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Chapter 10

CANTILEVER BIOSENSORS

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The fabrication of miniaturized and integrated devices as micro- and nano-electro-mechanical systems (MEMS and NEMS) has provided the development of an innovative family of biochemical sensors based on transducers involving mechanical phenomena. Biosensors based on microcantilevers have become a promising tool for directly detecting biomolecular interactions with great accuracy, especially when an optical read-out scheme is applied. The number of applications of these sensors has shown a fast growth in diverse fields, such as genomic, proteomic, environmental, or food quality control, being a promising alternative to the currently exploited biosensor techniques.

10.1. Technical concept

10.1.1. Introduction

This chapter describes the application of nano- and micro-electro-mechanical systems (NEMs and MEMs), and more specifically micro-cantilever structures, as transducers for highly sensitive biosensors. In these devices, named as "nanomechanical biosensors," a biomolecular

interaction produces a change in the mechanical behavior of the transducer (a movement at nanometer scale), which can be measured and analyzed in real time. Microcantilevers translate the molecular recognition of biomolecules into a nanomechanical motion that is commonly coupled to an optical read-out system. A rectangular beam, clamped at one end, is the most used transducer as it is the simplest mechanical structure that can be easily batch-fabricated at micrometer scale (Kim *et al.*, 2006). Microcantilevers can be fabricated in arrays of ten to thousands, and for that reason, they are a promising alternative to current biochips as they could permit the parallel, fast, and real-time monitoring of thousands of analytes (proteins, pathogens, DNA strands, etc.) without the need for labeling (Baller and Fritz, 2004).

Nanomechanical sensors are able to detect analytes with picomolar sensitivity and they have the ability for discerning single-base variations in DNA strands. Recently, microcantilever-based biosensors have arisen as a competitive biosensor alternative for measuring, in a direct way, extremely low forces and masses. When fabricated at the nanoscale (*nanocantilevers*), the sensitivity goes up and expected limits of detection are in the femtomole-to-attomole range with the possibility for detection at the single-molecule level in real time.

In this chapter, the main aspects regarding the physics of microcantilever sensors will be described as well the optical read-out techniques. We will review the state-of-the-art, and we will discuss the prospective future directions of this new family of biosensors.

10.1.2. Working principle

Nanomechanical sensors are derived from the microfabricated cantilevers used in atomic force microscopy (AFM) and are based on the bending or resonance change induced in the cantilever when, for example, a biomolecular interaction takes place on one of its surfaces. The cantilever response will depend on its mechanical properties, which are determined mainly by their spring constant and resonance frequency. Both parameters depend on the cantilever material and its geometry.

The spring constant, k, and resonance frequency, f_0, for a rectangular cantilever clamped at one end are given by

$$k = \frac{Ewh^3}{4L^3}$$

$$f_0 = \frac{1}{2\pi}\sqrt{\frac{k}{m}}$$

where E is the Young's modulus, m is the cantilever effective mass, w is the width, h is the thickness, and L is the length, respectively. Interaction or binding of molecules to one of the cantilever surfaces may lead to a change in the angle of cantilever bending or a shift in its resonance frequency. The detection of these responses is usually referred as *static* and *dynamic* modes of operation, respectively. But the mechanical response is also sensitive to different factors, such as temperature, heat, electromagnetic field, stress, and mass. These transducers can be employed for several applications besides biosensing. Figure 10.1
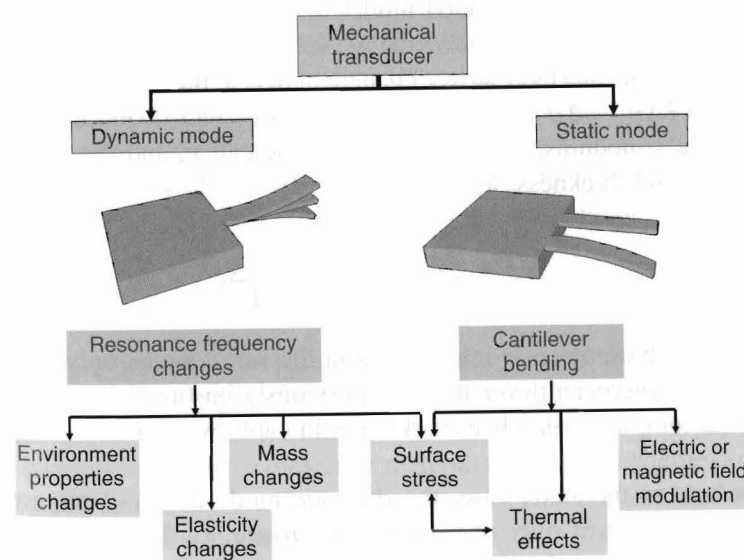

Figure 10.1   Diagram of the two nanomechanical response methods: static and dynamic; and the properties that could be measured with each of them.

shows a diagram with both transducer methods and its relation with the properties that could be measured.

In the sensors working in the *static* mode, the bending arises as a consequence of a surface stress change induced by any molecular reaction, which takes places on only one of the cantilever surfaces. The induced surface stress change could be positive or negative, depending on the surface deformation generated (Shuttleworth, 1950). The cantilever deformation depends mainly on the forces involved in the bioreaction process and is not directly related to the receptor–ligand binding energy. These forces could arise from the bond strength between the receptor and the surface (lineal response) and from the intermolecular interactions between neighboring molecules (non-lineal response). Although the factors and phenomena responsible for this change are still not fully understood, forces coming from electrostatic, steric, and van der Waals interactions, changes in the surface hydrophobicity or conformational changes of the adsorbed molecules could play an important role (Hagan *et al.*, 2002).

The easiest and most extended model to study the surface stress produced on cantilevers is based on the work by Stoney (1909). This model relates the total surface stress change between the top and the bottom sides $(\Delta\sigma_1 - \Delta\sigma_2)$ with the cantilever free end displacement, Δz, the Young's modulus, E, the Poisson coefficient, ν, and the cantilever length, L, and thickness, h, represented by

$$\Delta\sigma_1 - \Delta\sigma_2 = \frac{Eh^2}{3L^2(1-\nu)}\Delta z$$

For sensing biomolecular interactions in the *static mode*, only one surface of the microcantilever must be previously biofunctionalized. This can be a complex task when working with cantilever arrays.

In contrast to the static case, the *dynamic* mode does not require the functionalization of only one cantilever surface, as the resonance frequency depends on the total mass adsorbed on both sides. This mass could produce, at the same time, a change in the cantilever spring constant, affecting the final resonance frequency shift (Lu *et al.*, 2005;

Gupta *et al.*, 2006). In this mode, very high sensitivities can be obtained (in the attogram range), superior to those of other similar mass detectors, such as the quartz crystal microbalance (QCM) (Ilic *et al.*, 2004). The microcantilever resonator is also characterized by the quality factor (Q), which quantifies the energy dissipation and is defined as the ratio between the mechanical energy accumulated and dissipated per vibration cycle. The dissipative mechanisms could be both internal and external; the external damping is the dominant factor when working in air or liquids. The quality factor determines the frequency resolution of the system (Lavrik *et al.*, 2004):

$$\Delta f = \sqrt{3}\frac{f_0}{Q}.$$

Operating in liquids, the resonance frequency and the quality factor shift toward much lower values than in air, due to the damping effect of the viscous surround. The quality factor in liquids could be up to 100-fold lower than in air, reducing the frequency resolution. There are different ways to overcome this limitation: by fabricating cantilevers with higher resonance frequency and quality factors, by measuring the resonance frequency in air under controlled humidity before and after the biochemical recognition, by working with higher order vibration modes, or by using external excitations, among others. For the above reasons, this way of operation is more difficult to implement and most of the cantilever biosensors are based on the static mode.

10.1.3. *Beam design and sensitivity*

Typically, the cantilever dimensions range from tens to hundred of micrometers in length, some tens of micrometers in width, and hundreds of nanometer in thickness. In general, they are made of silicon, silicon nitride, polymers, or piezoelectric materials. The microcantilever properties and sensitivity are determined by the cantilever dimensions and material (Young's modulus and Poisson coefficient). The selection of the appropriate characteristics depends on the working detection method (static or dynamic), the final application, and the available fabrication technology. In general, smaller spring constants give softer cantilevers,

which are more sensitive to bending. Making thinner and longer cantilevers could improve the sensitivity, but they are also more fragile and therefore more difficult to handle. In addition, the thermomechanical noise increases.

As an example, the standard silicon microfabrication technology allows fabricating micrometer-sized cantilevers with a high length:thickness ratio in a reproducible and inexpensive way. However, the noise arising from the cantilever thermal motion could limit this ratio value (Alvarez *et al.*, 2006). On the other hand, reducing the cantilever length and thickness results in a sensitivity increase for the dynamic cantilever sensors, reducing the sensitivity for the surface stress sensors. Polymers, such as SU-8, have a lower Young's modulus than silicon and could be more sensitive for static deflection measurements (even if they do not have a high length:thickness ratio); their fabrication process is relatively inexpensive, fast, and reliable.

The final sensitivity could be determined by previously modeling the system to determine the range of dimensions needed for a specific material and working method. Modification of the microcantilevers' shape could also improve the sensor sensitivity, but the final sensitivity will also depend on the mechanical variabilities between microcantilevers in one array, the resolution of the detection set-up, and the total capacity for integration.

10.1.4. Detection method

A read-out system capable of monitoring changes with subnanometer resolution is crucial for detecting the nanomechanical motion induced by the biochemical recognition process. In addition to supplying high sensitivity and accuracy, the signal read-out is critical in the real-time measurements acquisition and, in the final biosensor, integration of microsystems. To avoid the influence of external factors, such as non-specific binding or temperature changes, a reference cantilever is typically used. For that reason, and for detecting several analytes at the same time, the read-out schemes have to enable the use of arrays of microcantilevers.

There are several techniques suitable for the cantilever response read-out: optical beam deflection, piezoresistivity, piezoelectricity, interferometry, and capacitance are among the most important (Lavrik *et al.*, 2004; Ziegler, 2004). However, the majority of the biochemical applications carried out with cantilever-based sensors are based on the *optical beam deflection method*, due to its high sensitivity. A variation of this optical method has been recently developed using an array of a new type of optical waveguide microcantilevers (Zinoviev *et al.*, 2007). These cantilevers act as a waveguide for conducting light, and it is possible to collect the exit light with a second waveguide or with a photodetector. In this case, the cantilever bending is related to the reduction in intensity of the collected light with respect to the input light.

10.1.4.1. Optical beam deflection

The *optical beam deflection* method is simple to implement and shows a linear response with sub-angstrom resolution. Movement of the cantilever's free end is detected by measuring the reflected laser beam displacement into a position-sensitive photodetector (PSD, Figure 10.2). The laser beam displacement over the photodetector, X, is related with the cantilever free end deflection, Δz, by a simple algebraic equation

$$\Delta z = \frac{XL}{2D}$$

where L is the cantilever length and D is the photodetector–microcantilever distance.

The read-out implementation of array platforms is technologically challenging, as it requires an array of laser sources with the same number of elements as the cantilever array. The laser's displacement could be measured using an array of photodetectors, adding alignment complications, or using just one photodetector and sequentially switching on and off each laser source to avoid the overlapping of the reflected beams (Lang *et al.*, 1998). Using a CCD camera and an array of microcantilevers with paddles at its end (Yue *et al.*, 2004) or a scanning laser source to sequentially illuminate each microcantilever (Alvarez *et al.*, 2004) are new ways to overcome the problem of overlap.
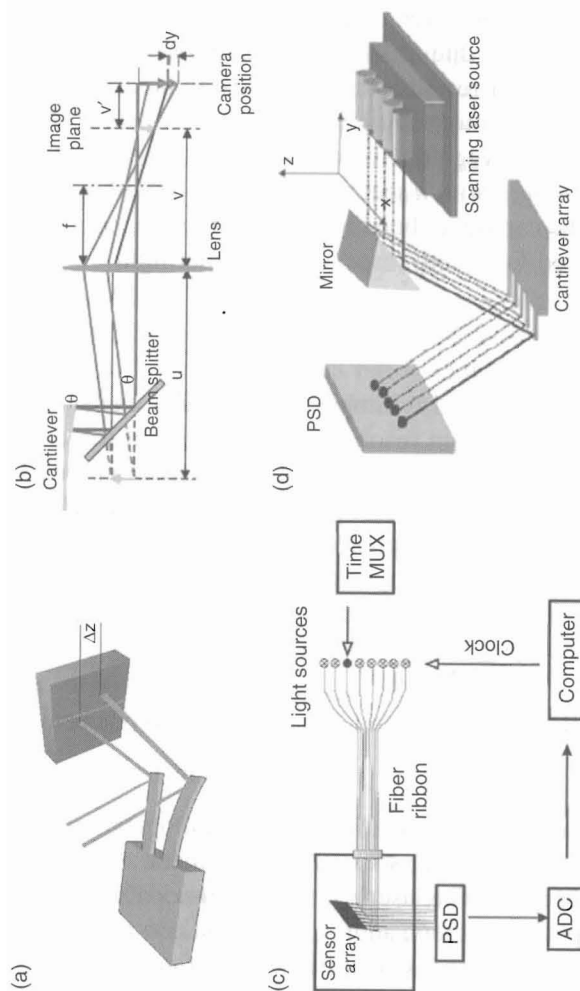
Figure 10.2 (a) Optical beam deflection read-out configuration. (b) Ray optics principles underlying optical detection of cantilever motion by using a CCD camera. (Reproduced with permission from TechScience Press [Yue *et al.*, 2004]). (c) Schematic of the quasi-simultaneous read-out of eight sensors achieved by time-multiplexing (MUX) of eight light sources which are guided by an optical fiber-ribbon onto the sensor array located in the analysis chamber. (Reproduced with permission from H.P. Lang [Lang *et al.*, 1998]. Copyright 1998, American Institute of Physics). (d) Scheme of the sequential illumination by using a scanning laser source (Alvarez *et al.*, 2004).

10.1.4.2. Optical waveguide microcantilever

This technique, based on butt-coupled optical waveguides, was proposed as a new alternative to solve some of the limitations of optical beam detection, the most serious of which is integration in array platforms. The use of a cantilever as a waveguide has been demonstrated by different groups (Wu and Frankena, 1992; Ollier *et al.*, 1999; Budakian and Putterman, 2002; Wang *et al.*, 2002; Xu *et al.*, 2005; Zinoviev *et al.*, 2006b; Nordin *et al.*, 2007). The sub-micron thickness of the cantilevers provides a high sensitivity, while the overall dimensions help to miniaturize the device and make it suitable for further integration in lab-on-chip applications. A schematic view of the optical sensor is shown in Figure 10.3. The device can be operated in both the visible and the infrared ranges.

The "heart" of the sensor is an optically transparent cantilever of sub-micron thickness. Light from the cantilever is injected into the output waveguide, called the "receptor," separated from the free end of the cantilever by a short gap (see Figure 10.3). Both the cantilever and the receptor are total internal reflection waveguides. If the gap is in the order of several microns, the energy transmitted into the receptor changes dramatically with the displacement of the cantilever's free end. The idea is to monitor the changes in the power transmitted into the output waveguide in order to register the deflection of the cantilever caused by any biomolecular interaction occurring on its surface. The device presented by Zinoviev *et al.* (2006a) was fabricated using standard silicon technologies. To obtain a straight cantilever free end aligned with the output waveguide, with precision better than 1 μm, a thermally grown silicon oxide film was used. The input waveguide used for delivering the light into the cantilever should be made out of high temperature low-pressure chemical vapor deposition (LPCVD) silicon nitride because the silica layer cannot work as an optical waveguide on the silicon substrate, due to its lower refractive index. If the input waveguide is fabricated thin enough and utilizes a taper, all the energy can be transmitted into the cantilever, due to the large evanescent field tail of the waveguide mode propagating along the silicon nitride layer.
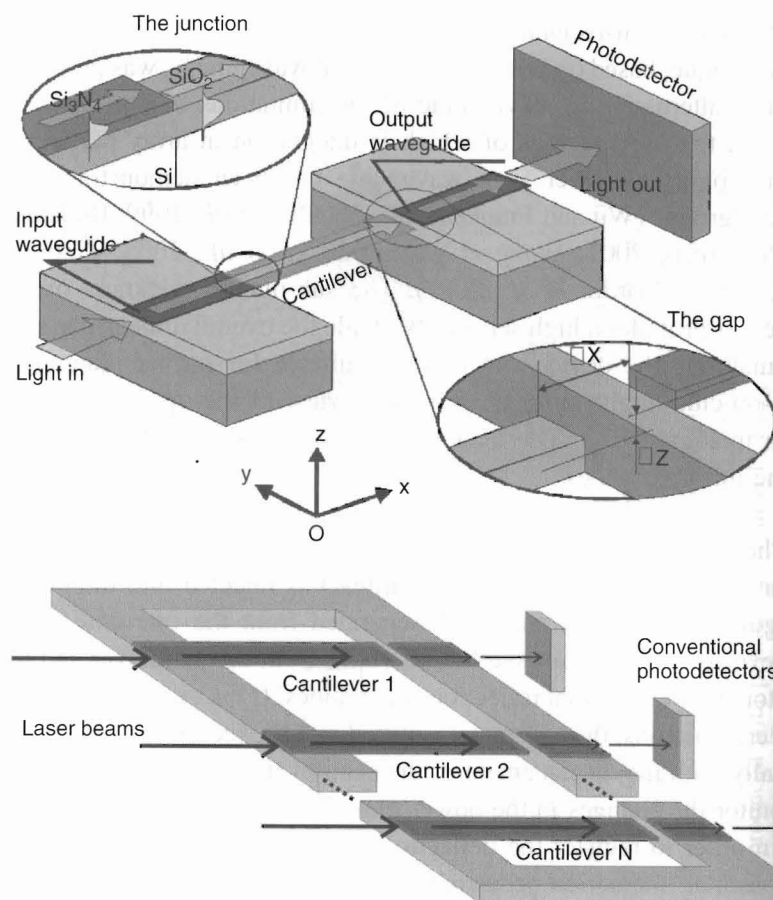
Figure 10.3 (*Top*) Schematic view of a waveguide optical microcantilever; (*bottom*) Schematic view of an array of optical cantilevers.

10.1.5. *Microcantilever surface functionalization*

Immobilization of bioreceptors on the cantilever surface strongly influences the quality of the bioanalysis to be performed. The efficiency of biomolecule attachment, the accessibility to its targets, and the degree of non-specific binding have to be taken into account. The immobilization process should avoid any change in the biological properties

of the receptors, but, at the same time, must keep the chemical and physical properties of the cantilever as uniform as possible in order to generate a large surface stress. The current interest in nanometer-sized cantilever is demanding new immobilization techniques for the manipulation at nanometer scales; newer strategies from synthetic chemistry will become essential for the manipulation of biosensor surfaces and subsequent assembly of receptors. Controlled chemical functionality can be accomplished using self-assembled monolayers (SAMs). The strong affinity of sulphur compounds (thiols, thioethers, and disulfides) for gold and other noble metals make them excellent candidates for nanomechanical biosensing (Nuzzo and Allara, 1983; Ferretti *et al.*, 2000), since the cantilever surface usually has one surface coated with a thin layer of gold (20–100 nm) to increase reflectance of the laser beam. The immobilization of both DNA and proteins on gold surfaces using thiol-SAMs has become the most widely employed method.

The covalent immobilization of proteins on the gold surfaces of the cantilevers can be achieved by a wide variety of chemical procedures, ensuring reproducibility and stability of the protein coating (see Figure 10.4). The main drawback is that some of the functional groups could randomize the orientation of the active sites of the protein. Thus, the appropriate immobilization procedure must be chosen in order to avoid inactivation or a decrease in the activity of the protein receptor. Several
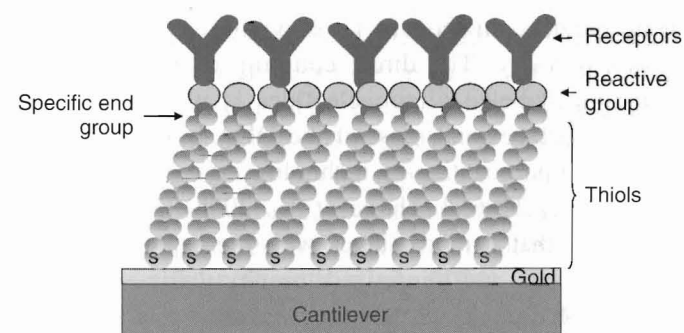

Figure 10.4   Scheme of covalent immobilization by using organic multilayers.

strategies could be employed which have already been useful in many other analytical systems, such as surface plasmon resonance (Boozer et al., 2004), QCM (Mannelli et al., 2005), or electrochemical measurements (Hianik et al., 2001). A very interesting one is to covalently immobilize carboxylate-terminated alkanethiols onto the gold surface, followed by the esterification of the carboxylic groups with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) (Staros et al., 1986; Grabarek and Gergely, 1990; Patel et al., 1997). Another alternative is using a cystamine modified with glutaraldehyde and the subsequent attachment of the protein through an amine group (Alvarez et al., 2003; Pavlov et al., 2004). In addition, we can immobilize biotinylated proteins on avidin monolayers previously attached to the surface using EDC or cystamine chemistry. The immobilization procedure developed by Park and Kim employs the reaction of sulfosuccinimidyl 6-[3-(2-pyridyldithio)pro-pionamido] hexanoate (sulfo-LC-SPDP) with the protein NH_2-groups to give amide linkages. The subsequent addition of dithiothreitol (DTT) reduces the disulfide to give a thiol, which finally self assembles onto the gold surface (Park et al., 2002). Alternatively, a protein, such as antibody, protein A, or avidin, can be thiolated, with the thiol group subsequently used for immobilization on the metal surface; however, there is a high risk of denaturing the protein and therefore losing its biological function (Pyun et al., 2005).

Nucleic acid immobilization is much easier, since chemical modifications can be included in their *in vitro* synthesis, avoiding the risk of losing its functionality. The direct coupling of DNA probes by self assembly of thiol-labeled oligonucleotides is widely used on microcantilever gold surfaces and most of the DNA biosensing applications performed with microcantilever technology are based on this strategy (Biswal et al., 2006; Lechuga et al., 2006). Herne and coworkers demonstrated that a mixed-layer with mercaptohexanol enhanced the hybridization rate and minimized the physical adsorption of DNA probes on gold surfaces by eliminating the weaker adsorptive contacts between the nucleotide chain and gold. In this way, the majority of the immobilized DNA probes are accessible for hybridization with the complementary strand. To prevent non-specific binding to the silicon side of the cantilever, a PEG-silane coating is used (Biswal et al., 2006).

For silicon optical waveguide microcantilevers, immobilization chemistry offers a multitude of well-established and relatively simple attachment strategies. Frequently used silicon modifications, such as aldehyde activation or coating with poly-lysine or nitrocellulose, are typical for the biomolecule attachment (MacBeath and Schreiber, 2000). The highly reactive epoxysilane surface, for example, reacts not only with amino groups, but also with other nucleophilic moieties on protein surfaces like alcohol, thiol, and acid groups, exhibiting a high binding capacity (Zhu et al., 2001). Other methods employ amine-terminal silanes such as aminopropyl-triethoxysilane to provide a reactive amine group at the nitride surface. Thus, alkylamine coupling using glutaraldehyde is simple and fast, giving an aldehyde derivative that can form an imine linkage with primary amines on the protein (Williams and Blanch, 1994). The direct immobilization via thiol-terminal silanes is also a well-known methodology. Using crosslinkers such as EDC and NHS, it is possible to control the directional immobilization with more than 90% of specific binding to the surface (Shriver-Lake et al., 1997).

The immobilization of different bioreceptors on each cantilever of an array is a complicated task. There are several commercial platforms devoted to the specific functionalization of individual cantilevers. Several commercial platforms have been reported using different strategies. For example, the *Autodrop* platform (Microdrop) (Bietsch et al., 2004; www.microdrop.com) uses the principle of the ink jet printing technology and employs up to eight dispensers in an area of $200 \times 200 \times 80$ mm. The core of the dispensing head consists of a glass capillary which is surrounded by a tubular piezo actuator. At one end, the capillary forms a nozzle. By applying a voltage pulse, the piezo actuator creates a pressure wave which propagates through the glass into the liquid. This platform has been used for uniform deposition of thiolated DNA and others alkenothiols (Bietsch et al., 2004). The *Nanojet* technology, also from Microdrop (www.microdrop.com), forms a liquid jet by means of a dispenser system controlled by time and pressure. Provided that the micro valve switches at a very fast rate, volumes down

to 200 nl can be dispensed. Moreover, a combination of the Autodrop and Nanojet systems, called "Dropjet," is also available, allowing the generation of a large number of drops with well-defined size (www.microdrop.com).

Cantion has developed the *Canti*™*Spot* platform, which can be used for delivering reagents onto individual cantilevers as well as onto controlled small areas. Its spotter, capable of delivering 100 pl droplets, is connected to a computer-controlled syringe pump for adjusted aspiration and dispensing of reagents (www.cantion.com). Although the *Cantisens*® *FU-401* platform from Concentris is currently used only for cantilever functionalization, it offers a novel way to immobilize biomolecules on cantilever arrays using capillary techniques; up to four cantilevers can be functionalized simultaneously (www.concentris.ch). Finally, a completely different approach under development in several laboratories uses a microfluidics system with an independent flow path for each cantilever in an array (Figure 10.5). With this type of system, immobilization can be performed on each cantilever independently by flowing the appropriate reagents through the individual flow cells.
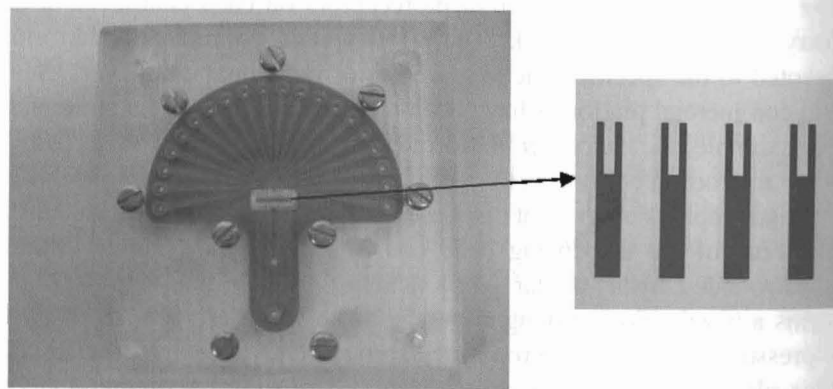

Figure 10.5 Flow cell with 20 independent flow channels for the measurement of an array of 20 microcantilevers with a pitch of 250 microns (flow cell photograph courtesy of ESD University of Southampton; microcantilevers photograph courtesy of CNM-CSIC).

10.2. History

The first chemical sensor based on a macroscopic bimetallic plate was proposed by Norton (1943) for hydrogen detection. In the following years, the concept of a device able to provide a direct conversion between the chemical stimulus and the mechanical energy was developed, focusing attention on miniaturized devices (Kuhn *et al.*, 1950; Steinberg *et al.*, 1966). At the end of 1960s, some integrated miniaturized devices were developed (Newell, 1968; Newell *et al.*, 1968). However, the idea of fabricating floating structures was given up due to the microelectronics fabrication limitations at that time. As early as 1986, with the appearance of AFM (Binnig *et al.*, 1986) and improvements in microelectronic technology, micro-mechanical transducers started to be used routinely and mass production at low cost was possible. Atomic force microscopy arose as a surface characterization technique, in air and subsequently in liquid, measuring the cantilever deflection induced by the interaction between a tip placed at its free end and the surface of the sample. Expansion of AFM techniques showed the microcantilever sensitivity for measuring intermolecular forces between two complementary biological molecules (Force Spectroscopy), such as the interaction between proteins (avidin/biotin) (Lee *et al.*, 1994), hybridization between complementary single-stranded DNAs (Lee *et al.*, 1994), or antigen–antibody interactions (Dammer *et al.*, 1996).

The sensitivity of microcantilevers for measuring intermolecular forces, the commercial availability of cantilevers, and their fabrication using standard microelectronic technology resulted, around 1994, in a new type of sensor where the transducer system is based on a silicon microcantilever with a tipless free end (Figure 10.6) (Gimzewski *et al.*, 1994; Chen *et al.*, 1995). Biochemical applications for this type of sensor have been specifically developed for bending-based modes of measurement, with an optical read-out, due to the complexity required for working with the dynamic mode in liquids. The first experiments in solution were focused on ion detection and measurement of changes in surface stress induced by changes in pH or ion concentrations. It was found that the cantilever response depended upon both pH and the ionic strength of the aqueous medium (Butt, 1996).
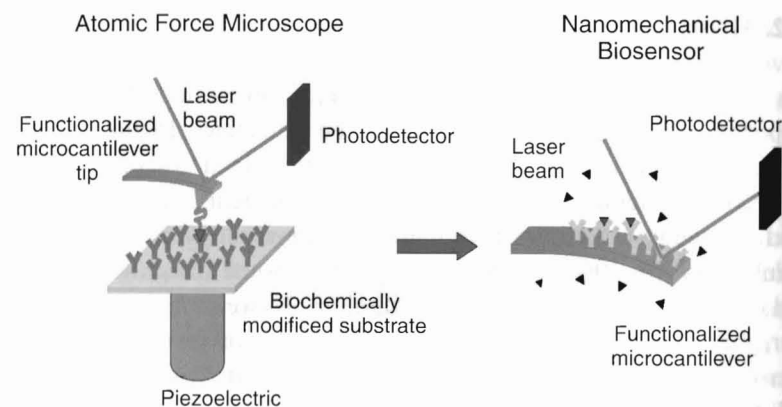
Figure 10.6 Schematic view of (*left*) force spectroscopy and (*right*) nanomechancial biosensor working principles.

Among other biochemical applications, induction of surface stress by formation of SAMs (Berger *et al.*, 1998; Datskos and Sauers, 1999; Fritz *et al.*, 2000b) or by non-specific adsorption of proteins such as BSA or lipoproteins has been studied (Moulin *et al.*, 2000). However, the largest growth in cantilever-based biosensor research has occurred after the landmark paper of Fritz *et al.* (2000a), in which sensitive discrimination of single-base variations in DNA strands was demonstrated without using fluorescent labels. This paper had a wide impact and marked the beginning of a major research effort in this field. Shortly afterwards, microcantilever sensors were also employed to detect diverse biomolecular interactions. Several commercial platforms (Carrascosa *et al.*, 2006) based on microcantilever array sensors are now available.

10.3. State of the art

The extensive development that cantilever-based biosensing has experienced during the last 10 years has been mainly focused on demonstrating its suitability and sensitivity in a wide range of different fields: genomic, proteomic, environmental control, clinical diagnosis, etc. This section reviews some of the biosensing applications of the

microcantilever-optical read-out configurations, as well as the different approaches proposed to increase cantilever biosensor sensitivity.

10.3.1. Biosensing applications: optical read-out configuration

The *bending* detection method is very sensitive to changes in the surrounding environment, and has been applied to detection of variations in pH or salt concentration using silicon microcantilevers modified and non-modified with SAMs (Butt *et al.*, 1995; Fritz *et al.*, 2000b; Ji *et al.*, 2001). The effect of functionalized cantilevers with SAMs, such as alkylthiols with different chemical termini or lengths, on surface stress has also been studied (Berger *et al.*, 1998; Datskos and Sauers, 1999; Godin *et al.*, 2004; Kohale *et al.*, 2007). Likewise, different functionalization schemes have shown that cantilevers were able to detect different ions, such as Ca^{2+} (Ji and Thundat, 2002) or CrO_4^{2-} in concentrations as low as 10^{-11} M (Zhang *et al.*, 2003), among many others.

Genomics is one of the fields where the cantilever-based biosensors have generated both interest and controversy. The first publication in this field demonstrated the detection of a single-base mismatch of single-stranded DNA chains comprised of 12 nucleotides, with a detection limit of 10 nM. An array of two cantilevers was used, employing one as a reference; the final signal was determined as the difference in bending of the two cantilevers (Fritz *et al.*, 2000a). A subsequent study reported the discrimination of single nucleotide polymorphisms with a single cantilever (Hansen *et al.*, 2001; Wu *et al.*, 2001b). This opened a discussion about the possibility of detecting the DNA hybridization and single nucleotide polymorphisms without a reference cantilever (Arntz *et al.*, 2003; Alvarez *et al.*, 2004).

McKendry *et al.* (2002) detected 75 nM of target oligonucleotide using an array of eight microcantilevers previously functionalized using microcapillaries containing a 40 μM solution of thiolated DNA probes. Thermal denaturation has also been checked (Biswal *et al.*, 2006), reporting a decrease in the melting temperature with chain length and salt concentration; lower melting temperatures were also reported for surface-grafted DNA than for DNA in solution. More recently, this technology has

been applied for the validation of mRNA biomarkers in total cellular RNA, with sensitivity in the picomolar range without target amplification (Zhang *et al.*, 2006). Another novel application is the design of synthetic DNA motors (Shu *et al.*, 2005), which generate a microcantilever nanoscale motion via controlled conformational changes (see Figure 10.7). The forces exerted by the conformational change of precise duplex to non-classical i-motif were shown to induce a compressive surface stress of 32 ± 3 mN/m (which means a single motor force of approximately 11 pN/m). The cantilever direction and amplitude were controlled by buffer pH and the ionic strength, signifying the importance of electrostatic forces in surface stress generation.

In the proteomics field, numerous studies have used antibodies as receptors for detecting their complementary proteins. Wu *et al.* (2001a) measured two isoforms of prostate-specific antigen (cPSA and fPSA) over a range of concentrations from 0.2 ng/ml to 60 μg/ml, in a background of human serum albumin (HSA) and human plasminogen (HP) at 1 mg/ml (Figure 10.8); these isoforms have proven to be a useful biomarker for early detection of prostate cancer. The limit of detection obtained was at the current limit of ELISA (0.2 ng/ml), with the advantage of being label free. Many other applications have been described, such as the detection of 100 nM of streptavidin with biotin functionalized cantilevers (Raiteri *et al.*, 2001) or the real-time recognition of multiple different cardiac biomarker proteins (creatin kinase and myoglobin) (Arntz *et al.*, 2003). A cantilever-based sensor sensitivity of ≈ 1 nM was achieved using single-chain Fv (scFv) antibody fragments as receptor molecules, covalently attached to the gold-coated sensor interfaces in directed orientation (Backmann *et al.*, 2005).

The potential of the cantilever-based biosensor for detection conformational changes in proteins has also been explored. Conformational change-induced surface stress was studied for the protein calmodulin. The cantilever bent when the calmodulin bound to Ca^{2+}, in contrast to the cantilevers modified with other proteins, such as hemoglobin and myoglobin, which do not exhibit change conformations upon binding with analytes (Yan *et al.*, 2006). The ability of cantilever-based sensors for detection of ligand–protein interactions and conformational
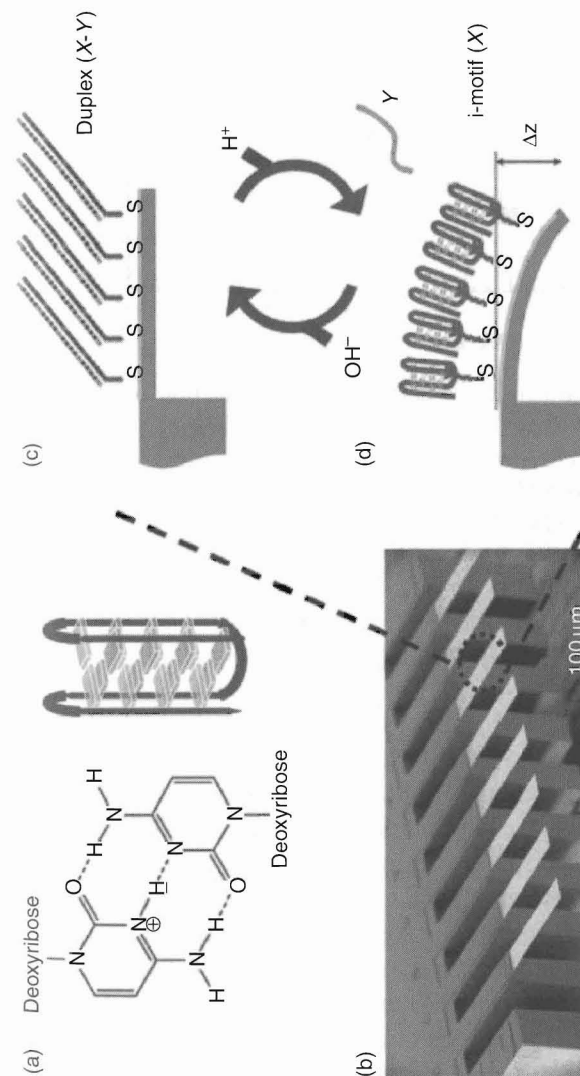

Figure 10.7 Conformational changes from duplex to i-motif. (a) Schematic diagram to show the intramolecular interdigitation of strand **X** to form the i-motif (at pH 5.0). (b) Scanning electron microscope image of an array of eight rectangular silicon cantilevers. (c) Schematic diagram to show the hybridization between the surface-tethered **X** and the strand **Y** in solution (at pH > 6.7). (d) Formation of the **X** strand self-folded i-motif, inducing repulsive in-plane surface forces (at pH 5.0). (Reproduced with permission from Shu *et al.* (2005). Copyright 2005, American Chemical Society.)
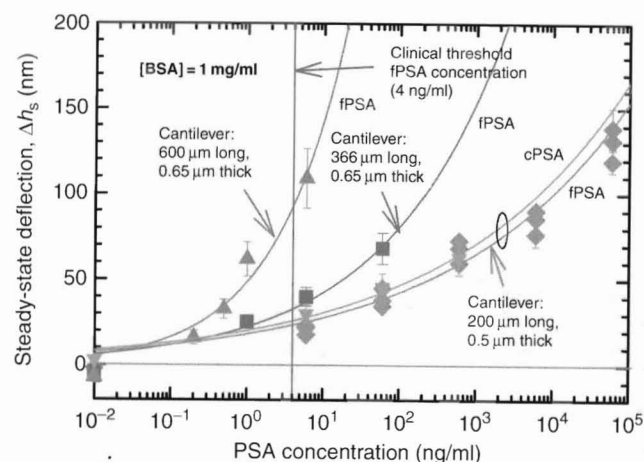
Figure 10.8 Steady-state cantilever deflections as a function of fPSA and cPSA concentrations for three different cantilever geometries (*longer cantilevers produce larger deflections for the same PSA concentration*). (Reproduced with permission from Macmillan Publishers Ltd: Nature Biotechnology [Wu *et al.*, 2001a], Copyright 2001.)

changes of membrane proteins (such as bacteriorhodopsin) has been demonstrated as well (Braun *et al.*, 2006).

Applications have expanded to new fields, such as detection of pathogens such as *Escherichia coli* for food quality control (Zhang and Ji, 2004) or environmental control. In this latter field, the interactions between the herbicide 2,4-dichlorophenoxyacetic (Raiteri *et al.*, 1999) and the organochlorine insecticide compound dichlorodiphenyl-trichloroethane (DDT) (Alvarez *et al.*, 2003) with their corresponding monoclonal antibodies have been measured. Direct detection of DDT was achieved with a competitive assay, measuring DTT concentrations as low as 10 nM. In addition, the novel development for early osteosarcoma discovery, sensing the interactions between vimentin antibodies and antigens with a single cantilever-based biosensor, proved that cantilever biosensors can provide a suitable platform for life sciences research (Milburn *et al.*, 2005). Also of interest is application of microcantilevers for detection of the feline coronavirus type I (Velanki

and Ji, 2006), which demonstrated the feasibility of detecting severe acute respiratory syndrome associated coronavirus (SARS-CoV).

As previously mentioned, the application of *resonant* mechanical sensors for biochemical recognition in a liquid medium is a great challenge, as a consequence of the cantilever's resonant frequency and quality factor reduction. In spite of this limitation, there are some works that demonstrate detection of biomolecules by measuring the cantilever resonance before and after the binding event. Using this methodology, DNA hybridization has been sensed using DNA strands linked to the cantilever by gold–thiol covalent bonding and gold nanoparticle-modified probes. After hybridization, the gold nanoparticles act as a nucleating agent for the growth of silver particles, which leads to a detectable frequency shift by increasing the cantilever effective mass. This method can detect at least 0.05 nM and is able to discriminate a single-base mismatch (Su *et al.*, 2003).

The number of applications performed using the dynamic mode grows when working with pathogens, cells, viruses, etc. that have a large mass. Ilic *et al.* (2001) demonstrated the ability to detect both an *E. coli* antibody monolayer and a single *E. coli* cell, operating in air and measuring the cantilever thermal noise with the optical bending method. The mass sensitivities for a 15 and 25 µm long silicon beam were 1.1 and 7.1 Hz/fg, respectively. Measurement of growth of pathogens such as *Aspergillus niger* spores (Nugaeva *et al.*, 2005) and *E. coli* cells (Gfeller *et al.*, 2005), in a humidity-controlled environment, has also been reported. In these experiments, the resonance frequency shifts as a function of the increasing mass on the cantilever.

Reducing the cantilever size is a widespread alternative for increasing the frequency resolution, in both air and liquid. With a silicon cantilever 4–5 µm long, 1–2 µm wide, and 20–30 nm thick, Gupta *et al.* (2004) established detection of a single vaccinia virus particle with an average mass of 9.5 fg (under ambient conditions, and by measuring the cantilever thermal noise). Later, they found that the protein attachment increases with cantilever size (Figure 10.9), resulting in an increase in the effective spring constant and a corresponding anomalous increase in frequency for a certain class of cantilevers (Gupta *et al.*, 2006).
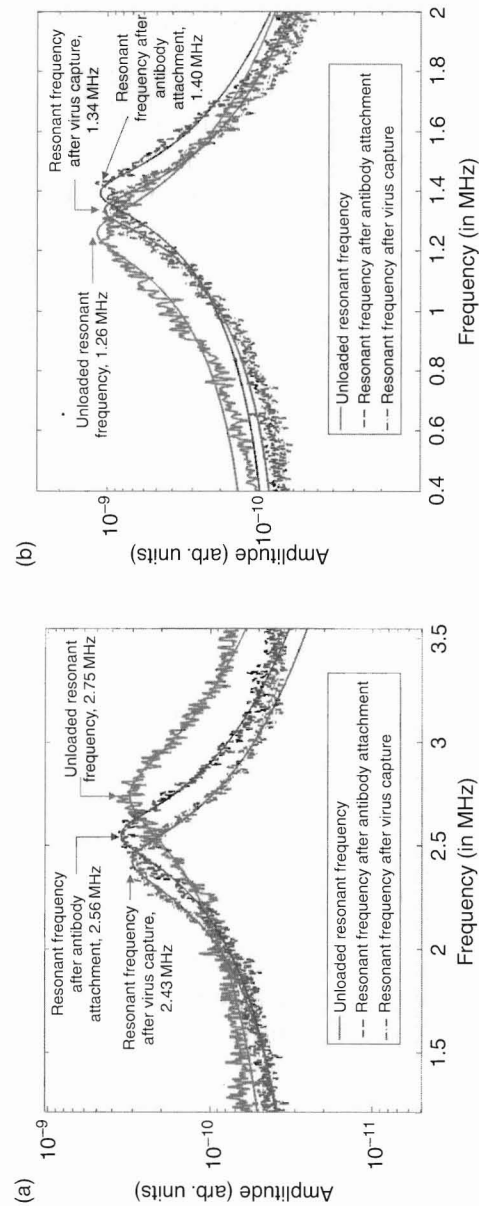
Figure 10.9 Thermal noise-induced frequency spectra of cantilever beams, at various stages of the biosensor analysis. For the same process, the resonance frequency decreases (a) or increases (b) depending on the cantilever dimensions. (Reproduced with permission from Gupta *et al.* [Gupta *et al.*, 2005], Copyright 2005, IEEE.)

The interaction of biotin with streptavidin has been measured in liquids by working with higher cantilever resonance harmonics (Ghatkesar *et al.*, 2004). As an alternative, the integrated piezoelectric microcantilevers are becoming increasingly popular, showing quality factors one order of magnitude greater than thermal-driven cantilevers, allowing for mass virus detection in the femtogram range (5–10 fg) in air (Johnson *et al.*, 2006). A nanomechanical lead zirconium titanate or PZT thin film cantilever, composed of $SiO_2/Ta/Pt/PZT/Pt/SiO_2$ on a SiN_x supporting layer, was used for detection of different concentrations (1–100 ng/ml) of prostate-specific antigen (PSA) in liquids (Hwang *et al.*, 2004). For these experiments, the antibody was previously immobilized with calixcrown SAMs on a gold surface deposited on the cantilever; the subsequent antigen detection was executed in a fabricated poly(dimethylsiloxane) (PDMS) flow cell with a 200 μm width channel and a 20 μl volume reaction chamber, by measuring the resonant frequency with a laser Doppler vibrometer (Figure 10.10).

10.3.2. Other cantilever-based biosensor approaches

Currently, there are many different and alternative ways to increase the sensitivity of cantilever-based biosensors, depending on the sensor working mode. For example, production of cantilever surface nanostructures has been demonstrated as a good method for amplifying the bending signal, due to the increased effective surface area (Lavrik *et al.*, 2001; Headrick *et al.*, 2003).

Exploitation of polymers for cantilever fabrication results in a cheaper alternative to increase the cantilever static deflection, as the Young's moduli of polymers are lower than that of silicon. At present, one of the most widely employed alternative materials for new sensor designs is the SU-8 polymer, due to its favorable properties. A non-vacuum fabrication process to produce arrays of SU8 cantilevers has been reported, demonstrating their application as chemical sensors (Ransley *et al.*, 2006). Zhang and Xu (2004) demonstrated the suitability of 6 μm thick polyethylene terephthalate films for microcantilever fabrication using laser micromachining techniques. In this work, DNA hybridization experiments showed the capability of detecting 12 base oligonucleotides
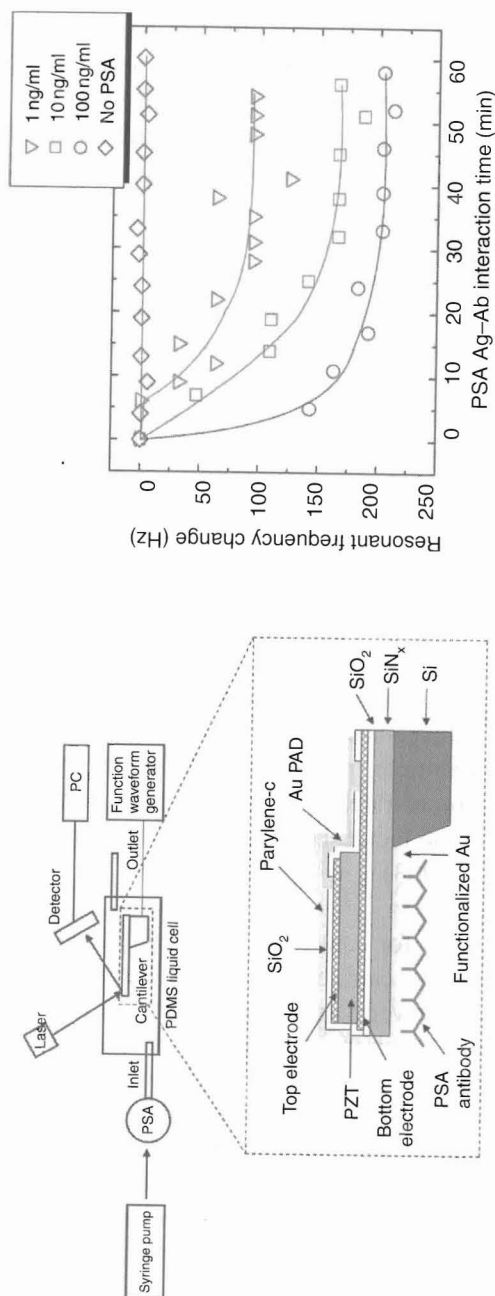
Figure 10.10 Schematics of the experimental measurement system and in-situ resonant frequency change as a function of PSA antigen concentrations (1, 10, and 100 ng/ml[1], respectively). ([Hwang et al., 2004] – Reproduced by permission of the Royal Society of Chemistry.)

at concentrations as low as 0.01 μm. Both experiments were carried out with the optical beam deflection method. Another widely employed polymer is PDMS. With this polymer, three-dimensional thin structures with different geometries on each side have been fabricated (Park et al., 2006). In addition to polymer cantilever fabrication, this work demonstrated the mechanical shear stress enhancement of self-organized cardiomyocytes on three-dimensional grooved surfaces compared to that on two-dimensional surfaces (Figure 10.11), revealing quantitative mechanical changes in cells in real time between the relaxed state and the contracted state of cardiomyocytes.

Other works propose new designs and cantilever configurations fabricated using standard silicon microtechnology to improve sensitivity or solve existing drawbacks. A configuration based on two adjacent cantilevers forming a sensor/reference pair was used to demonstrate the capability of a DNA aptamer–protein binding event to generate changes in surface stress (Savran et al., 2004). This configuration allowed the researchers to directly detect the differential tip deflection between the two cantilevers.

For sensors based on an array of microcantilevers and standard optical detection, either with a single or an array of PSD, a very low variability
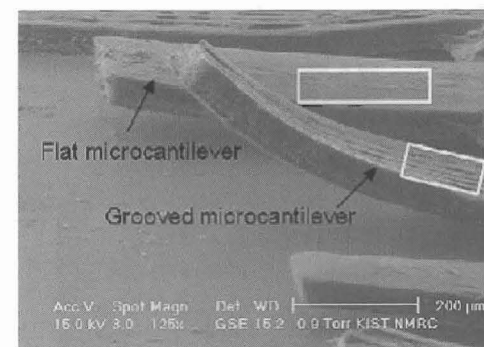

Figure 10.11 ESEM (environmental scanning electron microscope) image of cardiomyocytes cultured on flat and grooved microcantilever structures. [Reproduced with permission from IOP Publishing Limited (Park et al., 2006).]

in the initial position of cantilever array is desired. A reduction in the initial angular offset and angle deviation between the cantilevers of an array is achieved by fabricating T-shaped cantilevers (Plaza et al., 2006). This geometry consists of a sensing cantilever joined to a doubly clamped beam (Figure 10.12), which allows that all the cantilevers remain flat and parallel to each other in spite of possible non-uniform initial stresses at the anchor region.

New designs, such as an array of cantilevers with a common or discrete window (Lechuga et al., 2006) or the aforementioned optical waveguide microcantilevers, offer a new and interesting approach for further integration in "lab-on-a-chip" microsystems. Figure 10.13 shows the device produced by Zinoviev et al., which contains cantilever transducers with lengths of several hundreds of microns fabricated in an array of 20 (Zinoviev et al., 2006a, 2007). In this work, characterization
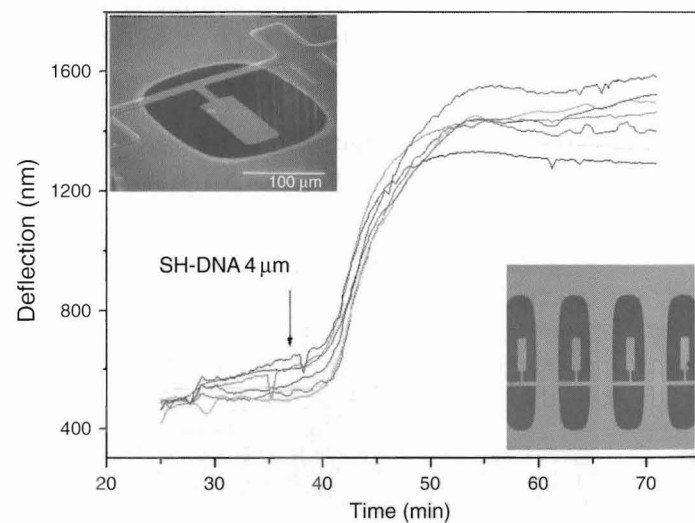
Figure 10.12 Real-time deflection of seven T-shaped microcantilevers due to the immobilization of thiol-modified DNA chains (SH ssDNA). Scanning electron microscopy images of T-shaped cantilevers. (Reproduced with permission from J.A. Plaza [Plaza et al., 2006]. Copyright 2006, American Institute of Physics.)
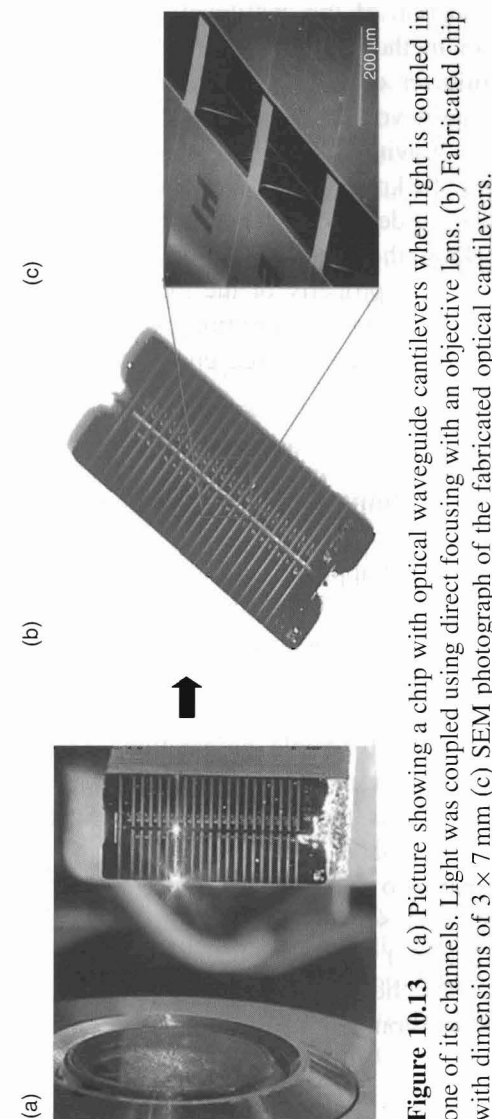
Figure 10.13 (a) Picture showing a chip with optical waveguide cantilevers when light is coupled in one of its channels. Light was coupled using direct focusing with an objective lens. (b) Fabricated chip with dimensions of 3 × 7 mm (c) SEM photograph of the fabricated optical cantilevers.

of the cantilevers' resonance frequency and bending was performed using the optical beam technique for monitoring the waveguide output signal as a function of the cantilever absolute displacement. The sensitivity of both methods, resonant frequency and bending, allows resolution of cantilever displacement to less than 0.1 nm. The effect of covering the cantilevers with gold for biosensing applications has also been studied, showing a strong adsorption depending on the cantilever length and thickness as well as the gold layer thickness. In contrast, the higher order modes are getting filtered, which has the advantage of making the cantilevers single mode in the transverse direction. An additional property of the metallized cantilevers is their ability to deform with light absorption, which helps to adjust the initial position of the cantilever free end with respect to the output waveguide.

10.4. Advantages and limitations for use in optical biosensing

The increasing number of applications of microcantilevers as biosensors has established these systems as a versatile platform for real-time and *in situ* measurements of physical, chemical, and biochemical interactions. As previously mentioned, one of the main advantages is their ability to detect molecular interactions without any kind of label, reducing the time and cost of sample preparation. Another advantage is the low cost of fabrication and mass production of extremely sensitive devices, as well as the flexibility in fabrication techniques to change the size and shape depending on the required application. Hence, it is possible to fabricate cantilevers with very low spring constants that are very sensitive for bending detection, or reduce its size (stiffer cantilevers) for mass changes measurements. A disadvantage related with the cantilever dimensions is the mechanical–thermal noise, which could limit the fundamental biosensor detection limit.

The microcantilever can be operated in vacuum, gasses, and liquids, although the dynamic resolution is reduced when working in liquids, due to the damping effect over the resonance response. This limitation

has rendered the static mode as the most attractive method for biosensing platforms. The main disadvantage related to bending detection is the complex relation between the measured signal and the factors producing it, due to the number of forces acting in the biorecognition processes. Although the working principle of mechanical response could be more difficult to understand than in other types of sensors, a new type of information is reported and represents a new alternative for the discrimination of interactions, such as DNA single-base mismatch polymorphisms or single-molecule detection, otherwise not possible with other established biosensing techniques. Among other advantages, microcantilevers present a reduced sensor area, is compatible with the complementary metal oxide semiconductor (CMOS) technology, and can be easily scaled up, being currently fabricated in arrays of tens to thousands of microcantilevers. This could allow the parallel, fast, and real-time monitoring of thousands of analytes (DNA strands, proteins, pathogens, cells, etc.) without the need for labels. Moreover, the bending and resonance response (frequency, amplitude, Q-factor or phase), when simultaneously detected, can supply valuable information.

Currently, excellent results have been obtained with the optical bending read-out configuration; however, this set-up is limited by environmental optical properties which confound its applications with real samples such as blood.

In order to obtain an industrial sensor system, low power consumption and compact portable devices are required. These requirements have led to an increase in the development of integrated detection read-outs with a CMOS electric circuitry incorporated (Lechuga *et al.*, 2006). In addition to an integrated read-out, the biotechnology field requires that biosensors be capable of working with small amounts of reagents, with highly reproducible surface functionalization and subsequent recognition processes. For this purpose, an integrated microfluidics system that allows liquid handling in an easy and reproducible approach is needed (Bietsch *et al.*, 2004; Yue *et al.*, 2004; http://www.protiveris.com/products/pages/sensorcartridge.html).

10.5. Potential for improving performance or expanding current capabilities

Some of the most desired improvements on the cantilever-based biosensors are the increase in the sensitivity and reproducibility, a more in-depth knowledge of the working principles, and a means of integration of these sensors into a portable and miniaturized system.

There are different ways to increase the biosensor sensitivity. Some are related to optimization of the cantilever material and dimensions, noise reduction, or improvements in the detection methods. The development of a highly sensitive and integrated detection method is one of the most important unresolved matters in this field where new approaches, such as the optical waveguide cantilevers, arise as a great alternative to standard optical beam methods. Other techniques based on piezoresistive and piezoelectric microcantilevers allow an easy and more integrated read-out and are becoming important during the past several years, although their sensitivities are presently inferior to those obtained with the optical method.

Other ways to improve sensitivity are focused on the biochemistry, looking for new methods to functionalize the cantilever reproducibly, reducing both the non-specific interactions and the volume of reagents. SAMs are especially useful for binding assays, allowing surface regeneration for subsequent assays. Development of an integrated microfluidic system would be especially valuable for improving the signal reproducibility, reducing the volume required and the total system size. The possibility of working with several cantilevers at the same time, in completely independent microfluidics channels, would allow the detection, in real time, of many different reagents simultaneously, providing this sensor with a greater capability of measurement and higher versatility than other current techniques (such as ELISA or DNA microarrays).

A more fundamental understanding about the cantilever working principles and the factors involved in the cantilever response, both static and dynamic, are needed. This knowledge will provide new useful

information for increasing the biosensor sensitivity and for controlling the binding process itself (Lu *et al.*, 2005; Reed *et al.*, 2006; Craighead, 2007; Watari *et al.*, 2007).

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Chapter 11

PROTEIN MICROARRAY TECHNOLOGIES: AN ARRAY OF APPLICATIONS

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Within the last years, protein microarray-based research has moved from being technology-based to application-oriented. Protein microarrays have been successfully used in a variety of applications such as the identification, quantification, and functional analysis of proteins in basic and applied proteome research. Array-based assay systems analyze hundreds of molecular ligand-binding events in a single experiment. Such miniaturized and parallelized assay systems have the potential to replace singleplex analysis systems. Numerous analytical platforms have been developed that are likely to evolve into key technologies for the characterization of complex samples. Many of the systems developed have demonstrated robustness, automation, and the