11

Nanomechanical Sensors for Biochemistry and Medicine

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11.1 Introduction

The purpose of biosensors in biochemistry and medicine is to provide a highly sensitive and selective method for reliable, rapid, and preferably continuous monitoring of certain chemicals and chemical processes in a biochemical and a physiological environment. In most cases, specific biochemical interactions between various biological ligands are utilized for the sensing of binding events. The sensor's input is a physical property or an interaction of a physical, chemical, or biochemical nature. This input is processed via the sensor's transduction element into a recordable signal.

Many different ways of transduction exist, such as electrochemical, electromechanical, electroacoustical, photoelectric, electromagnetic, magnetic, electrostatic, thermoelectric, electric, and mechanical transductions. In a biosensor, a biological transduction element is combined with a physicochemical detection element. The sensitive biological part may consist of biological materials such as tissues, microorganisms, organelles, cell receptors, enzymes, proteins, peptides, polysaccharides, antibodies, or nucleic acids. The sensor element responds with a specific signal, such as changes in electric potential, electrical current, conductance or impedance, the intensity and the phase of electromagnetic radiation, and also in mass, temperature, viscosity, strain, or stress.

Transduction methods comprise among others: (1) optical processes involving spectroscopy (absorption, fluorescence, phosphorescence, Raman) and refraction, (2) electrochemical processes like electrolysis and voltammetry, (3) mass detection via resonance frequency shifts in quartz crystal microbalances, surface acoustic wave devices, or microfabricated resonant structures, (4) array techniques, e.g., charge-coupled device camera readout of fluorescence-labeled spotted arrays, and (5) nanomechanical cantilevers, which are the focus of this article.

Biosensors have a major application potential in daily life, e.g., for bacteria detection to avoid contamination of food and for the detection of life-threatening bio-agents. While the main drivers are the health care and the food industry, authenticity issues (genuine products, detection of genetically modified food) are also of importance.

The total biochip market size is projected to grow to about $4.9 billion in 2012 with an annual growth rate of 12.3% (Fuji-Keizai 2008). The key requirements for biosensors are high selectivity, cost effectiveness,, speed, and reliability. Their main applications lie in the fields of medicine, health, diagnostics, in the food/beverage sectors, cosmetics, perfumes, and in safety and security issues (terrorism prevention). Biosensor strategies include bioinformatics, i.e., a multiple probe/measurement approach including statistical evaluation of acquired data.

Biosensors are most economically produced by batch microfabrication in large numbers and may include novel smart materials, functional coatings, or nanoparticles. Food contamination still poses a common problem, even in the most developed countries, and foodborne diseases (e.g., campylobacteriosis and salmonellosis) have reached epidemic proportions in several countries. Emerging health issues, such as contamination from acrylamide or dioxins, rotten meat, avian flu, bovine spongiform encephalopathy (BSE), and genetically modified organisms (GMOs) are creating additional concerns among both the public and the decision makers (WHO 2006).

11.2 Microcantilever Array Sensors

11.2.1 Sensor Concept

In this chapter, we focus on nanomechanical microcantilever sensor arrays for the detection of biochemical processes. Microcantilevers have been used for many years in atomic force microscopy (AFM), a technique pioneered by the IBM Zurich Research Laboratory in Switzerland (Binnig et al. 1986). AFM employs a microcantilever with a sharp tip to image a nonconductive surface with a lateral and a vertical resolution on the atomic scale. For the application as a sensor, neither the tip nor the surface are needed: the sensor response is generated by processes taking place on the surface of the microcantilever. These processes involve the adsorption of molecules on the microcantilever's surface, resulting in the bending of the cantilever beam via the generation of interface stress and strain. The specificity of the detection process is considerably enhanced if the specific probe receptor molecules are attached to one of the surfaces of the microcantilever to bind the target molecules.

Here, an array of eight microcantilevers is used (Figure 11.1a). Typically, the upper surface of a microcantilever is coated with a thin layer of a material that exhibits an affinity to molecules in the environment. This surface is referred to as the "functionalized" surface of the microcantilever. The other surface of the microcantilever (typically the lower surface) may be left uncoated or is coated with a passivation layer, which is either inert or does not show significant affinity to the molecules to be detected (Figure 11.1b). To functionalize a microcantilever surface, a metal layer is often deposited. Metal surfaces, such as gold, may be used to covalently bind a monolayer representing the chemical surface sensitive to the target molecules. Frequently, a monolayer of thiol molecules, offering well-defined surface chemistry, is formed on the gold surface, offering a template for subsequent molecule adsorption. The gold layer also often serves as a reflection layer in case the cantilever bending is read out optically.

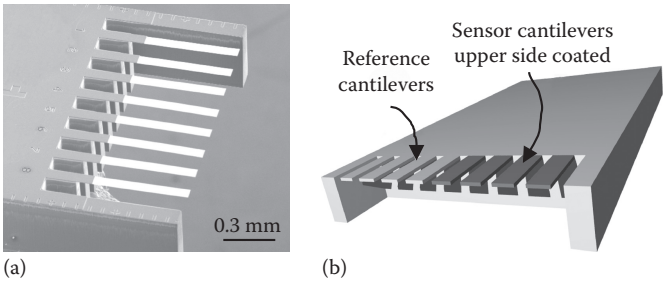


FIGURE 11.1 (a) Scanning electron microscopy image of a silicon microcantilever array. (b) Schematic drawing showing the upper functionalized and the lower passivated surfaces of four sensor cantilevers (darker color, right part of the array) and four passivated reference cantilevers (left part of the array). The thick solid side bars are on the one hand for mechanical protection of the cantilevers, on the other hand they represent a solid reference surface, e.g., for a reference baseline.

11.2.2 Compressive and Tensile Surface Stress

If molecules are adsorbed on the upper (functionalized) surface, then a downward bending of the microcantilever will result due to the formation of surface stress. The surface stress is called "compressive," because the adsorbed layer of molecules (e.g., a monolayer of alkylthiols) produces a downward bending of the microcantilever away from its functionalized side. In case of the opposite situation, i.e., if the microcantilever bends upward, one would speak of "tensile stress." If both the upper and the lower surface of the microcantilever are subjected to surface stress changes, then the situation is much more complex, because a predominant compressive stress formation on the lower microcantilever surface will appear as tensile stress on the upper surface. Therefore, it is extremely important to properly passivate the lower surface in such a way that, ideally, no stress-generating adsorption processes take place on the lower surface of the microcantilever.

Various strategies can be used to passivate the lower surface of microcantilevers. For biochemical systems, the application of a thin layer of 2-[methoxy-poly(ethyleneoxy)propyl]trimethoxysilane will create a pegylated surface that is almost inert toward the adsorption of biological layers. Only the actual experiment will show whether the passivation layer was really efficient, for, as such, passivated cantilevers will not show a substantial bending response upon exposure to an analyte.

11.2.3 Differential Stress Measurements

Single microcantilevers may bend due to effects other than the formation of surface stress during the adsorption of molecules. The major influences for such bending are a thermal drift, or an interaction with the environment, especially if the microcantilever is operated in a liquid. Furthermore, a non-specific physisorption of molecules on the cantilever surface or a nonspecific binding to the receptor molecules during measurements may contribute to the drift.

To exclude such influences, the simultaneous measurement of reference microcantilevers aligned in the same array as the sensing microcantilevers is crucial (Lang et al. 1998). The difference in responses from the reference and the sensor microcantilevers yields the net bending signal, and even small sensor signals can be extracted from large microcantilever deflections without being dominated by undesired effects. When only single

microcantilevers are used, no thermal-drift compensation is possible. To obtain useful data under these circumstances, both microcantilever surfaces have to be chemically well defined. One of the surfaces, typically the lower one, should be passivated; otherwise, the microcantilever response will be convoluted with undesired effects originating from uncontrolled reactions taking place on the lower surface.

With a pair of microcantilevers, reliable measurements are obtained. One of them is used as the sensor microcantilever (coated typically on the upper side with a molecule layer that shows affinity to the molecules to be detected), whereas the other microcantilever serves as the reference. The sensor microcantilever should be coated with a passivation layer on the upper surface so as not to exhibit affinity to the molecules to be detected. Thermal drifts are canceled out of difference responses, i.e., the difference in the deflections of the sensor and the reference microcantilevers are taken (differential measurements). Alternatively, both microcantilevers are used as sensors (sensor layers on the upper surfaces), and the lower surface is passivated.

The use of an array of microcantilevers is recommended in which some cantilevers are used either as sensor or as reference microcantilevers so that multiple difference signals can be evaluated simultaneously. The thermal drift is canceled out since one surface of all microcantilevers, typically the lower one, is left uncoated or coated with the same passivation layer.

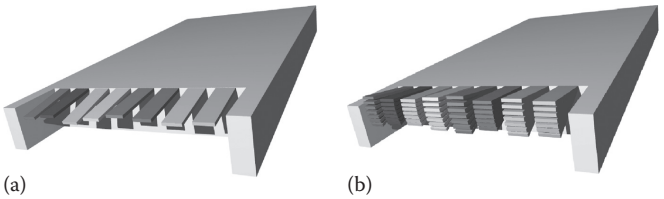
11.3 Modes of Operation

11.3.1 Static Mode

The gradual bending of a microcantilever, as a result of a progressing molecular coverage, is referred to as an operation in the "static mode" (Figure 11.2a). Various environments are possible, such as vacuum, ambient environment, and liquids. In a gaseous environment, molecules adsorb on the functionalized sensing surface and form a molecular layer, provided there is affinity for the molecules to adhere to the surface.

Polymer sensing layers show a partial sensitivity because molecules from the environment diffuse into the polymer layer at different rates, mainly depending on the size and the solubility of the molecules in the polymer layer. By selecting polymers out of a wide range of hydrophilic/hydrophobic ligands, the chemical affinity of the surface can be influenced, because different polymers vary in diffusion suitability for polar/unpolar molecules. Thus, for a detection in the gas phase, the polymers can be chosen according to the detection problem, i.e., what the applications demand. Typical chemicals that can be detected are volatile organic compounds (VOCs), such as solvent vapors.

A static-mode operation in liquids, however, usually requires rather specific sensing layers based on molecular recognition, like in DNA hybridization or in antigen-antibody recognition.



(a) (b)

FIGURE 11.2 (a) Static mode of operation. The individual cantilevers are bent down to a certain extent, depending on the magnitude of surface stress formed during adsorption of a molecular layer. (b) Dynamic mode of operation. The magnitude of oscillation might vary for each individual cantilever.

11.3.2 Dynamic Mode

Information on the mass change and the amount of molecules adsorbed on the microcantilever surface can be obtained by oscillating the microcantilever at its eigenfrequency (Figure 11.2b). However, the surface coverage is basically not known and molecules on the surface might be exchanged with molecules from the environment in a dynamic equilibrium.

Tracking the eigenfrequency of the microcantilever during mass adsorption or desorption is done to obtain information about these processes. The eigenfrequency is identical to the resonance frequency of an oscillating microcantilever if its elastic properties remain unchanged during the molecule adsorption/desorption process and if the damping effects are negligible. This mode of operation is called the "dynamic mode." The microcantilever is used as a microbalance, as with mass addition on the cantilever surface, the cantilever's eigenfrequency will shift to a lower value. The mass change on a rectangular cantilever is calculated (Thundat et al. 1994) according to

$$\Delta m = \left(\frac{k}{4\pi^2} \times 0.24\right) \times \left(\frac{1}{f_1^2} - \frac{1}{f_0^2}\right), \tag{11.1}$$

where

f_0 is the eigenfrequency before the mass change occurs
f_1 is the eigenfrequency after the mass change has happened

The cantilever spring constant k is calculated according to

$$k = \frac{Ewt^3}{4l^3}, \tag{11.2}$$

where

E is Young's modulus ($E_{Si} = 1.3 \times 10^{11}$ N/m^2 for Si(1 0 0))
w, t, and l are the width, the thickness, and the length of the cantilever

Dynamic mode operation in a liquid environment poses a challenge because of the strong damping of the cantilever oscillation due to the high viscosity of the surrounding media. This results in a low quality factor Q of the oscillation, and the resonance frequency shift is difficult to track with high resolution. The quality factor is defined as

$$Q = \frac{2\Delta f}{f_0}. \tag{11.3}$$

In air, a frequency resolution Δf of below 1 Hz is easily achieved, in contrast to a liquid environment, where resolution values of about 20 Hz are already considered very good. With damping or changes in the elastic properties of the cantilever during the experiment, e.g., a stiffening or softening of the spring constant by adsorption of a molecule layer, the measured resonance frequency will not be exactly the same as the eigenfrequency, and the mass derived from the frequency shift will be inaccurate.

Unlike in ultrahigh vacuum conditions (Ilic et al. 2004, Ekinci and Roukes 2005), where the resonance frequency is equal to the eigenfrequency, the two terms eigenfrequency and resonance frequency should be carefully distinguished for operation in a strong damping environment, as described in literature (Braun et al. 2005).

11.3.3 Further Modes of Operation

Microcantilevers coated with metal layers are also prone to thermal effects because thermal expansion differences in the cantilever and the coating layer will also contribute to bending when the temperature is varied. This effect is used in another mode of operation referred to as the "heat mode." There, cantilever bending occurs because of differing thermal expansion coefficients in the sensor layer and in cantilever materials (Gimzewski et al. 1994).

Heat changes are either caused by external influences, such as a change in temperature, and occur directly on the surface by exothermal, e.g., catalytic reactions, or are due to the material properties of a sample attached to the apex of the cantilever. The latter technique is known as micromechanical calorimetry. The sensitivity of the cantilever heat mode is in orders of magnitude higher than that of the traditional calorimetric methods performed on milligram samples, as it only requires nanogram amounts of sample and achieves nanojoules (Berger et al. 1996) to picojoules (Bachels and Schäfer 1999, Bachels et al. 1999) sensitivity. Static, dynamic, and heat measurement modes have established cantilevers as versatile tools to perform experiments in nanoscale science with very small amounts of material.

Mass-change determination can be combined with varying environment temperature conditions to obtain a method introduced in the literature as "micromechanical thermogravimetry" (Berger et al. 1998). The sample under investigation is mounted directly onto the cantilever. Its mass should not exceed several hundred nanograms. In case of adsorption, desorption, or decomposition processes, mass changes in the picogram range can be observed in real time by tracking the resonance-frequency shift.

In photon-absorbing materials, a fraction of energy is converted into heat. This photothermal heating can be measured as a function of the light wavelength to provide optical absorption data of the material. The interaction of light with a bimetallic cantilever creates heat on the cantilever surface, resulting in a bending of the cantilever (Barnes et al. 1994). Such bimetallic-cantilever devices are capable of detecting heat flows due to an optical heating power as low as 100 pW, being two orders of magnitude better than in conventional photothermal spectroscopy. Recently, this technique has been applied for reliable and quick detection of explosives (Krause et al. 2008, Van Neste et al. 2008).

11.4 Microcantilever Functionalization

For reliable operation of microcantilever sensors, it is essential that the surfaces of the cantilevers are coated in a reproducible and robust manner to provide suitable receptor surfaces for the analyte molecules to be detected. Such coatings should be specific, homogeneous, stable and reproducible. Microcantilever sensors might be designed to be either reusable or for single use only.

For static mode measurements, one side of the cantilever should be passivated to block undesired, unspecific adsorption. Often, the microcantilever's upper side, which will be referred to as the sensor side, is coated with a 20 nm thick layer of gold to provide a platform for the binding of the receptor molecules, for example, by means of thiol chemistry, whereas the lower side is passivated using silane chemistry to provide an inert surface such as poly-ethylene glycol silane. Silanization is performed first on the silicon microcantilever. Subsequently, a gold layer is deposited on the top side of the microcantilever, leaving the lower side unchanged. It is very important that the method for microcantilever coating is fast, reproducible, reliable, and allows one or both cantilever surfaces to be coated separately. Various ways are reported to coat a microcantilever cantilever with functional molecular layers. Here, two different strategies are highlighted.

11.4.1 Coating in Microcapillary Arrays

It is essential that every microcantilever in an array can be coated separately with a functional layer. This requirement can be achieved by confining each cantilever in a dimension-matched microcapillary filled with the liquid containing the molecules to be deposited on the microcantilever (Figure 11.3). Therefore, the microcantilevers of the array are inserted into disposable glass microcapillaries filled with liquid containing the probe molecules. The outer diameter of the glass capillaries is 240 μm so that they can be easily and neatly placed next to each other to accommodate the pitch of the cantilevers in the array (250 μm). Their inner diameter is 150 μm, allowing sufficient room to

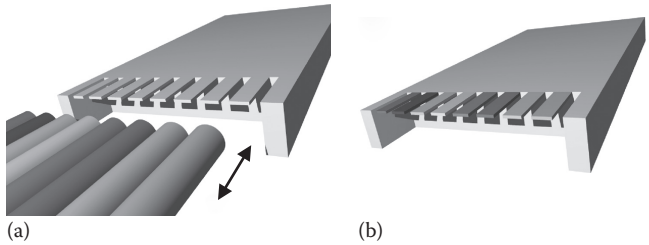


FIGURE 11.3 Functionalization of a microcantilever array in dimension-matched glass microcapillaries filled with a solution of probe molecules. (a) Before insertion and (b) after incubation.

insert the cantilevers (width: 100 μm) safely. This method has been successfully applied for the deposition of a variety of materials onto cantilevers, such as self-assembled monolayers (Fritz et al. 2000a), thiol-functionalized single-stranded DNA oligonucleotides (Fritz et al. 2000b, McKendry et al. 2002, Zhang et al, 2006), and proteins (Arntz et al. 2003, Backmann et al. 2005). Incubation of the microcantilever array in the microcapillaries takes from a few seconds (the self-assembly of alkanethiol monolayers) to several tens of minutes (coating with protein solutions). The microcapillary functionalization unit may be placed in an environment of saturated vapor of the solvent used for the probe molecules to avoid drying out of the solutions.

11.4.2 Coating Using an Inkjet Spotter

The disadvantage of coating in microcapillary arrays is that manual alignment of the micro-cantilever array and the functionalization tool is required, and therefore the technique is not suitable for coating large numbers of cantilever arrays. Moreover, the upper and the lower surfaces of the microcantilevers are exposed to the same solution containing the probe molecules. For ligands that bind covalently, e.g., by gold-thiol coupling, only the upper surface will be coated, provided the gold layer is only on the upper surface of the microcantilever. For coating with polymer layers, microcapillary arrays are not suitable, because both surfaces of the microcantilever would be coated with polymers. This would be inappropriate for static mode measurements, where an asymmetry between the upper and lower surface is required.

A method suitable for coating many cantilever sensor arrays in a rapid and reliable way is inkjet spotting (Bietsch et al. 2004a,b), see Figure 11.4. An *x*–*y*–*z* positioning system allows a fine nozzle (typical capillary diameter: 70 μm) to be positioned with an accuracy of approx. 10 μm over a cantilever. Individual droplets (diameter: 60–80 μm, volume 0.1–0.3 nL) can be dispensed individually by means of a piezo-driven ejection system in the inkjet nozzle. When the droplets are spotted with a pitch smaller than 0.1 mm, they merge and form continuous films. By adjusting the number of droplets deposited on cantilevers, the resulting film thickness can be controlled precisely. The inkjet-spotting technique allows a cantilever to be coated within seconds and yields very homogeneous, reproducibly deposited films of well-controlled thickness.

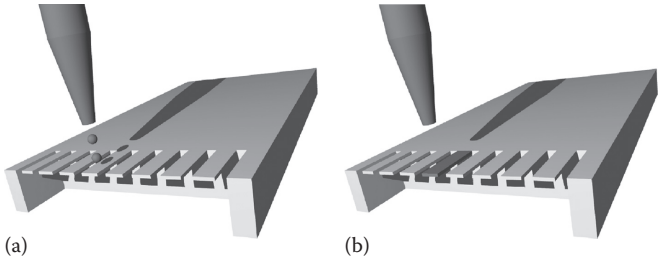


FIGURE 11.4 Functionalization of a microcantilever array using an inkjet spotting nozzle. (a) Individual droplets are ejected from the nozzle onto the upper surface of the microcantilever. (b) An individual microcantilever has been coated with a film of probe molecules.

The successful coating of self-assembled alkanethiol monolayers, polymer solutions, self-assembled DNA single-stranded oligonucleotides (Bietsch et al. 2004b), and protein layers has been demonstrated. In conclusion, inkjet spotting has turned out to be a very efficient and versatile method for functionalization that can even be used to coat arbitrarily shaped sensors reproducibly and reliably (Lange et al. 2002, Savran et al. 2003).

11.5 Experimental Setup

11.5.1 Measurement Setup for a Liquid Environment

In general, a measurement set-up for cantilever arrays consists of four major parts, see Figure 11.5: (1) the measurement chamber containing the cantilever array, (2) an optical or electrical system to detect the cantilever deflection (e.g., laser sources, collimation lenses and a position-sensitive detector [PSD], or piezoresistors and Wheatstone-bridge detection electronics), (3) electronics to amplify, process and acquire the signals from the detector, and (4) a gas- or liquid-handling system to inject samples reproducibly into the measurement chamber and purge the chamber. The cantilever sensor array is located in an analyte chamber with a volume of 3–90 μL, which has inlet and outlet ports for gases or liquids. The cantilever deflection is determined by means of an array of eight vertical-cavity surface-emitting lasers (VCSELs) arranged at a linear pitch of 250 μm that emit at a wavelength of 760 nm into a narrow cone of 5°–10°. The light of each VCSEL is collimated and focused onto the apex of the corresponding cantilever by a pair of achromatic doublet lenses, 12.5 mm in diameter. This size has to be selected in such a way that all eight laser beams pass through the lens close to its center to minimize scattering, chromatic, and spherical aberration artifacts. The light is then reflected off the gold-coated surface of the cantilever and hits the surface of a PSD. PSDs are light-sensitive photo-potentiometer-like devices that produce photocurrents at two opposing electrodes. The magnitude of the photocurrents depends linearly on the distance of the impinging light spot from the electrodes. Thus, the position of an incident light beam can easily be determined with micrometer precision. The photocurrents are transformed into voltages and amplified in a preamplifier. As only one PSD is used, the eight lasers cannot stay switched on simultaneously. Therefore, a time-multiplexing procedure is used to switch the lasers on and off sequentially at typical intervals of 10–100 ms. The resulting deflection signal is digitized and stored together with time information on a personal computer (PC), which also controls the multiplexing of the VCSELs as well as the switching of the valves for the liquid handling system. The measurement set-up for liquids (Figure 11.5) consists of a poly-etheretherketone (PEEK) liquid cell, which contains the cantilever array and is sealed by a viton O-ring and a glass plate. The VCSELs and the PSD are mounted on a metal frame around the liquid cell.

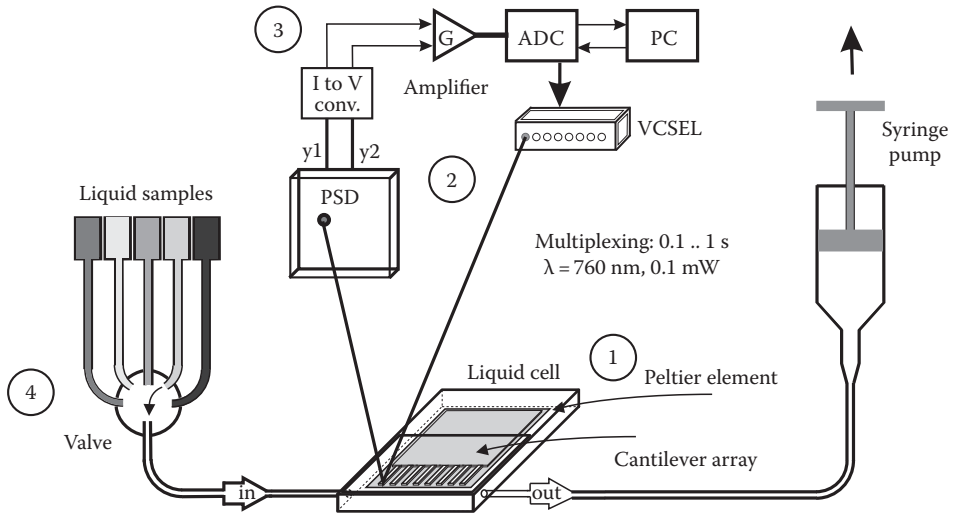


FIGURE 11.5 Schematic drawing of the measurement setup: (1) measurement chamber with microcantilever array, (2) deflection readout system (optical beam deflection), (3) amplification electronics, (4) liquid-handling system: the liquid is pulled from individual reservoirs through the measurement chamber using a motorized syringe.

After preprocessing the position of the deflected light beam in a current-to-voltage converter and amplifier stage, the signal is digitized in an analog-to-digital converter and stored on a PC. The liquid cell is equipped with inlet and outlet ports for liquids. They are connected via a 0.18-mm-inner-diameter Teflon tubing to individual thermally equilibrated glass containers, in which the biochemical liquids are stored. A six-position valve allows the inlet to the liquid chamber to be connected to each of the liquid-sample containers separately. The liquids are pulled through the liquid chamber by means of a syringe pump connected to the outlet of the chamber. A Peltier element is situated very close to the liquid-containing volume of the chamber to allow temperature regulation within the chamber. The entire experimental set-up is housed in a temperature-controlled box regulated with an accuracy of 0.01 K to the target temperature.

11.5.2 Application I: Patient's Breath Characterization

The first application discussed here is an experiment in a gaseous environment (Baller et al. 2000). The experimental setup is basically the same as the one in liquids, except for the fact that exhaled air collected from a patient is pushed by a syringe pump through the measurement chamber. Before using modern diagnosis tools, medical doctors examined the patient's breath to detect diseases, since certain diseases can be recognized by an examination of exhaled air. Examples of such illnesses are the following: (1) Diabetes mellitus (type II diabetes), a severe, chronic form of diabetes caused by insufficient production of insulin and resulting in abnormal metabolism of carbohydrates, fats, and proteins. This disease involves the presence of acetone in the patient's breath. (2) Uremia, a toxic condition resulting from kidney disease in which there is a retention of waste products in the bloodstream. These waste products are normally excreted in the urine. A compound found in a patient's breath associated with uremia is dimethylamine.

Breath samples of two patients suffering from diabetes mellitus and uremia were taken and stored in medical plastic bags for exhaled air samples. For a comparison, breath samples from healthy persons were also investigated for reference. For each measurement, 10 mL of exhaled air was removed from the medical plastic bag under temperature-controlled conditions, and injected into the microcantilever array measurement chamber. Each cantilever is coated with a different polymer and responds in its own characteristic way to the breath sample during the exposure time of 6 min because the rate of diffusion of the substances in exhaled air is different for each polymer. Also, during the purging process of the chamber (cleaning with dry nitrogen for 8 min from a second syringe) the desorption characteristics are unique to each polymer. All flow rates were set to 1 mL gas per minute. The microcantilever deflections were found to be very reproducible for samples from the same patient, but dissimilar for sick and healthy persons (Figure 11.6a through d). The amount of data was reduced by extracting the deflections of the eight microcantilevers at three different points in time (at 320, 420, and 520 s after start of the measurement, cf. vertical lines in Figure 11.6) during exposure to the exhaled air sample, and at four different points during the purging process of the measurement chamber with dry nitrogen gas (at 620, 720, 820, and 920 s). The reduced data set consisted of cantilever deflections of eight cantilevers at seven different points in time, i.e., $8 \times 7 = 56$ cantilever deflection values, which characterize one measurement in a 56 dimensional space. The mathematical method of principal component analysis (PCA) projects this 56 dimensional information into 2 dimensions, whereby the largest differences between measurements are determined in a least-square fit procedure. The two axis of a two-dimensional PCA plot are referred to as the principal components. The PCA reveals the most dominant

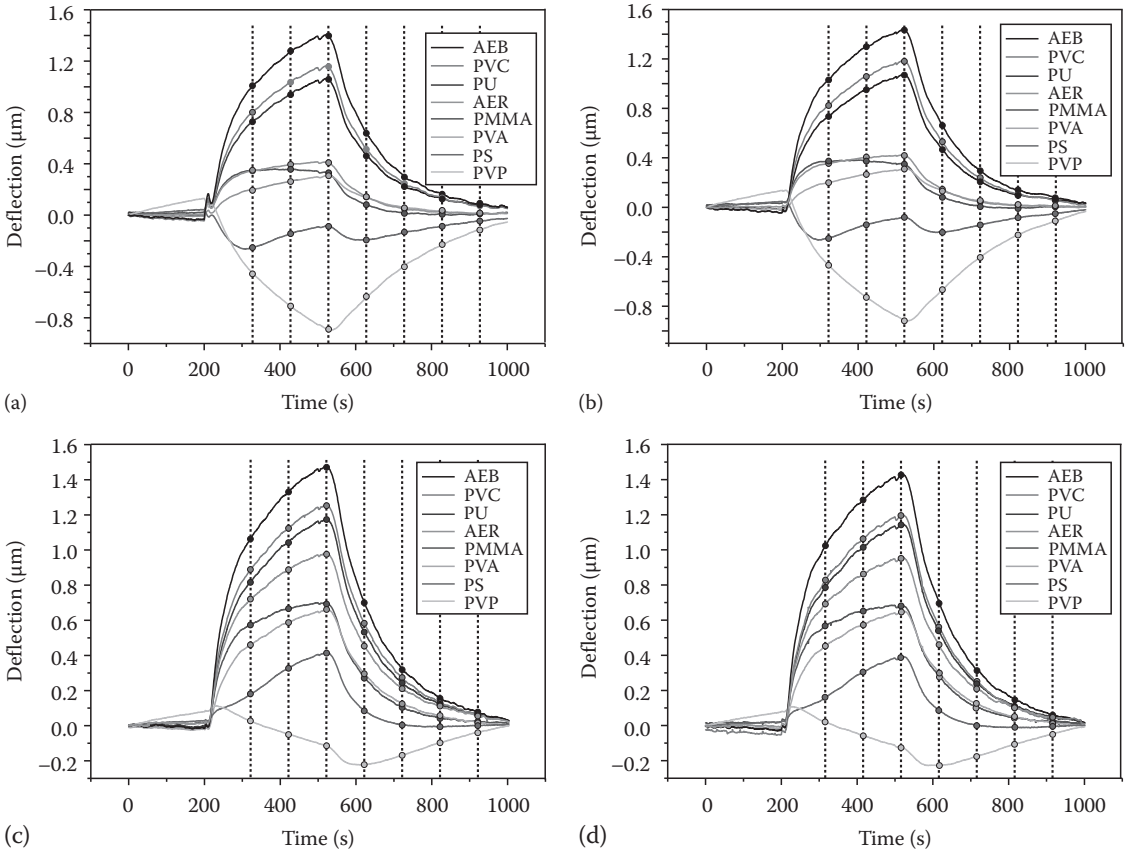


FIGURE 11.6 (a and b) Two independent measurements taken from a healthy person (cantilever deflection traces acquired during the injection of a breath sample into the measurement chamber). (c and d) *Ditto*, but for a patient suffering from uremia. Polymer coatings: AEB = araldite epoxy resin type B, PVC = polyvinyl chloride, PU = polyurethane, AER = araldite epoxy resin type R, PMMA = polymethylmethacrylate, PVA = polyvinyl alcohol, PS = polystyrene, PVP = polyvinylpyridine. (Data courtesy of Daniel Schmid, University Hospital Basel, Basel, Switzerland.)

deviations in the responses for the different patients' breath samples in measured data. Clear clustering of breath measurements of healthy persons and of patients with acetone breath (diabetes) and uremia is observed in Figure 11.7. The symbols in the PCA plot (Figure 11.7) indicate the individual measurements. Three different clusters of points are observed, allowing a distinction between healthy persons, acetone breath patients and dimethylamine breath patients. We conclude that the microcantilever technique allows a fast and a noninvasive detection of diseases in patients' breath samples (Schmid et al. 2008).

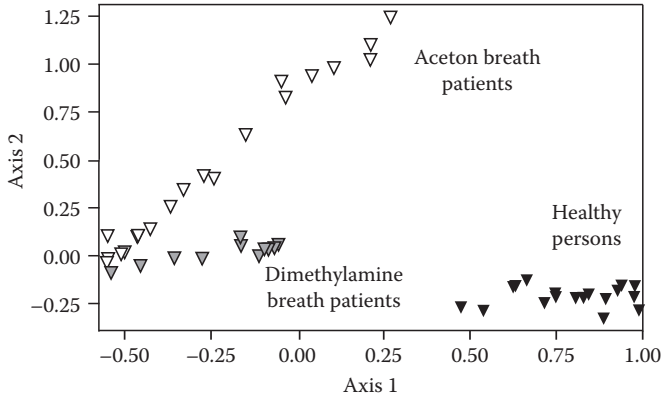


FIGURE 11.7 Principal component analysis plot revealing clearly separated groups of triangles (each symbol is a breath measurement) that allows a clear distinction between healthy persons from patients with acetone or dimethylamine in their breath. (Data courtesy of Daniel Schmid, University Hospital Basel, Basel, Switzerland.)

11.5.3 Application II: DNA Hybridization Sensing

This example demonstrates the capability of cantilever array sensors to detect biochemical reactions. Each cantilever is functionalized with a specific biochemical probe receptor, sensitive for detection of the corresponding target molecule. The main advantage of can-tilever-array sensors is that measurements of differences in the responses of sensor and reference cantilevers can be evaluated. Measuring the deflection of only one cantilever will yield misleading results that might give rise to an incorrect interpretation of the cantilever-deflection trace. Therefore, at least one of the cantilevers (the sensor cantilever) is coated with a sensitive layer that exhibits an affinity to the molecules to be detected, whereas other cantilevers are

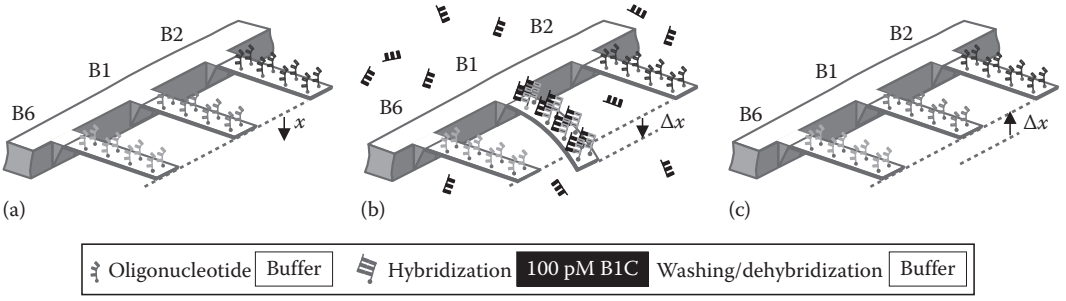


FIGURE 11.8 (a) Schematic drawing of a cantilever sensor array coated with single stranded DNA oligonucleotides. (b) Hybridization with the matching DNA sequence bends the second cantilever due to the formation of surface stress. (c) The initial state is restored after purging with a dehybridization agent.

coated with a molecular layer that does not show an affinity to them (reference cantilevers).

The biochemical system to be investigated here involves a DNA hybridization experiment in liquid using a thiolated 12-mer oligonucleotide sequence from the Bio B biotin synthetase gene (EMBL accession number: J04423). Three surface-bound probes were selected, Bio B1 (5′-SH-C_6-ACA TTG TCG CAA-3′, C_6 is a spacer), Bio B2 (5′-SH-C_6-TGC TGT TTG AAG-3′) and Bio B6 (5′-SH-C_6-TCA GGA ACG CCT-3′), which were immobilized by thiol binding onto the gold-coated upper surface of a cantilever in an array (Figure 11.8a).

Please note that the sequences are selected in length in such a way that stress generation is expected to occur close to the cantilever surface. With much longer sequences, the experiment would not necessarily work because the stress would be generated too far away from the surface (Alvarez et al. 2004).

The target complements called Bio B1C, B2C, and B6C are diluted in a 5× sodium saline citrate (ssc) buffer at 100 pM concentration. Upon injection of the matching sequence to Bio B1, i.e., Bio B1C, the sensor cantilever coated with Bio B1 is expected to bend, whereas the reference cantilever coated with Bio B2 as well as that coated with Bio B6 will not bend (Figure 11.8b). After thorough rinsing with an unbinding agent, the cantilever coated with Bio B1 will bend back to its initial position (Figure 11.8c). The bending is due to the formation of surface stress during the hybridization process because of steric crowding, because a double-stranded DNA requires more space than a single-stranded DNA.

The actual experiment proceeds as follows (Figure 11.9): First, the liquid cell with the functionalized cantilever array is filled with an ssc buffer. After a stable deflection base line has been achieved, the ssc buffer is injected after 4 min for 3 min. All cantilevers deflect, but once the injection is over, a stable baseline is reached again. At 18 min, the target Bio B1C is injected, which is supposed to hybridize with the Bio B1 probe, but not with the Bio B2 or the Bio B6 probe. All cantilevers deflect, but the deflection magnitude of the Bio B1-coated cantilever is much larger than those of the Bio B2- and the Bio B6-coated cantilever. Finally, at 37 min, the ssc buffer is injected again and a stable baseline is reached. From the deflection data shown in Figure 11.9a, it seems that no conclusive result can be obtained from individual cantilever responses only, as both the sensor and the reference cantilevers bend. However,

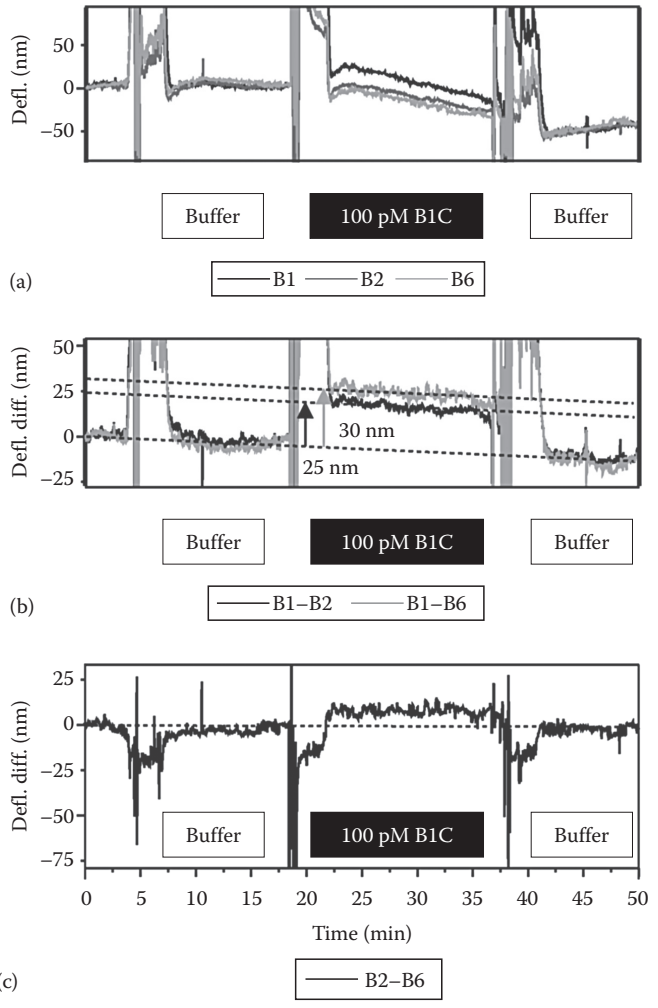


FIGURE 11.9 (a) Deflection traces of sensor (functionalized with DNA oligonucleotide sequence Bio B1) and reference cantilevers (functionalized with DNA oligonucleotide sequences Bio B2 and Bio B6, respectively). (b) Differences B1 to B2 (signal: 25 nm) and B1 to B6 (signal: 30 nm) of the bending responses of the sensor cantilever B1 and the reference cantilevers B2 and B6. (c) Difference in responses of the two reference probes B2–B6 (signal: <5 nm). (Data courtesy of Jiayun Zhang, University of Basel, Basel, Switzerland.)

a clear deflection signal is observed when calculating the difference in deflection responses from probes Bio B1 (sensor) and reference Bio B2 (Figure 11.9b), or the difference in deflection responses from probes Bio B1 and reference Bio B6. The differential deflection magnitudes obtained are 25 nm (B1–B2) or 30 nm (B1–B6), respectively. The difference in deflection responses between two reference cantilevers yields no signal or only a very small signal that can be attributed to an unspecific binding of B1C to one of the reference probes, supposedly to B2, as the difference B2–B6 yields a small positive signal of less than 5 nm, see Figure 11.9c. We conclude that it is absolutely mandatory to use at least two cantilevers in an experiment, a reference cantilever and a sensor cantilever, to be able to cancel out undesired artifacts such as thermal drift or unspecific adsorption.

11.6 Applications in a Biochemical Environment

The following sections give an overview on the research performed with microcantilevers in the field of biochemistry and medicine. The examples given only represent a selection of some of the publications in this field in the last few years, and are not meant to be comprehensive.

11.6.1 pH Sensing

Control of pH is often important in biochemical reactions. Hence, this section concerns the measurement of pH using microcantilevers by measuring their deflection as a function of pH. Microcantilevers coated with self-assembled monolayers of mercaptohexadecanoic acid (MHA, hydrophilic) and hexadecanethiol (HDT, hydrophobic) bend due to the presence of hydrogen ions, as interfacial stress develops depending on pH values and ionic strength (Fritz et al. 2000a). At a low pH, MHA is protonated, whereas at a high pH, MHA is deprotonated. SiO_2 and silicon nitride microcantilevers were also found to exhibit a deflection dependency with pH when coated with 4-aminobutyltriethoxysilane, 11-mercaptoundecanoic acid and Au/Al-coated over a pH range 2–12. Aminosilane-modified SiO_2/Au cantilevers performed robustly over the pH range 2–8 yielding 49 nm deflection/pH unit, while Si_3N_4/Au cantilevers performed well at the pH 2–6 and 8–12, producing a 30 nm deflection/pH unit (Ji et al. 2001a,b). Microcantilevers with poly(methacrylic acid) (PMAA) and poly(ethylene glycol) dimethacrylate coating were found to be sensitive to pH changes (Bashir et al. 2002). Hydrogel coatings were also found to be sensitive to pH (Zhang et al. 2004a). The dependence of the micromechanical responses to different ionic strength and ion species present in the aqueous environment is discussed in detail (Watari et al. 2007), highlighting the critical role of counter- and co-ions on surface stress.

11.6.2 Ion Sensing

Detection of ions using microcantilevers requires receptor molecules on their surface to be able to recognize ions selectively in solution. Coupling of the ions to the receptor sites involves conformational changes of the receptor and also a generation of interfacial stress that is transduced to the microcantilever, which, in turn, responds by bending. Using microcantilevers coated with a self-assembled monolayer of triethyl-12-mercaptododecylammonium bromide on gold CrO_4^{2-} ions are detected at a concentration of 10^{-9} M. Other anions, such as Cl^-, Br^-, CO_3^{2-}, HCO_3^-, and SO_4^{2-} do not deflect such modified cantilevers significantly (Ji et al. 2001b). Hg^{2+} has been measured at a concentration of 10^{-11} M using a microcantilever coated with gold. Almost no affinity to other cations exists, such as K^+, Na^+ Pb^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+}, Cu^{2+}, and Ca^{2+} (Xu et al. 2002). Adsorption characteristics of Ca^{2+} ions as a function of concentration in an aqueous $CaCl_2$ solution was investigated in the static and the dynamic mode (Cherian et al. 2002). Microcantilevers functionalized with the metal-binding protein, AgNt84-6, are able to detect heavy metal ions like Hg^{2+} and Zn^{2+}, but are insensitive to Mn^{2+} (Cherian et al. 2003). Hydrogels containing benzo-18-crown-6 have been used to modify microcantilevers for measurements of the concentration of Pb^{2+} in aqueous solutions (Liu and Ji 2004). Using different thiolated ligands as self-assembled monolayers (SAMs) functionalized on silicon microcantilevers coated with gold allows the detection of Cs^+, Co^{2+}, and Fe^{3+} (Dutta et al. 2005). In an electrochemical application, a gold coated microcantilever is utilized as the working electrode to detect Cr(VI) (Tian et al. 2005). Others use 11-undecenyltriethylammonium bromide (Boiadjiev et al. 2005) or sol–gel layers (Carrington et al. 2006) for detection of Cr(VI). Based on the EDTA–Cd(II) complex and its binding capability to bovine serum albumine (BSA), an antibody-based Cd(II) sensor using microcantilevers is presented (Velanki et al. 2007).

11.6.3 Glucose

Living cells use glucose as a source of energy. Chemically, glucose is a monosaccharide or simple sugar, also known as blood sugar. Detection of glucose concentrations is of outmost importance also to determine the medical condition of a patient. Glucose sensing via microcantilevers is achieved by coating the cantilevers with the enzyme glucose oxidase on gold (Subramanian et al. 2002) or via polyethyleneimine (PEI) conjugation (Yan et al. 2004). Glucose concentrations between 0.2 and 20 mM could be detected (Pei et al. 2004). In another study, a detection range between 2 and 50 mM is reported for glucose. No signal is observed for fructose, mannose, and galactose (Yan et al. 2005).

11.6.4 Hydrogen Peroxide (H_2O_2)

Hydrogen peroxidase provides oxygen in enzymatically controlled reactions. Hydrogen peroxide is detected at the nM level using multilayer modified microcantilevers functionalized through a layer-by-layer nanoassembly technique via intercalation of the enzyme horseradish peroxidase. The magnitudes of bending were found to be proportional to the concentrations of hydrogen peroxide (Yan et al. 2006a).

11.6.5 DNA, RNA

Observation of DNA hybridization using microcantilevers provides valuable information on the similarity of genetic sequences, whereby changes in single nucleotides are detectable. The microcantilever technique does not use an additional polymerase chain reaction (PCR) amplification and is label-free. Specific DNA hybridization detection was observed via surface stress changes related to the transduction of receptor-ligand binding into a direct nanomechanical response of microfabricated cantilevers without the need for external labeling or amplification. The differential deflection of the cantilevers was found to provide a true molecular recognition signal despite the large responses of individual cantilevers. The hybridization of complementary oligonucleotides shows that a single base mismatch between two 12-mer oligonucleotides is clearly detectable (Fritz et al. 2000b). The findings were confirmed or modeled by several groups (Hansen et al. 2001, Hagan et al. 2002). Hybridization in a complex nonspecific background was observed in a complement concentration range between 75 nM and 2 μM (McKendry et al. 2002) following the Langmuir model kinetics (Marie et al. 2002). Enzymatic processes were directly performed on a microcantilever functionalized with DNA incorporating a Hind III restriction endonuclease site, followed by digestion with Hind III to produce DNA comprising a single-stranded end on the cantilever surface. Ligase was used to couple a second DNA molecule with a compatible end to the DNA on the cantilever (Stevenson et al. 2002). Using a gold nanoparticle–labeled DNA, microcantilevers have been used to detect DNA strands with a specific sequence in the dynamic mode, whereby a concentration of 23 pM could still be detected, as well as, a single basepair mismatch (Su et al. 2003). Whereas the adsorption of a thiol functionalized single-stranded DNA is easily observed, hybridization cannot be observed if long hydrocarbon spacer molecules between a single strand DNA and a thiol anchor are used (Alvarez et al. 2004). A very high sensitivity is obtained by creating localized binding sites with gold nanodots. Consecutive selective bonding of double-stranded DNA molecules through a thiol linker allows the detection of a single 1587 basepair DNA molecule (Ilic et al. 2005). DNA hybridization is also observed using piezoresistive cantilevers (Marie et al. 2002, Gunter et al. 2004). A different technique to read out the microcantilever deflections in an array is reported (Alvarez and Tamayo 2005). There, the optical beam deflection technique is combined with the scanning of a laser beam illuminating the cantilevers of an array sequentially. DNA hybridization is also reported using polymer SU-8 cantilevers (Calleja et al. 2005). Mukhopadhyay et al. report 20 nM hybridization sensitivity using piezoresistive cantilevers and DNA sequences with an overhang extension distal to the surface (Mukhopadhyay et al. 2005a) A larger array comprising 20 microcantilevers is described in Lechuga et al., 2006. Moreover, the authors present integration of the array with microfluidics. Surface stress changes in response to thermal dehybridization, or melting, is reported (Biswal et al. 2006). The dependence of salt concentration and hybridization efficiency is discussed in detail (Stachowiak et al. 2006). Two different DNA-binding proteins, the transcription factors SP1 and NF-kappa B are investigated (Huber et al. 2006). Phase transition and stability issues of DNA are discussed in Biswal et al., 2007. A differential gene expression of the gene 1-8U, a potential marker for cancer progression or viral infections, has been observed in a complex background. The measurements provide results within minutes at the picomolar level without target amplification, and are sensitive to base mismatches (Zhang et al. 2006).

11.6.6 Proteins and Peptides

Proteins are larger organic molecules composed of amino acids arranged in a linear chain and connected by peptide bonds between the carboxyl and amino groups of adjacent amino acid residues. Proteins are involved in all vital metabolic processes in cells, providing, e.g., mechanical functions in muscle cells or, in the case of enzymes, catalyze biochemical processes. Further importance lies in cell signaling, immune responses, and cell adhesion processes, as well as digestion. Since proteins often also have a secondary and tertiary structure, i.e., they are folded in a complex way, it is essential to investigate protein interaction under conditions as close as possible to their native environment in the cell or organism. This requirement is fulfilled by using adequate buffers, pH, and temperature conditions. Microfabricated cantilevers were utilized to detect the adsorption of low-density lipoproteins and their oxidized form on heparin, and to detect the adsorption of bovine serum albumine and immunoglobuline G (IgG) (Moulin et al. 2000). The activity, stability, lifetime and re-usability of monoclonal antibodies to myoglobin covalently immobilized onto microfabricated cantilever surfaces was investigated (Grogan et al. 2002). Using piezoresistive microcantilevers, the interaction of the anti-bovine serum albumin (a-BSA) with the bovine serum albumin (BSA) was studied (Kooser et al. 2003). Continuous label-free detection of two cardiac biomarker proteins (creatin kinase and myoglobin) was demonstrated using an array of microfabricated cantilevers functionalized with covalently anchored anti-creatin kinase and anti-myoglobin antibodies (Arntz et al. 2003). Label-free protein detection was reported using a microcantilever functionalized with DNA aptamers receptors for Taq DNA polymerase (Savran et al. 2004). A label-free detection of the C-reactive protein (CRP) using a resonant frequency shift in piezoresistive cantilevers was described (Lee et al. 2004), utilizing the specific binding characteristics of the CRP antigen to its antibody, which was immobilized with Calixcrown SAMs on Au. Receptors on microcantilevers for serotonin, but insensitive to its biological precursor with a similar structure tryptophan were described (Zhang et al. 2004b). Using single-chain fragment antibodies instead of complete antibodies allowed a lowering of the limit of detection to concentrations of about 1 nM (Backmann et al. 2005). Wee et al. (2005) reported the detection of the prostate-specific antigen (PSA) and the C-reactive protein. The detection of the human oestrogen receptor in the free and the oestradiol-bound conformation could be distinguished (Mukhopadhyay

et al. 2005b). The Ca^{2+} binding protein calmodulin changed its conformation in the presence or absence of Ca^{2+} resulting in a microcantilver deflection change (Yan et al. 2006b). No effect was observed upon exposure to K^{+} and Mg^{2+}. The detection of the activated cyclic adenosine monophosphate (cyclic AMP)-dependent protein kinase was performed in the dynamic mode employing a peptide derived from the heat-stable protein kinase inhibitor (Kwon et al. 2007). The detection of streptavidin at a 1–10 nM concentration was reported using biotin-coated cantilevers (Shu et al. 2007). Using glutathione-*S*-transferase (GST) for the detection of GST antibodies, a sensitivity of 40 nM was obtained (Dauksaite et al. 2007). A two-dimensional multiplexed real-time, label-free antibody-antigen binding assay by optically detecting nanoscale motions of two-dimensional arrays of microcantilever beams was presented (Yue et al. 2008). The PSA was detected at 1 ng/mL using antibodies covalently bound to one surface of the cantilevers. Conformational changes in membrane protein patches of bacteriorhodopsin proteoliposomes were observed with microcantilevers through a prosthetic retinal removal, i.e., bleaching (Braun et al. 2006). Using an analog of the myc-tag decapeptide, binding of anti-myc-tag antibodies was reported (Kim et al. 2003).

11.6.7 Lipid Bilayers, Liposomes, Cells

Larger biochemical arrangements of molecules include lipid bilayers in biological membranes or whole cells, which can also be examined using microcantilevers. Cantilever array sensors can sense the formation by vesicle fusion of supported phospholipid bilayers of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) on their surface and can monitor changes in the mechanical properties of lipid bilayers (Pera and Fritz 2007). Liposomes were detected based on their interaction with the protein C2A, which recognized the phosphatidylserine exposed on the surface of the liposome (Hyun et al. 2006). Individual *Escherichia coli* (*E. coli*) O157:H7 cell-antibody binding events using microcantilevers operated in the dynamic mode were reported (Ilic et al. 2001) The contractile force of self-organized cardiomyocytes was measured on biocompatible poly(dimethylsiloxane) cantilevers, representing a microscale cell-driven motor system (Park et al. 2005). Resonating cantilevers were used to detect individual phospholipid vesicle adsorption in liquid. A resonance frequency shift corresponding to an added mass of 450 pg has been measured (Ghatnekar-Nilsson et al. 2005).

11.6.8 Spores, Bacteria, and Viruses

Even larger biological entities include fungal spores, whole bacteria, and viruses. Micromechanical cantilever arrays have been used for a quantitative detection of the vital fungal spores of *Aspergillus niger* and *Saccharomyces cerevisiae*. The specific adsorption and growth on concanavalin A, fibronectin or immunoglobulin G cantilever surfaces was investigated. Maximum spore immobilization, germination and mycelium growth was observed on the immunoglobulin G functionalized cantilever surfaces, as measured from shifts in resonance frequency within a few hours, being much faster than standard petri dish cultivation (Nugaeva et al. 2005). Short peptide ligands can be used to efficiently capture Bacillus subtilis (a simulant of Bacillus anthracis) spores in liquids. Fifth-mode resonant frequency measurements were performed before and after dipping microcantilever arrays into a static B. subtilis solution showing a substantial decrease in frequency for binding-peptide-coated microcantilevers as compared to that for control peptide cantilevers (Dhayal et al. 2006). A new approach for investigating antibiotic reaction mechanisms that could speed up the development of new antibiotics has been reported recently (Ndieyira et al. 2008) using microcantilever arrays to explore the mechanisms of antibiotic interactions with mucopeptides—components of bacterial cell walls—down to a sensitivity of 10 nM, and at clinically relevant concentrations in blood serum.

11.6.9 Medical

Diseases can often be identified or characterized by the presence of certain specific biochemical molecules. If receptor ligands exist for these target molecules, then these molecules are likely to be detected by receptor sites attached to a microcantilever, provided the binding events are transduced into a nanomechanical response, i.e., bending of the microcantilever. A bioassay of the PSA using microcantilevers has been presented (Wu et al. 2001), covering a wide range of concentrations from 0.2 ng/mL to 60 μg/mL in a background of human serum albumin (HSA). Detection has been confirmed by another group using microcantilevers in the resonant mode (Hwang et al. 2004, Lee et al. 2005). The feasibility of detecting severe acute respiratory syndrome associated coronavirus (SARS-CoV) using microcantilever technology was studied in a publication (Velanki and Ji 2006) by showing that the feline coronavirus (FIP) type I virus can be detected by a microcantilever modified by a feline coronavirus (FIP) type I anti-viral antiserum. A method for quantification of a prostate cancer biomarker in urine without sample preparation using monoclonal antibodies was described (Maraldo et al. 2007).

11.7 Outlook

Cantilever-sensor array techniques have turned out to be a very powerful and highly sensitive tool to study physisorption and chemisorption processes, as well as to determine material-specific properties such as heat transfer during phase transitions. Experiments in liquids have provided new insights into such complex biochemical reactions as the hybridization of DNA or molecular recognition in antibody–antigen systems or proteomics.

Future developments must go toward technological applications, in particular, to find new ways to characterize real-world samples such as clinical samples. The development of medical diagnosis tools requires an improvement of the sensitivity of a large number of genetic tests to be performed with small amounts of single donor-blood or body-fluid samples at low cost.

From a scientific point of view, the challenge lies in optimizing cantilever sensors to improve their sensitivity to the ultimate limit: the detection of individual molecules.

Several fundamentally new concepts in microcantilever sensing are available in recent literature, which could help to achieve these goals: the issue of a low-quality factor of resonating microcantilevers in liquid has been elegantly solved by fabrication of a hollow cantilever that can be filled with biochemical liquids. Confining the fluid to the inside of a hollow cantilever also allows a direct integration with conventional microfluidic systems, and significantly increases sensitivity by eliminating high damping and viscous drag (Burg and Manalis 2003) Biochemical selectivity can be enhanced by using enantioselective receptors (Dutta et al. 2003). Other shapes for micromechanical sensors like microspirals could be advantageous for biochemical detection (Ji et al. 2006). Miniaturization of microcantilevers into "true" nanometric dimensions, by using nanowires (Cui et al. 2001), single wall carbon nanotubes (Singh et al. 2007), or graphene sheets (Sakhaee-Pour et al. 2008) will further increase sensitivity.

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