was tested in a mixture including one other fungal species (*A. niger* + *Mucor hiemalis*, data not shown in the paper). We confirmed that chosen polyclonal IgG reacted specifically with *Aspergillus* spores in the mixture of two species. And as a result of this experiment we chose this IgG for specific (selective) immobilization of *Aspergillus* spores on the cantilevers. Generally, the IgG coating is presented in our paper as a model sensitization which is highly adaptable for future development. In future research, various specific antibodies to detect different fungal species on cantilever arrays in parallel could be applied.

Functionalization of the cantilever is very important for further development of the technique, in particular, if antibodies are used for molecular recognition then the orientation of antibodies coupled to the surface must be controlled (Arntz et al., 2003). We used protein A as ‘crosslinker’ on the interface between the silicon cantilever surface and IgG

to avoid random IgG variable domains orientation. The efficiency criteria evaluated for our tested coatings were: (1) the density of spores immobilized on the interface; (2) the percentage of germinating spores; (3) the mycelial growth rate (see Table 1). All three studied protein functionalizations showed a good efficacy for spore deposition on cantilevers. However, sufficient density of immobilized spores ~ 640 CFU (mm²)⁻¹, maximum germinated spores $\sim 30\%$ and fastest mycelial growth ~ 4 μm h⁻¹ were observed on IgG functionalized cantilever arrays.

To summarize, all studied protein coatings were reproducible, selective and convenient for fungal spore immobilization, germination as well as for resonance frequency measurements of micro-fabricated cantilevers in humid air. The IgG coated cantilever exhibited the best growth conditions of the three tested types of protein coatings.

4. Conclusions

This paper presents the development and optimization of protein coating procedures for fungal spore immobilizations and mycelial active growth detection on microcantilever surfaces. The immobilization of the spores and cells is based on surface grafted proteins with affinities to cell-surface receptors of the fungal cell wall. Microorganism specific depositions were enabled via concanavalin A, fibronectin and highly specific immunoglobulin G protein activating of the cantilever interfaces. We revealed that these proteins had different affinities and efficiencies to bind fungal spores (IgG > Con A > Fn). Maximum spore immobilization, germination and mycelium growth were observed on IgG functionalized cantilever surfaces. The possibility for fast adaptation of our protein coating protocol toward fungal species of interest is an advantage of the methods presented in this paper.

Our results show that cantilever arrays operated in dynamic mode in humid air allow a quantitative and qualitative detection of single fungal spores as well as selective detection of vital functionalized spores in situ within ~ 4 h which is more than ten times faster than current applied standard procedures for fungal detection.

Acknowledgements

We acknowledge Dr. V. Barwich and G.M. Krishna for helpful discussion, Dr. M Chiquet for the generation of purified fibronectin and J.P. Ramseyer for the help provided

to assemble the instrumentation parts. This research work was supported by the TopNano 21 program (Grant KTI 5956.2 TNS).

References

- Abadal, G., Davis, Z.J., Helbo, B., Borrise, X., Ruiz, R., Boisen, A., Campabadal, F., Esteve, J., Figueras, E., Peres-Murano, F., Barniol, N., 2001. Electromechanical model of a resonating nano-cantilever-based sensor for high-resolution and high sensitivity mass detection. *Nanotechnology* 12, 100–104.
- Arntz, Y., Seelig, J.D., Lang, H.P., Zhang, J., Hunziker, P., Ramseyer, J.P., Meyer, E., Hegner, M., Gerber, C.H., 2003. Label-free protein assay on a nanomechanical cantilever array. *Nanotechnology* 14, 86–90.
- Baller, M.K., Lang, H.P., Fritz, J., Gerber, Ch., Gimzewski, J.K., Drechsler, U., Rothuizen, H., Despont, M., Vettiger, P., Battiston, F.M., Ramseyer, J.P., Fornaro, P., Meyer, E., Güntherodt, H.J., 2000. A cantilever array-based artificial nose. *Ultramicroscopy* 82, 1–9.
- Battiston, F.M., Ramseyer, J.P., Lang, H.P., Baller, M.K., Gerber, Ch., Gimzewski, J.K., Meyer, E., Güntherodt, H.J., 2001. A chemical sensor based on a microfabricated cantilever array with simultaneous resonance-frequency and bending readout. *Sens. Actuators B: Chem.* 77, 121–131.
- Becker, J., Reeke, G., Cunningham, B., Edelman, G., 1976. New evidence on the location of a saccharide-binding site of concanavalin A. *Nature* 259, 406.
- Bittiger, H.S., Schneble, H.P., 1976. *Concanavalin as a Tool*. Wiley-Interscience, London.
- Bhalerao, K.D., Mwenifumbo, S.C., Soboyejo, A.B.O., Soboyejo, W.O., 2004. Bounds in the sensitivity of BioMEMS devices for cell detection. *Biomed. Microdevices* 6 (1), 23–31.
- Chen, G.Y., Thundat, T., Wachter, A., Warmack, J., 1995. Adsorption-induced surface stress and its effect on resonance frequency of microcantilevers. *J. Appl. Phys.* 77 (8), 3618–3622.
- Davet, P., Rouxel, F., 2000. *Detection and Isolation of Soil Fungi*. Science Publishers Inc., Enfield.
- Davis, Z.J., Abbatial, G., Kuhn, O., Hansen, O., Grey, F., Boisen, A., 2000. Fabrication and characterization of nanoresonating devices for mass detection. *J. Vac. Sci. Technol. B* 18 (2), 612–616.
- Guar, N.K., Klotz, S.A., 1997. Expression, cloning, and characterization of a *Candida albicans* gene, ALA1, that confers adherence properties upon *Saccharomyces cerevisiae* for extracellular matrix proteins. *Infect. Immun.* 65 (12), 5289–5294.
- Gupta, A., Akin, D., Bashir, R., 2004. Single virus particle mass detection using microresonators with nanoscale thickness. *Appl. Phys. Lett.* 84 (11), 1976–1978.
- Hermanson, G.T., 1996. *Bioconjugate Techniques*. Academic Press, San Diego.
- Hoog de, G.S., Guarro, J., Gene, J., Figueras, M.J., 2000. Atlas of Clinical Fungi. In: *Centraalbureau voor Schimmelcultures*, second ed. Universitat Rovira i Virgili, Reus, Utrecht.
- Ilic, B., Czaplowski, D., Craighead, H.G., 2000. Mechanical resonant immunospecific biological detector. *Appl. Phys. Lett.* 77 (3), 450–452.
- Ilic, B., Czaplowski, D., Zalalutdinov, M., Craighead, H.G., 2001. Single cell detection with micromechanical oscillators. *J. Vac. Sci. Technol. B* 19 (6), 2825–2828.
- Ilic, B., Craighead, H.G., 2004. Attogram detection using nano-electromechanical oscillators. *J. Appl. Phys.* 95 (7), 3694–3703.
- Ivnitski, D., Abdel-Hamid, I., Atanasov, P., Wilkins, E., 1999. Biosensors for detection of pathogenic bacteria. *Biosens. Bioelectron.* 14, 599–624.
- Lang, H.P., Baller, M.K., Berger, R., Gerber, Ch., Gimzewski, J.K., Battiston, F.M., Fornaro, P., Ramseyer, J.P., Meyer, E., Güntherodt, H.J., 1999. An artificial nose based on a micromechanical cantilever array. *Anal. Chim. Acta* 393, 59–65.
- Lang, H.P., Hegner, M., Gerber, Ch., 2002. Nanomechanics—the link to biology and chemistry. *Chimia* 56, 515–519.
- Lima, O.C., Figueiredo, C.C., Previato, J.O., Mendonça-Previato, L., Morandi, V., Bezerra, L.M., 2001. Involvement of fungal cell wall components in adhesion of *Sporothrix schenckii* to human fibronectin. *Infect. Immun.* 69 (11), 6874–6880.
- London, K.W., Cook, A.P., Burnie, J.P., Oppenheim, B.A., Morris, C.Q., 1996. Kitchens as a source of *Aspergillus niger* infection. *J. Hosp. Infect.* 32, 191–198.
- Madigan, M.T., Martinko, J.M., Parker, J., 2003. *Biology of Microorganisms*, 10th ed. Pearson Education Inc., Upper Saddle River.
- Meng, J.H., Zhao, S.H., Doyle, M.P., Kresovich, S., 1996. Polymerase chain-reaction for detecting *E. coli* O157:H7. *Int. J. Food Microbiol.* 32 (1/2), 103–113.
- Menning, M., Donnelly, J.P., Verweij, P.E., 2004. Detection of circulating galactomannan for the diagnosis and management of invasive aspergillosis. *Lancet Infect. Dis.* 4, 349–357.
- Melitiadis, J., Meis, J.F.G.M., Mouton, J.M., Verweij, P.E., 2001. Analysis of growth of filamentous fungi in different nutrient media. *J. Clin. Microbiol.* 39 (2), 478–484.
- Mislovicova, D., Masarova, J., Svitel, J., Gemeiner, P., 2002. Influence of mannane epitopes in glycoproteins-Concanavalin A interaction comparison of natural and synthetic glycosylated proteins. *Int. J. Biol. Macromol.* 30, 251–258.
- Muramatsu, H., Kajiwara, K., Tamiya, E., Karube, I., 1986. Piezoelectric immunosensor for the detection of *Candida albicans* microbes. *Anal. Chim. Acta* 188, 257–261.
- Pinnaduwa, L.A., Hawk, G.E., Boiadjev, V., Yi, D., Thundat, T., 2003. Use of microcantilever for the monitoring of molecular binding to self-assembled monolayers. *Langmuir* 19, 7814–7844.
- Sader, J.E., 1998. Frequency response of cantilever beams immersed in viscous fluids with applications to atomic force microscope. *J. Appl. Phys.* 84 (1), 64–76.
- Sader, J.E., Chon, J.W.M., Mulvaney, P., 1999. Calibration of rectangular atomic force microscope cantilevers. *Rev. Sci. Instrum.* 70 (10), 3967–3969.
- Severo, L.C., Geyer, R.G., Porto, N.S., Wagner, M.B., Landero, A.T., 1997. Pulmonary *Aspergillus niger* intracavitary colonization. Report of 23 cases and review of the literature. *Revta Iberoam. Micol.* 14, 104–110.
- Sperveslage, J., Stackerbrandt, E., Lembke, F.W., Koch, C., 1996. Detection of bacterial-contamination, including bacillus spores, in dry growth media and in milk by identification of their 16S rDNA by polymerase chain-reaction. *J. Microbiol. Methods* 26 (3), 219–224.
- Stygen, D., Goris, A., Safrati, J., Latge, J.P., 1995. A new sensitive sandwich enzyme-linked immunosorbent assay to detect galactofuran in patients with invasive aspergillosis. *J. Clin. Microbiol.* 33, 497–500.
- Talbot, G.H., Weiner, M.N., Gerson, S.L., Provencher, M., Hurwitz, S., 1987. Serodiagnosis of invasive aspergillosis in patients with hematologic malignancy: validation of *Aspergillus fumigatus* antigen radioimmunoassay. *J. Infect. Dis.* 155, 12–27.
- Vivas, C., 1998. Endocarditis caused by *Aspergillus niger*: case report. *Clin. Infect. Dis.* 27, 1322–1323.
- Wasylnka, J.A., Moore, M.M., 2000. Adhesion of *Aspergillus* species to extracellular matrix proteins: evidence for involvement of negatively charged carbohydrates on the conidial surface. *Infect. Immun.* 68, 3377–3384.
- Yeldandi, V., Laghi, F., McCabe, M.A., Larson, R., O'Keefe, P., Hussain, A., Montoya, A., Carrity, E.R., 1995. *Aspergillus* and lung transplantation. *J. Heart Lung Transpl.* 14, 883–890.
- Ziegler, Ch., 2004. Cantilever-based biosensor. *Anal. Bioanal. Chem.* 379, 946–959.