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An Antibody-Sensitized Microfabricated Cantilever for the Growth Detection of *Aspergillus niger* Spores

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Abstract: We demonstrate a new sensitive biosensor for detection of vital fungal spores of *Aspergillus niger*. The biosensor is based on silicon microfabricated cantilever arrays operated in dynamic mode. The change in resonance frequency of the sensor is a function of mass binding to the cantilever surface. For specific *A. niger* spore immobilization on the cantilever, each cantilever was individually coated with anti-*Aspergillus niger* polyclonal antibodies. We demonstrate the detection of single *A. niger* spores and their subsequent growth on the functionalized cantilever surface by online measurements of resonance frequency shifts. The new biosensor operating in humid air allows quantitative and qualitative detection of *A. niger* spores as well as detection of vital, functional spores *in situ* within ~4 h. The detection limit of the sensor is 10^3 CFU mL^{-1}. Mass sensitivity of the cantilever sensor is ~53 pg Hz^{-1}.

Key words: biosensor, microfabricated cantilever array, fast fungal growth detection, anti-*Aspergillus niger* polyclonal antibodies

Introduction

Airborne fungus *Aspergillus niger* is gaining importance as a spoilage agent, main air contaminator in industry, and serious pathogen of humans. Most of the fungal detection and identification approaches are based on conventional microbiological methods, which are time consuming and require high professional skills (Hoog et al., 2000).

The development of real-time, sensitive, and selective detection methods for specific fungi will enhance our ability to effectively track microorganisms in different environments. Micro- and nanofabricated cantilever sensors with selective coatings for target immobilization represent a promising new technique for sensing applications in the field of chemistry and biology (Lang et al., 1998). These types of sensors have been successfully applied in the gas-sensing field (Baller et al., 2000), in genomics (Fritz et al., 2000; Hansen et al., 2001; McKendry et al., 2002), and proteomics (Moulin et al., 2000; Arntz et al., 2003; Liu et al., 2003; Backmann et al., 2005). The mass resolution obtained with cantilevers in air is in the femto- to picogram range (Lang et al., 1999; Baller et al., 2000; Ilic et al., 2001; Weeks et al., 2003; Bhalerao et al., 2004; Gupta et al., 2004; Gfeller et al., 2005).

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In microbiology, oscillating cantilevers have been applied as a microbalance for bacteria and virus particles in vacuum or dry air (Ilic et al., 2000, 2001; Gupta et al., 2004). These experiments were performed with inactivated microbes that did not allow determination of microbe viability and, therefore, the use of adequate inhibitors for treatment.

Our aim was to selectively detect vital fungal spores of *A. niger* using microcantilever arrays. To reach this goal, first, we applied a specific cantilever coating to provide binding sites for *A. niger* spores, and, second, we used sensitized sensors and reference cantilevers *in situ* to eliminate external influence during our measurements and to reveal online viable and inactive/dormant spores.

Materials and Methods

Reagents

All chemicals and buffer components were purchased from Sigma–Aldrich Chemie GmbH (Buchs, Switzerland). Anti-*Aspergillus niger* polyclonal antibodies (IgG) were purchased from Virostat (Portland, ME).

Cantilever Arrays

Silicon cantilever arrays were fabricated in the Micro/Nanomechanics group, IBM Zurich Research laboratory, Switzerland (Fig. 1a). Eight cantilevers are linearly arranged

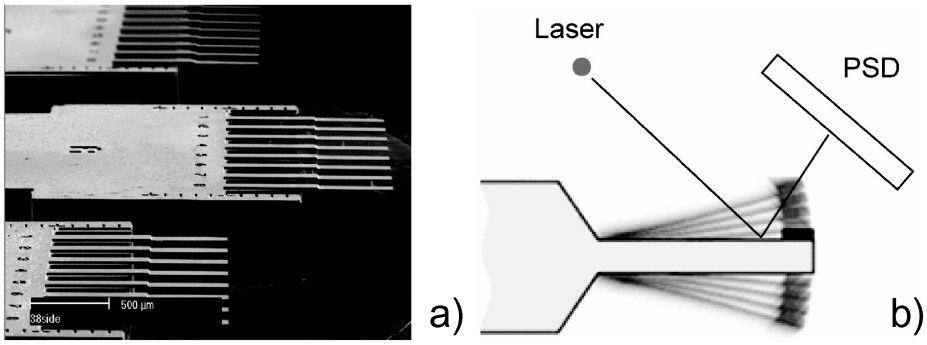

Figure 1. IBM microbabricated cantilever array. Dimensions: 500 μm long, 100 μm wide, and 4 μm thick (**a**). Detection scheme (**b**).

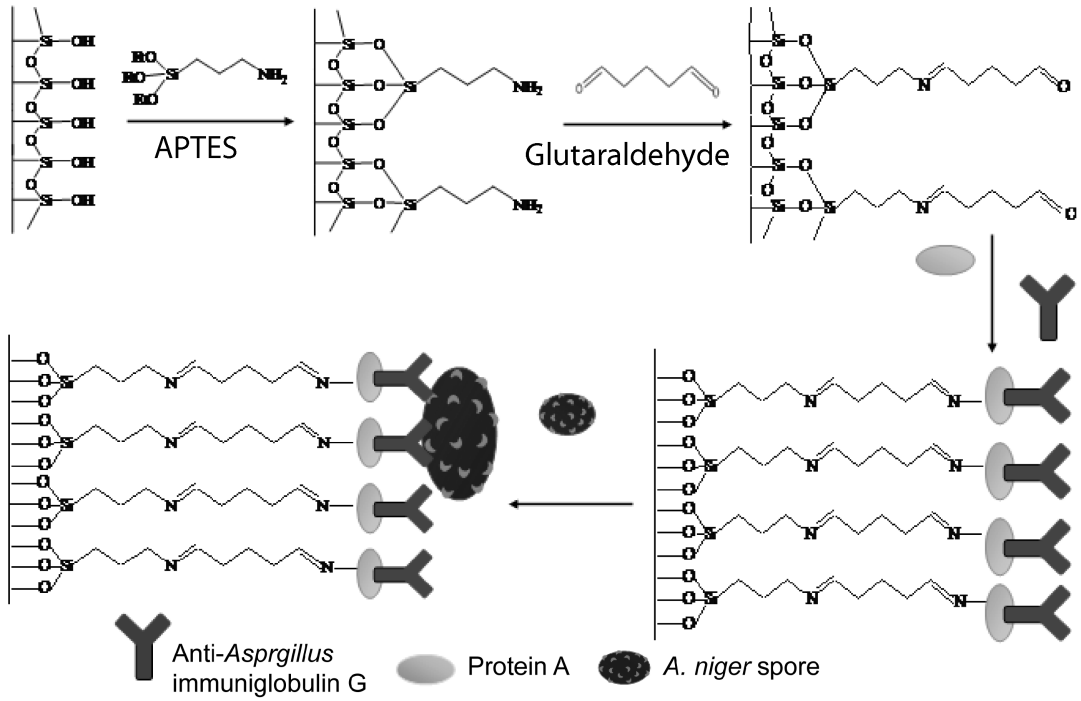

Figure 2. Scheme of cantilever functionalization.

in an array at a pitch of 250 μm, each of them 500 μm long, 100 μm wide, and 4 μm thick. Before the functionalization, the cantilever arrays were cleaned to remove contaminations from the silicon surfaces in Piranha solution (97% H_2SO_4 conc. in 30% H_2O_2, 1:1) for 20 min and, afterward, rinsed twice with sterile-filtrated water and once with deionized water. The cleaning procedure was performed twice.

Instrumentation

The frequency response of the microcantilever during excitation was measured using a MultiMode™ AFM head (Digital Instruments, Santa Barbara, CA). The light of the laser diode was focused on the apex of the cantilever, from where it was reflected onto a position-sensitive detector. Afterward, the signal was transmitted to a frequency generator and frequency analyzer (Fig. 1b). The resulting resonance spectrum of the excited cantilever was analyzed with the AFM control software (NanoScope® control software). To keep temperature and humidity constant, the MultiMode™ head was placed in a humid flow chamber in a temperature-controlled box (Intertronic, Switzerland). The parameters were monitored with a data acquisition board (Type: 6036E, National Instruments, Austin, TX). LabView software allowed regulation of temperature and air flow (EL-Flow, Bronkhorst HI-TEC, Reinach, Switzerland).

Procedure of Cantilever Fuctionalization

Immobilization of fungal spores was performed via anti-*Aspergillus niger* polyclonal antibodies (IgG) (Fig. 2) anchored in an oriented manner on protein A activated silicon

cantilevers. Anti-*Aspergillus niger* polyclonal antibodies and protein A were adjusted to 100 μg ml^{-1} in 0.15 M PBS (pH 7.4). Protein A, glutaraldehyde, and (3-Aminopropyl)-triethoxysilan depositions had to be performed before the cantilevers could be activated with IgG. The silicon arrays were silanized using a 1% (3-Aminopropyl)-triethoxysilan solution in toluene for 2 h at 24°C. The amino-groups of the newly silanized layer were activated by 2.5% glutaraldehyde (in PBS buffer) for 1 h at room temperature and then washed in PBS (in our experiments all samples were washed three times in buffer). Afterward, the prefunctionalized surface of the cantilever arrays were incubated in a protein A solution overnight at 4°C and washed in PBS.

To functionalize the surface with IgG each cantilever was individually placed in a microcapillary (Garner Glass, Inc., Clarmont, CA; Bietsch et al., 2004) containing IgG solution, and incubated for 2 h at room temperature. After incubation the arrays were washed in PBS.

To confirm functionalization of the cantilever surface, contact angle measurements were done using the sessile drop method (de Gennes, 1985) after cleaning in Piranha solution, APTES, glutaraldehyde depositions, and after IgG immobilization.

Fungi

Fungal strain *A. niger* (#1988) was purchased from DSMZ (Germany). The revitalization of dry fungal culture was performed according DSM recommendations. To prepare final spore suspension for spore immobilization, 3-week-old *A. niger* culture was diluted with 10 ml sterile 0.15 M PBS (pH 7.4), mixed for 20 s, and filtered through nitrocellulose filters (Sartorius, Switzerland; pore diameter 8 μm) to separate spores from mycelia. Antibody-functionalized cantilevers in the same array were incubated in quartz microcapillaries. The sensor cantilevers were exposed to an *A. niger* suspension in PBS and reference cantilevers were immersed in PBS without *A. niger* spores simultaneously. After incubation in microcapillaries for 45 min, microarrays were first rinsed in PBS buffer and then in a nutritive solution (PBS/Malt-extract mixture, 1:1). Afterward, the functionalized cantilevers were mounted into the MultiMode device for the measurement.

The final spore concentration was 17×10^3 CFU ml^{-1}.

Visualization of immobilized spore and fungal growth was done using a Philips XL 30 ESEM.

Results and Discussion

Cantilever Silicon Surface Functionalization

We developed a new coating protocol for specific *A. niger* spore immobilization. Our approach was based on an aminosilan film, which self-assembled onto the hydroxylated surface of a silicon cantilever surface. Functional amino groups of the silanized cantilever surface were modified with glutaraldehyde, which can, in turn, react with amines on the antibody or with other biomolecules (Baselt et al., 1997). The immobilization of antibodies was a critical component in the experiment. We used anti-*Aspergillus niger* polyclonal antibodies to exploit their affinity to specific binding sites of fungal membrane surfaces. In our first experiments, we immobilized IgG directly on the functionalized by aminosilan and glutaraldehyde silicon surface of cantilevers. However, better results were achieved when protein A was applied as a crosslinker between the modified interface of the silicon cantilever surface and anti-*Aspergillus niger* polyclonal antibodies (Fig. 2). Attachment of antibodies directly to substrates frequently reduces their ability to bind antigen. Binding antibodies in controlled orientation and/or allowing them mobility on the end of a "tether" molecule (Hinterdorfer et al., 1996) can enhance the fraction of antibodies that remain fully functional, potentially improving the sensitivity of the biosensor. We used protein A to avoid random IgG variable domain orientation and, therefore, to provide more binding sites for antigens of outer fungal membrane.

Table 1. Hydrophobicity of Silicon Surface after Functionalizations; Contact Angle of Silicon Surface, in Degrees, after Functionalization with Reagents

Piranha solution (H_2SO_4 and H_2O_2, 1:1)	APTES and glutaraldehyde deposition	IgG deposition
26.7 ± 2.4	54.7 ± 4.2	57.6 ± 2.1

The efficiency of the IgG coating was characterized by the following parameters using scanning electron microscopy (SEM): density of immobilized *A. niger* spores (642 ± 34 CFU (mm^2)$^{-1}$), number of germinated spores (32 ± 7%), fungal mycelial rate (32 ± 7 μm(h)$^{-1}$). The comparison with the same parameters based on concanavalin A and fibronectin coatings applied for spore *A. niger* loading demonstrated that IgG is more effective for spore immobilization (Nugaeva et al., 2005).

Results of the contact angle measurements (Table 1) confirmed the surface modification due to the silanization process and protein coating of the silicon cantilevers.

Thicknesses of protein deposits were in a range of 20–25 nm as measured by scanning probe microscopy (data not shown).

Generally, any fungal species could be detected using the above described scheme. The elegance of the proposed method is the possibility of easy modification of the final coating layer for targeting species, which is highly species/genus specific.

The IgG coating was easy to reproduce and convenient for *A. niger* spore immobilization and germination.

Detection of Spores and Their Germination

Our experiments demonstrated for the first time that an AFM-based technique allows (1) detection of single fungal

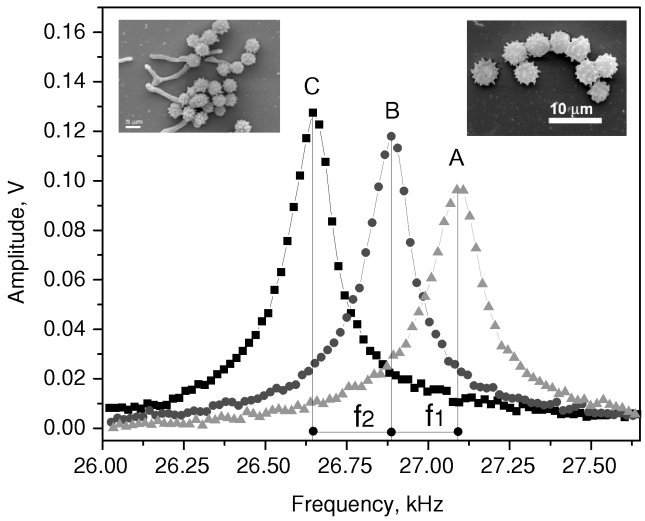

Figure 3. Typical experiments showing a first frequency decrease due to spore immobilization (Δf_1) and a second owing to germination and growth of the spores (Δf_2) in humid air. Point A: Initial frequency of the uncoated cantilever without IgG and spores (f_0). Point B: Frequency of the IgG functionalized cantilever with spores (f_1). Point C: Frequency of the functionalized cantilever with spores that started to germinate (f_2). Right and left images are related to graphs B and C relatively—B: spores after immobilization, C: spores started to germinate and to spread mycelium onto the cantilever surface after exposition in humid air for 4 h.

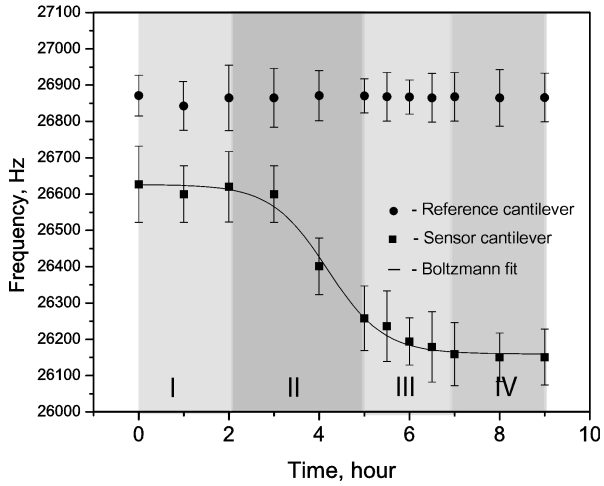

Figure 4. Detection of fungal growth. Resonance frequency changes of reference (without fungal spores) and sensor (with immobilized fungal spores) cantilevers. Both cantilevers were sensitized identically with IgG. The phases I, II, III, and IV correspond to lag phase (spore stage), exponential (log phase), deceleration, and stationary (mycelial stage) phases respectively. Bars correspond to the standard deviation.

spores immobilized on cantilever surfaces functionalized with IgG, (2) to distinguish active, functional fungal spores from dormant/inviable spores within a few hours.

We observed a first resonance frequency shift ($\Delta f_1 = f_0 - f_1$) in 1 h after exposure of the functionalized cantilever to a spore suspension due to binding of the spores on the IgG-sensitized cantilever interface (Fig. 3). A second resonance frequency shift ($\Delta f_2 = f_1 - f_2$) was registered after 4 h when the activated cantilevers with immobilized spores were placed in a humidity chamber, thus providing favorable conditions for spore germination (27°C, 96% RH). In all experiments where a second resonance frequency shift was registered, we observed by SEM that the spores were germinating and mycelia were growing.

The calculated mass sensitivity of our cantilever sensor was determined: for the first mode 52.7 ± 5.7 pg/Hz, the second mode 8.8 ± 0.7 pg/Hz, and the third mode 3.3 ± 0.2 pg/Hz. By monitoring the quality factor, Q, while assuming a constant cantilever spring constant, k, during experiments, we measured the additional mass of spores and growing microfungi on the interface, due to decreasing resonance frequencies (Chen et al., 1995).

The calculated mass of an *A. niger* spore was 47 pg. The minimal time for spore detection on the protein-coated cantilever surface was ∼1 h. The minimal time to detect vital, functional spores was ∼4 h.

Differential Measurements

Sensor (coated with IgG and with immobilized spores) and reference (coated with IgG and without spores) cantilevers in parallel (in the same array) were used to eliminate external influence during our measurements (Fig. 4). No decrease of the resonance frequency of the reference cantilever was detected over a period of 14 h. In contrast to the reference lever, the resonance frequency of a sensor lever with immobilized spores dropped exponentially during the first 5 h and then slowly leveled out to a constant value.

We compared the observed resonance frequency shift versus time curve with conventional fungal growth curve (Deacon, 1997). All of the characteristic growing phase can be observed (Fig. 4, sensor cantilever): an initial lag phase (I), a phase of exponential growth (II), and, afterward, a deceleration phase (III) and stationary phase (VI).

We assume that the sensor lever exhibits an increased initial fungal exponential growth rate that reaches a steady state. During exponential growth, maximum fungal activity is observed: Swelling spores start to germinate and spread mycelium along the cantilever. Growing mycelia consume (assimilate) mainly water (as water content is ∼90–94% of fungal cell) from the coating layer, which compensates the surrounding humid air to regain equilibrium with the environment. Thus, cantilever mass is increased. This continued during 7 h until essential nutrients became limiting (thickness of coating layer containing trace of nutrition is ∼25 nm) or until metabolic products accumulated to inhibitory levels. The obtained curve based on resonance frequency changes of sensor cantilever was identical to the curve describing growth behavior of *A. niger* (Viniegra-González et al., 1993).

Due to the very short lag phase (spore stage, duration ~2 h), the following fast exponential growth (mycelial stage, duration ~3 h) can be detected easily after 4 h of measurement as a differential shift in the resonance frequency. Therefore, application of cantilever biosensors allowed us for the first time to detect active fungal growth of single spores in less than 5 h.

Conclusion

By modifying the sensor surface with functional layers on the cantilever interface, they can be designed as a receptor surface to direct and specifically adsorb a target species without the need of labeled antibodies.

We have developed protein coating procedures for microcantilevers for specific *A. niger* spores immobilization via IgG and active mycelial growth detection. We have shown that cantilever arrays operated in dynamic mode in humid air allow vital spores to be detected within ~4–5 h as well as single fungal spores within ~1 h. The biosensor allows the use of different types of highly specific protein coatings simultaneously, including reference cantilevers to detect various fungal spores.

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