# Cantilever biosensors

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This review will provide a general introduction to the field of cantilever biosensors by discussing the basic principles and the basic technical background necessary to understand and evaluate this class of sensors. Microfabricated cantilever sensors respond to changes in their environment or changes on their surface with a mechanical bending in the order of nanometers which can easily be detected. They are able to detect pH and temperature changes, the formation of self-assembled monolayers, DNA hybridization, antibody–antigen interactions, or the adsorption of bacteria. The review will focus on the surface stress mode of microfabricated cantilever arrays and their application as biosensors in molecular life science. A general background on biosensors, an overview of the different modes of operation of cantilever sensors and some details on sensor functionalization will be given. Finally, key experiments and current theoretical efforts to describe the surface stress mode of cantilever sensors will be discussed.



## 1. Introduction to biosensors

Biosensor research is a vital and rapidly progressing field. There seems to be a never-ending demand for novel biosensors in order to detect a growing number of different molecules at increasingly lower concentrations, to reduce sample volumes by miniaturization, to record an increasing number of signals in parallel or to implant sensors in the body.[1] Major areas of biosensor applications are basic research in the life sciences, health care and medical diagnostics, environmental screening, drug screening or process control in industry, but also the detection of harmful substances for military applications. An ideal biosensor would detect molecular species from a single molecule up to high concentrations and identify the molecular composition of a sample in real-time without influencing the sample. In doing so it should be reliable, cheap, small and portable, and usable also for untrained personnel. Clearly, biosensor research is a highly interdisciplinary endeavour, bringing together physicists and engineers for developing hardware parts of a sensor, chemists to modify sensor surfaces and to synthesize sensor labels, and biologists or doctors interested in specific biological samples or biotechnological processes.

What exactly is a biosensor? A biosensor is a device which detects the presence or activity of molecules with the help of biomolecules. A biosensor consists of two basic elements (see Fig. 1). The first is a layer of biomolecules which can bind or interact with sample molecules and serves as the recognition element. This sensing layer defines the specificity of the sensor.[2] The second element is a physical transducer, a solid state device

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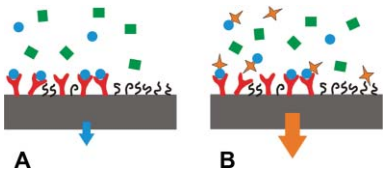


**Fig. 1** Biosensing principle. A biomolecular layer of receptor molecules (red) recognizes the target analyte (blue circles) but does not bind background molecules (green squares). A molecular layer (black lines) protect the sensor against unspecific adsorption. The receptor molecules are attached to a sensor (grey) which transduces the presence of the analyte into a measurable signal. (A) Label-free detection. The sensor detects analyte molecules directly. (B) A label is attached to the target analyte and the sensor detects the presence of the label close to its surface. Signals from labels are normally stronger than those coming directly from analytes, but labeling might disturb the recognition process or free labels might increase the signal noise.

which is able to detect the interaction between the sensing layer and the sample molecules. The interaction event is then transduced into a convenient electronic signal for further processing. Examples of physical signals which can report the presence of molecules are fluorescence signals from dyes, electric fields from molecular charges, or mass changes or refractive index changes from the adsorption of molecules onto sensor surfaces.[1] Depending on the detection principle one can differentiate between label-free and non-label-free sensors. Label-free sensors detect the original and unmodified molecules and can be used for on-line monitoring or fast and direct detection (Fig. 1A). But molecules are often easier to detect when first tagged with a molecular label. The presence of the label acts then as an indicator for the presence of the molecule (Fig. 1B). An important example is fluorescence microscopy where molecules are located by signals from their fluorescent labels attached. Disadvantages of such methods are that the target molecules are chemically modified *before* they are investigated. Labeling normally improves detection limits but the label might interfere with the function of the molecule and labeling is time- and cost-consuming.

Since the first classical biosensor from Clark and Lyons in 1962 – an amperometric sensor detecting glucoses level in blood[3] – many different biosensors have been developed, but only a limited number fulfill the stringent requirement for a reliable commercial sensor or find their place outside specialized research labs. Here, I will report on a biosensing principle based on mechanical stresses produced in a sensor upon molecular binding. This stress bends the sensor mechanically and can easily be detected. I will focus on biosensing applications of such cantilever sensors in aqueous environments. In the last ten years these sensors have attracted more and more interest from a growing number of researchers and have impressed by their wide range of applications: to date, cantilever sensors have been used as artificial noses to detect gaseous analytes, they have detected pH, temperature changes, and ions; in the life sciences they have been used to detect nucleic acids, proteins, and bacteria; not to mention applications for infrared detection or the identification of explosives. But it must also be mentioned that a cantilever array sensor, as we discuss it here, is more of a diagnostic instrument, which detects what it is optimized for, rather than being a general analytical instrument, such as a mass spectrometer, where samples without prior knowledge of their composition can be analyzed.

Since this tutorial review cannot cover all applications and technical details, the interested reader is referred to some recent reviews and the references therein.[4–6]

## 2. Model systems for biosensor research

First let us discuss some basic applications and standard samples for biosensors in molecular life science. In the life sciences, a biosensor can give answers to questions like: Is a specific sequence present in a large sample of DNA? How high is the concentration of certain proteins in the blood? Which proteins does a bacterium produce under specific environmental conditions? However, before being able to answer such questions, a biosensor has to be tested and characterized by investigating its response to well known standard samples. These tests start usually with simple physical stimuli, move on to robust biological model systems and end with complex and real-life samples. At each step unexpected strengths and weaknesses of a new sensor might be discovered and at the end one has to decide if a sensor can compete with existing technologies or if it offers new insights into biosystems. In the most basic tests, a new biosensor has to prove that it is working in an aqueous environment and that the influence of buffers (salts and pH) and temperature on the sensor signal is small or at least well defined, because these signals might later interfere with signals from molecular recognition. After testing the unspecific adsorption of molecules to the sensor, first model systems for molecular recognition such as nucleic acids or antibodies can be investigated.[1] When attaching short, single-stranded DNA (ssDNA) molecules to the sensor surface, the binding of the complementary target DNA to the sensor can be investigated. Such DNA oligonucleotides are quite robust with respect to buffer conditions and degradation, they are easy to synthesize and to handle, and their properties and interactions are well known. Another robust ligand receptor system is the biotin–avidin system, a small organic molecule binding to a protein. This system has the strongest binding constant known and is again simple to obtain and easy to handle.[7] Much harder to prepare and investigate are antibody–antigen interactions, for which advanced knowledge on sensor surface preparation, handling of proteins, and buffer conditions is necessary. Complex systems which need sophisticated sample preparation and the special tailoring of biosensor surfaces are, for example, ligand receptor systems or protein channels located in cellular membranes.[2]

Whereas first proof-of-principle experiments are performed with synthesized or highly purified samples, real-life samples normally show orders of magnitude higher concentrations of unwanted molecules than target molecules. This can give rise to unspecific binding or large background signals which could hide a small specific signal. Here are some typical concentration values for the above-mentioned model systems:[8] for the biosensing of nucleic acids (oligonucleotides) one should aim for the lower pM range, and for antibody–antigen interactions, ng $mL^{-1}$ is a typical antigen concentration; metabolites in the blood are present in higher concentrations, such as around mg $mL^{-1}$ for blood glucose; viruses might be present in blood with a typical low copy number of only several hundred per mL. Many times the detection of molecules in a sample can be improved by choosing an appropriate sample pre-treatment such as amplification of nucleic acids by polymerase chain reaction (PCR), or concentrating and labeling proteins.

## 3. Cantilever sensor hardware

Cantilever sensors emerged from atomic force microscopy (AFM),[9] which is an offspring from the scanning tunneling microscope (receiving the Nobel Prize in 1986 together with electron microscopy). AFMs can image surfaces, nanosystems or single molecules with Ångström resolution, manipulate molecules, or measure forces between individual molecules. In particular, their operation under physiological buffer conditions makes them well suited to the investigation of biomolecular systems in their native environment with molecular resolution.[10] A thin flexible

 

cantilever beam with a sharp integrated tip is the sensing element of an AFM. When the tip comes into contact with an object a force is applied to the cantilever and it bends. Compared to AFM cantilevers, the cantilever sensors we will discuss in this review are free-standing beams, without a sharp tip, which bend in response to different stimuli from the environment (see Fig. 2). Microfabricated cantilevers are made of silicon or silicon nitride using basically the same micromachining methods as for computer chips.[4,11] Their dimensions are in the micrometer range as illustrated in Fig. 2. Owing to their nanometer-scale bending, these sensors are sometimes called *nano*mechanical sensors (despite they are of *micro*meter dimensions).

Cantilevers bend when a force is applied to their end. This can be described by Hooke's law: $F = -k_{spring}\Delta z$. Their deflection $\Delta z$ is directly proportional to the applied force $F$, and the proportionality factor $k_{spring}$ is called the cantilever spring constant. The spring constant determines the flexibility and sensitivity of a cantilever and is defined by its dimension and material constants:

$$k_{spring} = \frac{Ewt^3}{4L^3}.$$

Here, $E$ is the elasticity or Young's modulus, $w$ is the cantilever width, $t$ its thickness, and $L$ its length. Typical values for $E$ are in the order of $2 \times 10^{11}$ N m$^{-2}$ for silicon or silicon nitride. With dimensions of $500 \times 100 \times 1$ μm, this results in a spring constant of about 0.05 N m$^{-1}$ and a resonance frequency of about 6 kHz for standard rectangular cantilevers. Typical errors in cantilever characterization (for their dimensions or material constants) result in an uncertainty of the cantilever spring constant of about 10%.

The sensitivity of cantilever sensors to tiny forces could not be exploited without a reliable readout of cantilever bending. The most commonly used readout scheme for cantilever bending is the beam deflection or optical lever method introduced in 1988[4,12] and shown in Fig. 3. A laser beam is focused on the flexible end of a cantilever and is reflected off onto a position-sensitive detector (PSD). The change in position of the reflection spot on the light-sensitive detector can easily be calculated back into a cantilever deflection. To improve this readout scheme, cantilevers can be covered with a thin reflecting metal layer (typically gold) on one side. The gold coating is normally applied directly before the functionalization of a cantilever to avoid contaminations by a longer exposure to air. Thereby, a nanometer-thin adhesion layer of Ti or Cr ensures a proper adhesion of the several 10 nm thick gold layer to the cantilever. With such a setup, cantilever deflections down to 0.1 nm can be measured routinely (resulting in a change of laser spot position on the detector of some micrometers). To use the same PSD for several cantilevers in parallel, as indicated in Fig. 3, each cantilever can be illuminated by a different laser which are then switched sequentially on and off with a repetition rate of several milliseconds.

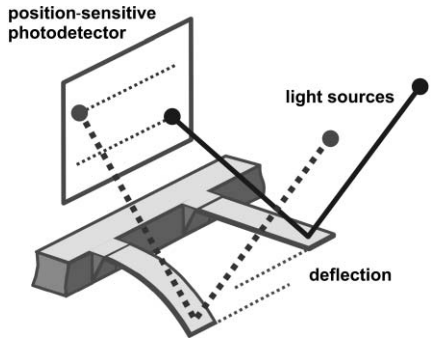


**Fig. 3** Cantilever array readout scheme by optical beam deflection. Nanometer deflections of cantilevers are easily translated into a change of position of the reflected laser spot on a position-sensitive detector by several micrometers.

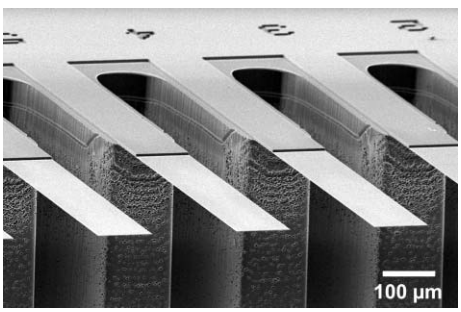


**Fig. 2** Scanning electron micrograph of a part of a commercial microfabricated cantilever array. The silicon cantilevers are 1 μm thick, 100 μm wide and 500 μm long. They have a pitch of 250 μm. The thin and flexible cantilever beams (the brightest parts in the micrograph) are not directly fixed to the bulk silicon. Instead, they are attached *via* a thicker bar to a solid platform having the same width as the cantilever. Such a geometry facilitates cantilever functionalization and prevents spreading of functionalization liquids from one cantilever to the other (see text).

## 4. Cantilever sensor modes of operation

We now will turn to the different *modi operandi* of cantilever sensors which have been proposed already more than ten years ago,[13,14] and which are shown in Fig. 4. In the dynamic or resonance mode, cantilevers are excited close to their resonance frequency, which is typically in the kHz or even MHz range. When an additional mass is attached to the oscillating cantilever, its resonance frequency changes (for adding a mass it lowers the resonance frequency). This is not surprising since at a first approximation cantilevers behave like a harmonic oscillator, an ideal oscillating spring–mass system,

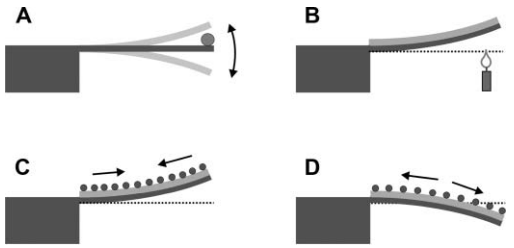


**Fig. 4** Cantilever sensor modes of operation: (A) dynamic mode detecting mass changes on the cantilever by changes in resonance frequency; (B) bimetallic mode detecting temperature changes by a static bending due to different thermal expansion of the metal layer and silicon cantilever; and (C,D) surface stress mode, where asymmetric molecular binding to the cantilever's top or bottom surface leads to an overall cantilever bending. For example, adsorption on the top surface can either cause tensile stress (C), bending the cantilever upwards, or compressive stress (D), bending the cantilever downwards.



with a basic resonance frequency of

$$f = \frac{1}{2\pi}\sqrt{\frac{k_{\text{spring}}}{m*}},$$

where $k_{\text{spring}}$ is the spring constant and $m*$ an effective mass (taking into account the cantilever geometry and mass distribution along the cantilever). With optimized cantilever geometries and under ultra-high vacuum one can measure mass changes in the resonant mode down to the single molecule level.[15] Unfortunately, applying this mode to detect biomolecules in solution is hindered by viscous damping of the oscillation, which decreases mass resolution and requires a more sophisticated setup.[5] A recent and interesting development in this area avoids viscous damping by not having the cantilever oscillating *within* a liquid environment, but rather putting the liquid sample *inside* a hollow cantilever. This eliminates viscous damping and preserves at the same time mass resolution of cantilever sensors.[16]

Cantilevers which are coated by a thin metal layer can also act as bimetallic sensors, which defines another mode of operation: the bimetal or heat mode (see Fig. 4B). Heating up such a composite metal–silicon cantilever structure will lead to different thermal expansions of the two materials and the beam will bend. Cantilevers as shown in Fig. 2 typically bend some 100 nm for a 1 K temperature difference when covered with some 10 nm of gold. With optimized setups, temperature changes smaller than $10^{-5}$ K can be detected and calorimetric measurements with a sensitivity of $10^{-15}$ J are possible (see citations within refs 4 and 5).

The third mode is by far the most common mode for biosensing experiments. It is the so-called 'surface stress' or 'static' mode: changes in the environment around or directly on the surface of the cantilevers create a mechanical stress in the surface which leads to an expansion or contraction of the cantilever surface. If this stress acts only on one side of the cantilever, then the asymmetrically stressed structure will bend and the cantilever will deflect. For simple isotropic materials, the surface stress $\sigma$ relates the reversible work d$w$ of deforming a surface (elastically) to the change in surface area d$A$ by: $\mathrm{d}w = \sigma \times \mathrm{d}A$.[17] For spontaneous processes, that is $\mathrm{d}w < 0$, and assuming a positive stress ($\sigma > 0$), d$A$ has to be negative, the surface wants to contract and the stress is said to be tensile. For a negative surface stress ($\sigma < 0$), d$A$ has to be positive, the surface wants to expand and the stress is said to be compressive. In short, expansion of a surface is defined as *compressive* surface stress and contraction as a *tensile* surface stress. Fig. 4C and 4D illustrate the concept: compressive and tensile stress have always to be related to one specific surface of a cantilever since an overall upward bending can be caused by either a tensile stress at the top surface (as in Fig. 4C) or by a compressive stress at the bottom surface (not shown). It has to be emphasized that when talking about surface stresses detected by cantilever sensors one always refers to a *change* in surface stress, but not to an absolute stress. Surface stresses can be caused, for example, by changes in buffer composition or adsorption of molecules to the cantilever surface. More details will be discussed below.

Surface stresses or film stresses are a major issue in microfabrication where one grows well defined thin films on a substrate, *e.g.* by chemical vapour deposition. During these processes one has to avoid substrate bending or film cracking.[18] Unfortunately, for cantilever sensor applications, still little is known on the detailed mechanism of the surface stress mode. Some developments on its theoretical description will be discussed below. The most commonly used formula to relate cantilever deflections to surface stresses is Stoney's formula:[15]

$$\Delta\sigma = \frac{Et^2}{3(1-\nu)L^2}\Delta z.$$

It holds for stresses from thin films (compared to the cantilever thickness) and for small deflections (assuming a circular shape of the cantilever bend). The formula connects the difference in surface stress $\Delta\sigma$ (given in N m$^{-1}$) between the bottom and top surfaces of a cantilever to the detected cantilever deflection $\Delta z$. Their dependence is determined by the material constants of the cantilever, the elasticity modulus $E$, and the Poisson ratio $\nu$ (which is about 0.3 for silicon), and its dimensions, the thickness $t$ and length $L$. $L$ is an effective length, that is the distance from the base of the cantilever up to the point where its deflection is read out by a laser (and not the full geometrical length of the cantilever). Reporting the surface stress values of an experiment instead the absolute deflections allows the direct comparison of results from cantilever sensors with different geometries or materials. The surface stress value might then be used to relate interactions on cantilever surfaces to surface free energy values (see discussion below). Here, a subtle point is worth mentioning, namely that the shape of the curvature along a bent cantilever beam differs if the beam is bent by a *force* applied to its end (as in AFM) or by a *bending moment* acting over its full length (for the surface stress mode). Since the local curvature of the beam affects also the deflection angle of the readout laser, one has to be careful by just using a classical AFM setup calibrated for force detection for cantilever sensor experiments. Details

 

on the calibration of cantilever deflection can be found in ref. 19. Because

$$\Delta z \sim \frac{L^2}{t^2}\Delta\sigma$$

it is obvious that for a given surface stress a cantilever deflects more and is more sensitive the thinner and longer it is. On the other hand, increased flexibility will also increase the thermal oscillation amplitude of the cantilever and the noise level of deflection detection. The future of cantilever array sensors might lay in a combination of all the mentioned detection modes within the same cantilever array.

Independent of the different modes it is always of advantage to use several cantilevers in parallel, as indicated in Fig. 2 and Fig. 3. It is evident that one can get more information out of a single experiment when using more sensors in parallel. A more sophisticated argument for parallel sensing lies in the way biologists deal with the fact that their systems under investigation (biomolecules and cells) cannot be as general and ideal as typical physical systems (*e.g.* atoms and crystals). Biological systems are normally more complex and more unique than physical systems and their properties depend much more strongly on their history and actual environment. Therefore, well thought out control experiments are an especially important part of any experiment in the life sciences. From the discussion above we know already that cantilevers are temperature-sensitive sensors and might also respond to changes in buffer composition. In addition, several other molecules in the sample and not only the target molecules might interact with the sensor. One therefore needs a strategy to subtract these unwanted background signals from the 'real' signal. In particular, first-time users want to see quick results with their samples, but are often not aware that signals from the environment in which the target molecules are presented can be much stronger than signals from the target molecules themselves. Therefore, researchers use arrays of cantilevers with several sensors in parallel: all cantilevers are physically identical and only differ in their surface coating. Their physical uniformity can be checked before an experiment by either applying a well defined heat pulse and recording the thermal responses of the cantilevers or by measuring their individual resonance frequencies which should be identical to within at least about 5%. Some cantilevers are then made sensitive for the target molecules whereas others act as a reference for unspecific binding or for physical signals such as temperature, refractive index changes or different buffers. Experiments with complex biological samples are nowadays performed with cantilever arrays of up to eight cantilevers in parallel.

## 5. Sensor surface functionalization

This section addresses the question of how an ordinary solid state surface can be transformed into an intelligent sensor surface, recognizing and identifying complex biological systems. Coating or functionalizing a sensor is a critical preparation step because the recognition layer will define the application and performance of a sensor. One of the major challenges in biosensor research today is to tailor biosensor coatings such that the biomolecules are tightly attached to the sensor surface but are still flexible and functional as in their natural environment.[2]

Operating cantilever biosensors in the surface stress mode is based on the binding of target molecules on only one side of a cantilever, otherwise the additional stress from the opposing surface would cancel and the cantilever will not bend. One surface should provide the receptors for the target molecules, whereas the other should prevent any specific or unspecific adsorption of sample molecules. Fig. 5 gives an example of cantilever functionalization. Surface coatings have to be reliable, they should be robust against changes in buffer and temperature and ideally withstand repetitive detection and cleaning cycles. Especially for cantilever sensors, interactions on top of the sensing layer should be fully transferred to the underlying substrate favouring a dense and covalent surface functionalization with receptor molecules close to the surface. For this task, experts in surface chemistry and biochemistry are needed. Here, we will discuss only on the most basic strategies.

Since cantilevers already show two distinct surfaces, *e.g.* a silicon dioxide bottom and a gold top surface (see Fig. 5), either silane or thiol chemistry, respectively, can be used to cover the two sides

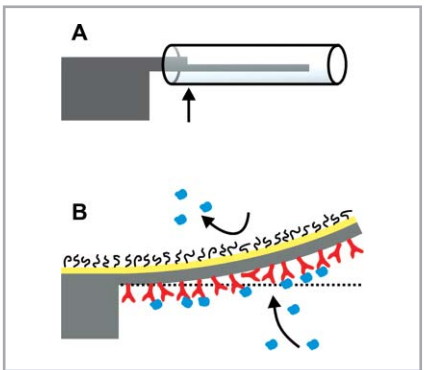


**Fig. 5** Cantilever functionalization. (A) Individual cantilevers can be functionalized by incubation in thin glass capillaries or pipette tips. In particular, the hinge region (arrow) has to be functionalized properly. (B) Example of functionalization and expected bending of cantilevers exposing a gold surface (top) and a silicon oxide surface (bottom). The gold surface is blocked by a protein resistive monolayer coating and the receptor molecules (red) are bound to the bottom surface. Binding of ligands (blue) to the receptors on the bottom surface leads in this case to a compressive surface stress (that is, the surface with the sensing layer expands).

with different receptor layers or inert coatings. Because of the high affinity of sulfur groups for gold, the gold surface can, for example, be modified by thiolabeled nucleic acids or proteins exposing cysteines (showing sulfur groups) at their surface.[20] On the other hand, thiolated poly(ethylene glycol)s can act as inert layers, preventing molecular adsorption.[21] The silicon oxide surface can be modified, for example, by amino- or mercapto-silane monolayers whose end groups can be further cross-linked to receptor molecules (*e.g.* ref. 22). In addition, highly positively charged molecules can be electrostatically bound to the negatively charged silicon dioxide. But there are many more sophisticated methods for surface functionalizations and many research groups work on optimizing surface coatings for different biosensing applications. An overview on surface biofunctionalizations can be found in ref. 2.

There are several approaches to exposing individual cantilevers of a cantilever array to specific biomolecules. The microfabricated cantilevers can, for example, be incubated in individual glass capillaries or standard pipette tips filled with coating molecules (see Fig. 5). They can be dipped

in the channels of an open microfluidic network, or can by spotted with microliter drops of receptor molecules using an ink jet type of device.[23] Special care should be taken for a homogeneous coating at the hinge region, where the flexible cantilever beam is connected to the bulk silicon (see Fig. 2 and Fig. 5A). Bending in this area will influence the deflection of the cantilever's free end much more strongly than a bending somewhere in the middle or close to the end of the cantilever. A closer look at Fig. 2 reveals some details which help to guarantee a homogeneous functionalization of the hinge region: the etch defining the cantilever width is actually extended deep into the solid part of the silicon chip such that cantilevers can be dipped deep into small capillaries or microchannels so that the entire flexible part is well immersed in the liquid (see Fig. 5A) and further spreading of the liquid from one cantilever across the wafer to another cantilever is prevented.

## 6. Model experiment

After a cantilever array has been cleaned, covered with gold and functionalized, the array is transferred to a liquid cell with an inlet and an outlet and a volume of typically less than 100 μL. The cell is closed and buffer is injected either by hand or *via* an automated delivery system with pumps and valves. Then, the readout lasers are aligned onto the end of the cantilevers and onto the position-sensitive detector. Temperature control is an advantage since we already know that slight changes in temperature might bend the silicon–gold structure by unwanted bimetallic effects.

Since the cantilevers have so far been subjected to several changes from ambient to liquid environments due to cleaning or functionalization they are most likely not in an equilibrium state and will show a strong drift of approximately several nanometers per minute. Normally, an experiment is started when the drift reduces to about a few nanometers per hour or when all cantilevers drift nicely in parallel, which can take up to several hours. Drift mechanisms are still under discussion but they might be caused by slight conformational changes in the sensing layers or by slow reactions at the cantilever–liquid interface on either of the two surfaces.

Fig. 6 shows the different steps of a model experiment with a cantilever array.

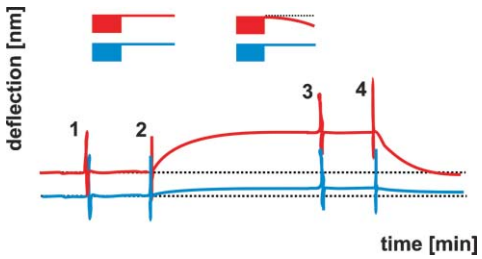


**Fig. 6** Deflection *versus* time graph of a typical model response from a biosensing experiment with two cantilever sensors in parallel. One cantilever (red) is functionalized with receptor molecules, the other serves as a reference cantilever (blue). The different steps are: (1) injection of a negative control; (2) injection of sample molecules leading to a strong bending of the red cantilever responding to the molecular interaction, but only a small bending of the blue reference cantilever; (3) the same injection as (2) to verify saturation or completion of the reaction; (4) buffer injection leading to unbinding of molecules from the sensor.

For simplicity we show only the idealized signals from two cantilevers. We assume that the cantilevers have been covered with different sensing layers, for example, with two different sequences of short, single-stranded DNA. First, the cantilever response upon injecting of sample buffer is checked. Flushing the liquid cell with the same buffer used for equilibration should cause no deflection or the deflection should go back to its initial value within seconds. Having verified this, a negative control should be performed by injecting a sample with molecules which are as identical to the sample molecules as possible but which should not interact with the sensing and reference layers, such as inactive ligands or mutants of the target molecules. In our case this could be a DNA with a totally random sequence or even just with one wrong base. Again, no signal or only very small signals from both the sensing and reference layers are expected. Then, the sample is injected (in our case containing complementary single-stranded DNA) and only the cantilever with the appropriate receptor molecules should respond (Fig. 6, step 2). The reference cantilever might show no signal or only a small unspecific signal. Typical timescales to reach saturation for standard receptor ligand binding are around several minutes to tens of minutes, showing deflections of 10 nm or more. These signals are clearly above the noise level of typically less than 1 nm peak-to-peak. Injecting the sample again (Fig. 6, step 3) should either show another signal increase or indicate saturation. Finally, flushing the sample molecules out of the liquid cell (step 4) can give indications of the nature of the binding: no signal decrease would indicate a strong and irreversible binding, whereas some signal decrease would indicate a reversible bond between receptor and target molecule. To analyze the data one now subtracts the unspecific signal (of the blue reference cantilever in Fig. 6) from the specific signal (of the red sensing cantilever in Fig. 6) which is then called the *differential* sensor response. Another characteristic of an ideal biosensor is that it can be cleaned, reset and used again several times without degradation of its functional layers. Doing a series of measurements at identical concentrations will therefore give valuable insights into the reliability of the sensor and especially the stability of its surface coating. By measuring the sensor response for different sample concentrations, the detection limits (lower and upper) and useful concentration range for the sensor can be determined. Finally, thermodynamical parameters such as the binding constant or on- and off-rates can be derived from signal *versus* concentration or signal *versus* time measurements.

## 7. Cantilever biosensor applications

The first applications of cantilever sensors for biological systems were reported in 1996 with single cantilevers.[14] The first biosensing experiments with cantilever arrays were demonstrated in 2000 showing the proof-of-principle for DNA detection and the ability to identify single-base mismatches between sensing and target DNA oligonucleotides.[24] This was soon followed

 

by more detailed experiments showing the dependence of cantilever bending on the length, grafting density and orientation of DNA oligonucleotides on surfaces, and demonstrating the ability to detect different sequences in parallel and within a high background of unspecific sequences.[25,26] The most elaborate experiments with nucleic acids to date report the detection of non-amplified RNA in total RNA from a cell with a detection limit of 10 pM.[27] These experiments were done within a high background of unspecific molecules and showed for the lowest concentration a differential deflection of 10 nm, or a surface stress of 1 mN $m^{-1}$ ('millinewtons per meter'). Another report addressed the monitoring of digestion and ligation of DNA on surfaces and the activity of a polymerase enzyme by cantilever sensors.[28] Applications of cantilever sensors for proteins have been reported for prostate cancer antigens,[29] for detecting glucose using glucose oxidase enzymes,[30] and for cardiac biomarkers (which are proteins indicating a heart attack).[31] By using single-chain antibody fragments immobilized on cantilever surfaces the detection of peptides at concentrations as low as 20 ng $mL^{-1}$ (corresponding to nM concentrations) have been demonstrated.[32] It seems that the use of DNA aptamers – short DNA sequences which have been evolved artificially to bind proteins – can improve the detection limit of proteins (in this case a DNA polymerase) to 1 pM or 100 pg $mL^{-1}$.[33] Another report aims to investigate the bending of artificial cellular membranes immobilized on cantilever surfaces. Several natural processes influencing membrane mechanics and bending cellular membranes seem to be well suited to be investigated with cantilever sensors.[34] Table 1 summarizes some key surface stress values reported so far. Stresses from more 'simple' interactions such as the adsorption of oxygen, the self-assembly of monolayers and the changes in pH show quite large changes in surface stress from several tens to thousands of mN $m^{-1}$. Compared with these the binding of biomolecules to surfaces resulted in nearly all cases in a surface stress of around 1–10 mN $m^{-1}$. Or in other words, the standard of surface stress detection with cantilevers is at the moment around 1 mN $m^{-1}$. Advances can be expected in transducing this stress into a larger cantilever deflection or improving surface coatings so that even lower molecular concentrations create stresses in the 1 mN $m^{-1}$ region. Following this idea, it has already been demonstrated that by using a nanostructured cantilever surface, such as a dealloyed silver–gold layer revealing a colloidal-like morphology, the bending signal can be enhanced by several orders of magnitude compared with a smooth gold surface.[35] Another recent development is to label biomolecules such that their binding to cantilevers can be used to amplify *mechanical* signals from cantilever sensors: when magnetic beads are attached to biomolecules bound to cantilever surfaces, an external magnetic field can be used to pull on the cantilevers and amplify the binding signal.[36]

Taking these results (and others reviewed in refs 4–6) together, one can say that cantilever array biosensors have mastered the basic tests mentioned in Section 2 and that their sensitivity is comparable with existing label-free technologies (*e.g.* refs 27 and 32). They now have to prove that their every-day performance and ease of handling can also compete with established methods outside the specialized labs where they have been developed.

## 8. Theory of surface stress sensor operation

The results above have been achieved without a well defined theory on the transduction principle of cantilever biosensors. A naïve calculation, such as one directly correlating the binding energy of analytes (to the receptor layer on the cantilever surface) with the mechanical energy stored in a bent cantilever, does not work and the different energy values are normally off by several orders of magnitude. The fact is that in cantilever sensor research, the theoretical description is at the moment lagging behind the technical developments and the progress in applications – especially for the surface stress mode. This is unfortunate because to finally optimize a sensor and extrapolate its potential a good theory on its working principle is needed.

The lack of theoretical description of cantilever biosensors is primarily based on the complexity of the transducer system: different cantilever materials, fluctuations in material parameters and different readout methods have to be taken into account; the morphology of the sensor surfaces such as its roughness and cleanliness are important; the sensing layer, especially its density, homogeneity, and immobilization procedure and accessibility of receptor molecules will influence the sensor response; the entire solid surface/molecules/liquid interface including ion distribution, hydrophobic or entropic interactions can effect sensor signals; and the binding of molecules to the sensor surface can lead to conformational changes, changes in surface charge or molecular density in the sensing layer. Since the surface stress is a cooperative phenomenon of many atoms and molecules, it is clear that both the interactions of molecules with the

**Table 1** Surface stress values and lowest detected concentrations for different biomolecular interactions compared to stresses from unspecific adsorption (data from cantilever experiments except for oxygen on silicon)

| Analyte system | Comment | Lowest concentration | Typical stress/mN $m^{-1}$ | Ref. |
|---|---|---|---|---|
| Self-assembly on Au | Dodecanethiol in gas phase, dependent on Au structure | — | 200–16 000 | 43,47 |
| Oxygen on Si(111) | In gas phase, capacitive measurement | — | 7200 | 48 |
| $Ca^{2+}$ ions | Bare silicon nitride/Au cantilever | 1 mM | 1–450 | 49 |
| Supported lipid bilayer | Vesicle fusion on cantilever | — | 30–220 | 34 |
| pH | With thiol-modified cantilevers, pH 4.5–9 | — | 1–30 | 37,38 |
| DNA | Oligonucleotide hybridization | 100 pM | 1–30 | 27,41 |
| Protein | With antibodies | 20 μg $mL^{-1}$ | 1–6 | 31 |
| Peptide | With antibody fragments | 20 ng $mL^{-1}$ | 1–10 | 32 |
| Protein | With oligonucleotide aptamers | 100 pg $mL^{-1}$ | 1–10 | 33 |

surface and also lateral interactions in the molecular layers parallel to the surface are important. All these effects can contribute to the finally measured 'surface stress' which obviously represents only a very simplified picture of what is actually going on in a cantilever biosensor experiment.

So far, some basic effects have been identified which clearly cause cantilever deflections,[4] such as increasing the surface charge on one side of a cantilever, *e.g.* by changes of pH followed by a protonation or deprotonation of the surface: this leads to an electrostatic repulsion of surface groups which leads to an expansion of a cantilever surface as illustrated in Fig. 7B.[37,38] The same mechanism also holds in part for the hybridization of DNA to a cantilever surface: the binding of negatively charged DNA increases the number of negative charges on a surface and again causes a compressive stress.[24] But, in addition, hybridization of DNA can also lead to tensile stresses by conformational changes of DNA molecules, *e.g.* when DNA transforms from a flexible, single-stranded random coil to stiff, hybridized, double-stranded DNA (dsDNA). This reduces lateral steric interactions between DNA molecules and the DNA layer on the surface contracts (see Fig. 7A).[39] The influence of lateral interactions was also investigated in experiments with DNA where changes in the grafting density and ionic strength modified DNA hybridization signals.[40,41]

To further elucidate different bending mechanisms, researchers have moved away from detecting complex molecular systems and are instead investigating the formation of well defined self-assembled monolayers on clean and well characterized cantilever gold surfaces, the response of cantilevers to pH changes, or the electrochemical responses of cantilevers.[37,42] From the investigation of the formation of self-assembled alkylthiol monolayers on gold-covered cantilevers, it was found that the longer the alkylthiol chain length the stronger the resulting surface stress, indicating the importance of lateral chain–chain interactions.[43] As mentioned above, an increased surface roughness can increase the sensor response, but also the cleanliness and especially the 'freshness' of a gold surface plays a critical role.[35,42] The cleaner the gold, the stronger the bending response from molecular self-assembly. In general, it seems that simple adsorption of molecules on a bare surface creates in most cases a compressive surface stress.[4]

Besides these experiments, an electrochemical approach offers an additional view on the connection between surface properties, surface stress, and surface energy changes.[42] The key formula from surface thermodynamics for this relation is the simplified Shuttleworth equation:

$$\sigma = \gamma + \frac{\partial \gamma}{\partial \varepsilon}.$$

It states (here, for the simplified case of isotropic materials) that the surface stress $\sigma$ (typically in units of N m$^{-1}$) equals the surface free energy $\gamma$ (typically in units of J m$^{-2}$) plus a term which describes the change of the surface free energy with the elastic strain $\varepsilon$ (the relative change in surface area). The right-hand side of the formula can be investigated by applying different electric potentials to cantilevers in solution and measuring the resulting bending.[42] Hopefully, by following the approach above it will be possible to sum up all the different free energy terms which might contribute to molecular binding and changes of surface properties and then correlate them with surface stress results.[40] More information about the ongoing discussion on the concepts of surface thermodynamics and the theory of surfaces stresses can be found, for example, in refs 44 and 45.

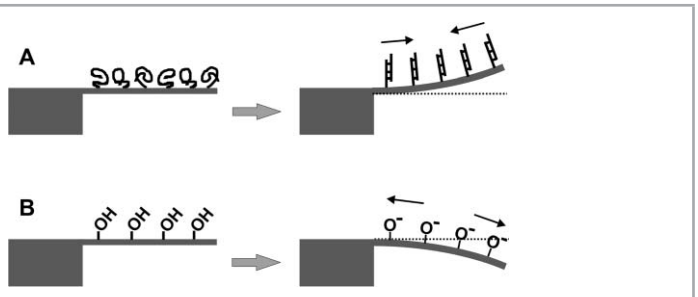


**Fig. 7** Most basic models of molecular interactions causing cantilever bending in the surface stress mode. (A) Conformational changes: when ssDNA is immobilized on a cantilever surface within distances corresponding to its random coil structure, the hybridization to a stiff dsDNA relaxes the repulsive steric interactions and the cantilever might bend in a tensile direction. (B) Surface charge: deprotonation of molecular groups on the cantilever surface (*e.g.* by pH changes) creates negative surface charges and an electrostatic repulsion. The cantilever bends in a compressive direction.

## 9. Conclusions and outlook

Cantilever sensors have already shown an impressive performance and a move to more advanced applications. Their major advantages are their wide field of application, that they are label-free, can be microfabricated, and need only small sample volumes for their operation. Their response is, to date, comparable with other established label-free biosensing methods and a general setup can be easily modified to detect a variety of different parameters and substances such as temperature, mass, gases, biomolecules or cells. Problems which still delay a general use of cantilever sensors are, in particular, their need for a sophisticated surface functionalization and the lack of a theoretical description. But these are not fundamental problems and they are currently being addressed by researchers. For predicting the direction and magnitude of cantilever bending one needs to identify all of the different contributions, *e.g.* by investigating simple model systems, and then combine them to a general theory. Another indicator of the advanced state of cantilever sensor technology and its potential is the existence of small companies which provide already for several years cantilever sensors as their core technology, as, for example, Concentris in Switzerland or Cantion in Denmark.[5]

Further technical progress is expected from another bending readout mechanism, the piezoresistive readout, where bending of a cantilever alters its electric resistance.[46] Recently, this detection method showed comparable sensitivity as the optical readout for protein detection. Still, the isolation of the electrical parts of the cantilever from the buffer solution

 

poses some problems, but the piezoresistive readout eliminates the alignment of laser beams to cantilevers and detectors so that sensors could be packaged in smaller volumes. Scaling up the number of cantilevers by using 2D cantilever arrays and their readout has already been demonstrated (see, for example, the citations in ref. 5).

When ignoring all the technical details and theoretical discussions, the simple but fascinating fact remains that *thin layers of soft molecules are able to bend solid state devices*. And we can watch these molecules doing so and exploit their activity to develop novel sensors or, from a more general point of view, might even use them to create novel actuators which might open little boxes or move tiny paddles.



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