Nanomechanics for specific biological detection

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ABSTRACT

Nanomechanical biosensors have emerged as a promising technology for measurement of biomolecular interactions. Among the advantages are direct detection without need of labelling with fluorescent or radioactive molecules, small sensor area, high sensitivity and suitability for integration using silicon technology. Here we present two important applications: i) study of DNA immobilization for nucleic acid detection and ii) direct detection of the harmful pesticide dichlorodiphenyltrichloroethane (DDT). Single-stranded oligonucleotides were derivatized with thiol molecules for self-assembly on the gold-coated side of a microcantilever. The geometry of the binding and the surface density were studied and controlled by mixing derivatized oligonucleotides with spacer self-assembled monolayers. The hybridization signals were smaller than 10% of the immobilization signal. The molecular mechanisms responsible of the nanomechanical response due to hybridization are discussed. On the other hand, herbicide DDT was detected by performing competitive assays, in which the cantilever was coated with a synthetic DDT hapten, and it was exposed to different ratios between the monoclonal antibody and the DDT. The relevance of this technique in functional genomics and environmental control will be discussed.

Keywords: Nanotechnology, biosensors, microcantilevers, nanomechanics, DNA, biochips

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1. INTRODUCTION

Biosensor devices detect molecules with high selectivity on the basis of biomolecular recognition1. The sensing surface usually is a solid support, in which receptor biomolecules such as nucleic acids, antibodies, enzymes have been immobilized. Bio-recognition gives a change of the physical properties of the sensor surface that it is translated into electronic information. There is a wide variety of suitable electrical transducers such as amperometric electrodes that monitor changes in the electrochemistry of the sensor surface, quartz crystals that monitor changes of mass and surface plasmon resonance devices that measure variations of the optical properties to name a few. The biomolecular interactions that can be measured include protein-protein, protein-nucleic acid and nucleic acid-nucleic acid, which are relevant in biological research, clinical diagnosis, environmental monitoring and for rapid detection of biological weapons. However several limitations must be overcome before the practical implementation of the biosensor devices. Thus many applications demand low sample consumption, short analysis time, direct detection without need of labelling with fluorescent or radioactive dyes, small size and low cost. Recent progress in micro- and nanotechnology has been significant. Implementation of these technologies will result in both miniaturization and an enhanced sensitivity of the bioassays.

Nanomechanical biosensors is an excellent example of the application of micro- and nanotechnologies in new biosensors2-4. Receptor molecules are immobilized on the surface of a micro-cantilevered structure. When molecular recognition is produced on the microstructure surface, a nanomechanical response is obtained, that consists of a strain of the structure and a shift of the resonant frequency of the vibration modes. Most of the works measure the deflection of the free end of a clamped microcantilever. The motion of the free end is usually detected by means of the well-known optical beam deflection method employed in most of the atomic force microscopes. Recent works have shown that this
technique can specifically detect single-base mismatches in oligonucleotide hybridization as well as minute amounts of proteins with extreme sensitivity\(^3, 5, 6\). The potential of this technique lies on i) the small sensor area requiring low amount of sample, and ii) the nature of the nanomechanical response which allows direct detection and real-time monitoring.

The most common detection technique is the measuring of the microcantilever deflection when only a single side of the lever is functionalized with receptor molecules. Molecular recognition produces a change of the surface stress of the sensitised cantilever side with respect to the other side, giving a cantilever deflection of few nanometers. Surface stress mainly arises from electrostatic, van der Waals, configurational and steric interactions between the adsorbed molecules. This method, which is often called DC, has allowed the measurement of submonolayer adsorption of different molecular species, changes of pH and salt concentration, and biomolecular interactions\(^3, 5, 6\). A less extended detection technique, called AC, consists of using microcantilevers as oscillators, and monitoring parameters of the oscillators such as the resonant frequency and quality factor, which depend on the mass adsorbed on the cantilever, environment viscosity, surface losses and surface stress. However the sensitivity of these measurements is limited by the low quality factor (Q) due to the hydrodynamic forces between the cantilever and the liquid. Thus the quality factor in air is of about 50-500 and decreases to 2-10 in liquid environment. AC detection has been recently enabled with the development of feedback driving techniques that allow a quality factor enhancement of about two orders of magnitude\(^10-12\).

Here we describe the theoretical and experimental principles of the DC nanomechanical response. Then experimental examples of biological detection measuring the DC nanomechanical response are presented. Firstly measurements of oligonucleotide immobilization and hybridization on microcantilevers are shown. The aim of these experiments is to develop sensitive DNA nanomechanical chips. Secondly the first results of pesticide detection are presented, which are relevant for environmental control.

2. MATERIALS AND METHOD

2.1. Materials and reagents

The cantilevers used in this work are fabricated in silicon nitride with V-shape (Digital Intruments, Veeco). The cantilever dimensions are: arm width of 40 µm, thickness of about 600 nm and length of 200 µm. The nominal spring constants is 0.12 N/m. For the experiments, the original gold coating of the reflective cantilever side was removed by immersion in a mixture of hydrochloric acid and nitric acid (3:1). This cantilever side was coated with 5 nm of chromium and 25 nm of gold using thermal evaporation the day before the experiment. The chromium layer was used to improve the adhesion between the gold and the silicon nitride.

DNA oligonucleotides were commercially prepared by Integrated DNA Technologies. DNA probes were 25 nucleotides in length and synthesized with a thiol linker group (HS-(CH\(_2\))\(_6\)), in order to form a self assembled monolayer onto the cantilever gold-coated side. Cleland’s Reductacryl Reagent from Calbiochem has been used for disulfide bond reduction prior to immobilization assays. 6-Mercapto-1-hexanol (MCH) was purchased from Sigma Chemical Co. For MCH treatment, a 1mM solution was prepared and injected into de liquid cell after oligonucleotide immobilization. The immobilization and hybridization buffer were prepared with 50 mM phosphate buffered solution (PB), pH 7.0, from Fluka, and 0.5 M and 1 M NaCl respectively .

For the detection of the pesticide DDT, cystamine dihydrochloride and glutaraldehyde were purchased from Sigma Chemicals Co. The synthetic hapten 4-{4-[1-(4-chlorophenyl)-2,2,2-trichloroethyl]phenyl}butanoic acid (DDT5), the assay conjugate with bovine serum albumin (DDT5-BSA), and the anti-DDT monoclonal antibody (LIB-DDT5-25) were prepared and characterized by enzyme-linked immunosorbent assay as previously described\(^13\).

2.2 Instrumentation

Figure 1 shows a scheme of the experimental set-up. Cantilever bending (deflection) measurements were carried out by using the well-known optical beam deflection method employed in most of the atomic force microscopes (fig. 1a). A laser beam is focused on the free end of the cantilever and the deflection of the reflected beam, which is
proportional to that of the cantilever, is measured with a four-segment photodetector. The laser beam deflection is proportional to the difference in the photocurrents generated in the upper and lower segments. The ratio between the deflections of the reflected laser beam and cantilever is equal to $L/2l_{\text{eff}}$, where $L$ is the length of the optical path of the reflected laser beam (about 14 cm) and $l_{\text{eff}}$ is the effective cantilever length. Since the position accuracy of the photodetector is of about 100 nm, the deflection resolution is of about 0.1 nm. Thus, the reflected spot is distributed evenly on the four segments for the cantilever rest position, and displaces vertically as the cantilever bends. To avoid cross-talk of the deflection signal with the cantilever torsional modes, the cantilever longitudinal axis and the direction between the upper and lower segments of the photodetector must be in the plane formed for the incident and reflected laser beams. The photocurrents of the upper and lower segments are amplified and connected to a A/D data acquisition card (National Instruments) for deflection monitoring in real-time. Acquisition software was programmed in Labview (National Instruments). The deflection signal is calibrated in nanometers by measuring the deflection signal as a function of the displacement of the photodetector, which is placed on a micrometric positioning stage.

![Diagram](image)

Figure 1. (a) Cartoon of the optical method used to measure the cantilever motion with sub-nanometer resolution. (b) Scheme of the experimental set-up.

The experiments were carried out in a commercial fluid cell (Digital Instruments, Veeco) with an inlet and a outlet for liquid flow. The top part of the fluid cell is made out of glass and the bottom is closed with a coverslip using a rubber O-ring. The configuration of the diode laser, photodetector and fluid cell is shown in fig. 1b. The system is isolated from mechanical vibrations. Flow and injection of sample is performed by using a peristaltic pump (Gilson). The volume enclosed in the fluid cell is of about 50 µl and the flow rate in these experiments was 0.3 µl/s, approximately. The sample delivery was controlled by an injector valve, which allows to switch between continuous buffer solution flow and analyte injection without mechanical perturbations of the fluid cell.

3. THE DC NANOMECHANICAL RESPONSE. DNA IMMOBILIZATION

3.1 Theory

Surface stress is the reversible work per unit area required to strain a surface elastically. The adsorption of molecules on a surface gives rise a change of surface stress due to two effects: i) the redistribution of electronic charge of the surface around each adsorbate atom, and ii) the intermolecular interactions between neighbouring adsorbate atoms. If the surface stress change due to adsorption is positive the surface tends to shrink and the surface stress is called “tensile”. In the opposite situation, the stress is called “compressive”. Most of the experimental knowledge about adsorbate-induced surface stress comes from the cantilever bending method, which measures differences in surface stress between the opposite sides of a microcantilever that are different in composition. The difference of surface stresses will be balanced via unequal changes of area of both surfaces giving a cantilever bending. The resulting cantilever deflection $z$ at the cantilever end is:
\[ z \equiv 3 \frac{(1-\nu)L^2}{Et^2} (\Delta \sigma_t - \Delta \sigma_b) \]  

(1)

where \( L \) and \( t \) are the cantilever length and thickness, \( E \) and \( \nu \) are the Young’s modulus and Poisson coefficient of the cantilever material, and \( \Delta \sigma_t \) and \( \Delta \sigma_b \) are the change of surface stress of the top and bottom surfaces of the cantilever, respectively. For the derivation of eq. (1), it is assumed that the cantilever bending is small compared with the cantilever length, and that the cantilever length is large compared with its width, which itself is large compared to its thickness. These conditions are approximately fulfilled in the experiments.

### 3.2. Characterization of DNA immobilization and hybridisation

The important role of DNA in life and nanotechnology lies in its structure, which arises from the specific binding through hydrogen bonds between complementary bases; between adenine (A) and thymine (T), and between cytosine (C) and guanine (G); which holds the two DNA single strands together and forming a linear double helix with a thickness of about 2 nm. Thus, base pairing is the fundamental of DNA-based computation and fabrication of DNA-mediated nanostructures. There is also intense research for using DNA as a molecular nanowire. Moreover attachment of single stranded (ss) DNA to solid supports is the base of nucleic acid sensors, which are crucial for detection of harmful pathogens and for the study and analysis of the genomes of organisms. Here nanomechanical biosensors are applied for characterization of the immobilization of single-stranded DNA (ssDNA) derivatized with a thiol linker on gold surfaces. The aim is twofold. Firstly, optimization of ssDNA immobilization techniques on solid supports to achieve a maximum efficiency in the hybridisation with complementary nucleic acids. This study is important in the development of DNA-based nanodevices such as those mentioned above. Secondly, nanomechanical biosensors have been proposed as a promising alternative to current technologies, such as DNA chips, for nucleic acid detection with sensitivity to single base mismatches. However it is necessary a better understanding of the molecular mechanisms responsible of the nanomechanical response to maximize the hybridisation signals.

![Figure 2. Cartoon of the experimental procedure for the measurement of thiol-derivatized ssDNA with nanomechanical biosensors.](image)

The scheme for study of the immobilization of DNA is shown in figure 2. A 25mer ssDNA with the sequence 5’-CAC GAC GTT GTA AAA CGA CCG CCA G-3’ was derivatized with the alkylthiol SH-(CH\(_2\))\(_6\) in terminal 5’. Thiols spontaneously form self-assembled ordered monolayers in metals such as gold, silver and platinum because of the covalent bond formed between the metal and the sulphur atom. To study the immobilization of thiol derivatized ssDNA (-SH-ssDNA) on gold, microcantilevers were coated with a 20 nm thick gold layer. As it is shown in fig. 2, besides the covalent bond between the gold and sulphur group, there can also be non specific interactions between the nucleotides and the surface. Non specific interactions play an important role in the geometry of the anchored –SH-ssDNA with respect to the gold surface, and they can impede the hybridisation with the complementary sequence.
To determine the role of the thiol linker and the nucleotide backbone in the attachment to the gold surface, the adsorption of thiol-derivatized ssDNA was compared to that of ssDNA with the same sequence but without a thiol linker. Figure 3 shows the cantilever deflection as a function of the time in an experiment where the non-derivatized oligonucleotide was injected before the thiol-derivatized one. The adsorption of the unmodified ssDNA gives an initial slight upwards deflection smaller than 1 nm that can be hardly discerned of the drift of the deflection signal. The main source of drift is the temperature variation. Indeed the microcantilever behaves as a bimetal and a temperature variation gives a cantilever bending due to the difference in thermal expansion coefficients of the gold thin layer and silicon nitride. Experiments and calculations indicate that the bimetallic effect on the deflection is of about 20-40 nm/°C for the cantilevers used in this work. The resulting small change of the deflection when unmodified ssDNA is injected has two plausible explanations. One is that the ssDNA attach on both cantilever sides evenly, i.e., the non specific interaction of the nucleotide chain with the gold is similar to that with the silicon nitride. The other explanation is that ssDNA adsorption is very low on both sides of the cantilever. We attribute the negligible deflection variation to the second explanation. To discard first explanation we performed experiments (not shown here) where the gold-coated cantilever side was protected with the hydroxyl-ended alkylthiol 6-mercapto-1-hexanol whose formulae is SH-(CH\textsubscript{2})\textsubscript{6}-OH. It is known that -OH functionalised surfaces exhibit a negligible interaction with DNA, inhibiting non-specific adsorption.

Exposure of these microcantilevers to non-modified ssDNA resulted into a negligible nanomechanical response, implying low adsorption of the ssDNA on the silicon nitride side of the cantilever.

![Figure 3. Immobilization experiment where the cantilever deflection is monitored during injection of 25-mer ssDNA without thiol linker (first arrow) and subsequent exposure to 25-mer thiol-derivatized ssDNA (second arrow). The oligonucleotide concentrations were about 4.5-4.8 µM and the experiments were performed in 50 mM phosphate buffer with 0.5 M of NaCl. The fluid cell is rinsed after 25 minutes of the analyte flowing over the cantilever.](image)

Injection of the –SH-ssDNA after injection of the unmodified ssDNA gave a pronounced downwards deflection of about 12 nm. This confirms that non specific adsorption is very low and it does not hinder covalent attachment of –SH-ssDNA to the gold surface (top cantilever side) through the sulphur atom. This is in agreement with fluorescence experiments where the measured surface density of derivatized oligonucleotides is of about 8-10 times that of the oligonucleotide without thiol linker. The downwards deflection produced by the covalent binding of the –SH-ssDNA implies compressive surface stress on the gold surface. The surface stress variation can be split into two terms, one that arises from the interactions between neighbouring DNA molecules and the other due to the interactions between the DNA and the gold surface. Intermolecular interactions between neighbouring DNA molecules are mainly dominated by the repulsive electrostatic forces between the negatively charged phosphate groups and the repulsive
steric force, giving a compressive contribution to the stress. On the other side, the main contribution to the gold-DNA interaction is the covalent bond between the sulphur and gold atoms, in which the negatively charged S group create a positive image charge in the Au surface that gives a compressive surface stress.

After –SH-ssDNA immobilization, the cantilever was exposed to 6-mercaptop-1-hexanol (MCH), a 6-carbon chain molecule terminated with thiol (−SH) and hydroxyl (−OH) groups on each of the extremes. In this treatment developed by Herne and Tarlov, thiol group of MCH rapidly displaces the possible weaker adsorptive contacts between the nucleotide chain and gold. Since hydroxyl group negligibly interacts with the nucleotide chain, MCH treatment assures that the immobilized ssDNA is only attached to the gold surface through the sulphur atom. This treatment enhances the accessibility of gold-tethered ssDNA molecules for base pairing with complementary nucleic acids, increasing the hybridisation efficiency from less than 10% to 80% approximately. The adsorption of MCH after –SH-ssDNA immobilization was monitored in real time as it is shown in figure 4. MCH adsorption produces a compressive surface stress, giving a cantilever deflection of about 10 nm that is of about 75% of the cantilever deflection resulting from the immobilization of –SH-ssDNA. This observation confirms that MCH molecules covalently attach to interstitial gold regions between neighbouring surface-tethered ssDNA, removing the non specific interactions between the nucleotide chain and the gold. The large amount of interstitial regions available for covalent binding of the MCH molecules is deduced from fluorescence experiment that gives a gold-tethered ssDNA density of about $\sim 10^{11}$ molecules/cm$^2$ in the cantilever, about two orders of magnitude lower than that reached by MCH on bare gold.

![Figure 4](image.png)

Figure 4. Experiment of treatment of the cantilever with mercaptohexanol after immobilization of the thiol-derivatized oligonucleotide. The oligonucleotide and MCH concentrations were about 4.5 µM and 1 mM respectively. The experiments were performed in 50 mM phosphate buffer solution with 0.5 M of NaCl. The fluid cell was rinsed after 25 minutes of the analyte flowing over the cantilever.

Strikingly the cantilever deflection was below 10% of the immobilization-induced bending when the cantilever is exposed to the complementary DNA sequence. This cantilever bending is close to the detection limits, and it is in contrast to fluorescence experiments performed on gold surfaces that give significant hybridisation signals. This result can be tentatively explained as following. The complementary ssDNA forming a duplex is separated from the gold surface by the thiol linker and MCH molecules, thereby a change of surface stress due to charge redistribution on the gold can be neglected. The main contribution to the hybridization-induced surface stress is a change of the interaction between neighbouring DNA molecules, mainly of electrostatic and steric nature. In agreement with McKendry et al, MCH treatment could arrange the immobilized DNA molecules with a geometry where the intermolecular interactions...
due to the hybridization are small. Thus it may occurs that a highly packed monolayer of immobilized ssDNA would produce a higher hybridisation-induced surface stress, although the density of hybridized DNA molecules would be lower. Further studies are being performed in order to elucidate the intermolecular mechanisms that govern the surface stress during DNA hybridization.

3.3. Detection of the pesticide DDT

Nanomechanical biosensors have been applied for detection of the pesticide DDT by performing competitive immunoassays using a synthetic hapten conjugated with BSA and its specific monoclonal antibody. DDT is a chlorinated compound with insecticidal properties that has been used worldwide for controlling insect pests. Its high hydrophobicity together the great stability to physical, chemical, and biological degradation have resulted in the accumulation of its residues in animal and human tissues, as well as in the environment. Development of cost-effective and rapid methods for monitoring exposure to DDT are needed. Immunochemical techniques are emerging as alternative to the expensive and time-consuming chromatographic techniques. We prove that nanomechanical biosensors allow rapid, highly sensitive, and direct detection of DDT. Briefly the immunoassays developed to monitor this pollutant require the use of three components. The most critical is the monoclonal antibody (LIB-DDT5-25) that is responsible of the sensitivity and specificity of the assay. The second component is the DDT, which is unable to produce an immune response due to its low molecular weight (354.5 D). Therefore it is necessary to design a third compound, the hapten, that can covalently bind a carrier, usually a protein, thereby the hapten-carrier can produce animal immunization. The hapten used here has similar geometry and structure to that of the DDT, and contains appropriate groups (-COOH, -NH₂) for attachment to the carrier protein bovine serum albumin (BSA).

The gold-coated cantilevers were exposed to 2 mM cystamine dihydrochloride during 30 min and washed in milliQ water. Cystamine is an amine-terminated thiol that produce a densely packed layer on the gold-coated surface. Then 2.5% glutaraldehyde was flowed over the cantilevers for 30 min. Rinsing was performed with milliQ water. The glutaraldehyde, with an aldehyde group in both extremes of the molecule, acts as covalent linker between the amine-functionalised gold surface and primary amine groups of the hapten protein carrier.

![Graph](image-url)

Figure 5. Real-time monitoring of the covalent attachment of the DDT hapten conjugated with BSA on the functionalised cantilever side, and the specific hapten/antibody binding. The experiment was performed in PBS solution.
A synthetic hapten of the pesticide DDT conjugated with BSA was flowed over the cantilever (fig. 5). The injection of the DDT hapten derivative produced a downward bending of the cantilever, indicating a compressive surface stress acting on the gold layer. The DDT hapten is strongly attached to the sensitised cantilever side through the covalent bond between the aldehydes coating the gold surface and the primary amine groups of BSA. Before the pesticide detection assay, the cantilever was exposed to 1M ethanolamine to saturate remaining aldehyde sites and to minimize no specific adsorption on the bottom surface of the cantilever. After rinsing with PBS, the cantilever was exposed to the monoclonal antibody to the synthetic hapten. The antibody/hapten recognition on the cantilever is again translated into a downward bending. Injection of non-specific antibodies did not produce a significant cantilever deflection. The clean detection signal for an antibody concentration of 25 nM indicates that pesticide detection below the nanomolar range is suitable.

Figure 4. Real-time monitoring of a competitive immunoassay (see related text). The cantilever surface was regenerated with 100 mM HCl (100 µl) to break the hapten/antibody complex.

The top surface of the cantilever could be regenerated a few times by rinsing with 100 µl of 100 mM HCl to dissociate the hapten/antibody complex. Regeneration was employed for direct detection of DDT by performing competitive assays. Figure 6 shows the biosensor response in a competitive assay. Firstly, the cantilever was exposed to 33 nM of the monoclonal antibody, and a deflection change of about 3 nm was obtained. Then, the cantilever surface was regenerated, and the cantilever deflection recovered to the same value approximately. Then a mixed solution of 33 nM of the monoclonal antibody with 10 nM of DDT was injected, resulting in a deflection variation of about the half obtained before. This indicates that the DDT in the solution competes with the hapten on the cantilever surface to bind the antibody. To check that the decrease of the deflection change is due to this two competing immunoreactions, the surface was again regenerated and the assay was performed only with the antibody as in the first assay. A deflection change of about 3.1 nm was obtained, indicating that the regeneration did not produce a degradation of the cantilever surface. The regeneration procedure depends critically on the hapten/antibody pair used. For the hapten and antibody used in this work, treatment of the surface with 100 mM HCl was the most reproducible method.
4. CONCLUSIONS

In this work, we have presented two important applications of biological detection based on nanomechanics. First application is the development of nanomechanical biosensors for nucleic acid detection, which is relevant to determine gene function and alterations responsible of disease as well as for rapid pathogen detection. Here it was characterized the covalent attachment of thiolated DNA. The aim is to control the geometry of the DNA binding to maximize the hybridization-induced cantilever bending. Hybridization signals were less than 10% of the immobilization signal for oligonucleotides attached only through the sulphur atom of the thiol linker. These results indicate that further investigation is required to elucidate the intermolecular mechanisms that govern the surface stress during hybridisation. Second application is a novel technique for direct immunodetection of pesticides by measuring the nanomechanical response of a microcantilever. Development of new techniques for rapid, sensitive and cost-effective detection of pesticides is needed for environmental and health control. These results indicate that nanomechanical biosensors can achieve subnanomolar sensitivity, without need of labelling with fluorescent and radioactive molecules.

Nanomechanical biosensors can become ultrasensitive devices for biological detection. The small sensor area (∼100 µm²) allow label-free detection in real time with high sensitivity and low reagent consumption. However, future technological developments will be required to exploit the potential of these sensors. Implementation of micro-nanofluidics is necessary to achieve femtomolar sensitivity and faster responses. Advantageously, microcantilevers are fabricated using standard silicon technology, which allows integration into microsystems, high throughput analysis though microarrays of cantilever, low cost and portability.

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