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Label-free protein assay based on a nanomechanical cantilever array

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Abstract
We demonstrate continuous label-free detection of two cardiac biomarker proteins (creatin kinase and myoglobin) using an array of microfabricated cantilevers functionalized with covalently anchored anti-creatin kinase and anti-myoglobin antibodies. This method allows biomarker proteins to be detected via measurement of surface stress generated by antigen–antibody molecular recognition. Reference cantilevers are used to eliminate thermal drifts, undesired chemical reactions and turbulences from injections of liquids by calculating differential deflection signals with respect to sensor cantilevers. The sensitivity achieved for myoglobin detection is below 20 μg ml^{-1}. Both myoglobin and creatin kinase could be detected independently using cantilevers functionalized with the corresponding antibodies, in unspecific protein background. This approach permits the use of up to seven different antigen–antibody reactions simultaneously, including an additional thermomechanical and chemical *in situ* reference. Applications lie in the field of early and rapid diagnosis of acute myocardial infarction.

1. Introduction

Biosensing tools are currently undergoing a further stage of development. Important requirements for a future generation of biosensors are

(a) combination of a biologically sensitive part with a physical transducer for specific and quantitative detection of analytes,
(b) the ability of label-free detection of the biological interaction,
(c) the scalability of the sensors to allow massive parallelization, and
(d) sensitivity of the detection range applicable for *in vivo* problems.

Three types of instruments are currently being developed to meet the requirements mentioned above:

(1) surface plasmon resonance (SPR) detectors [1],
(2) quartz crystal microbalances (QCM) [2], and
(3) cantilever array biosensors [3, 4].

Some SPR-type sensors suffer from the instrumental problem that there is no parallel reference detector to compensate for the background. QCM offers only little potential in scalability, because the mass-detection capability scales with the QCM sensor surface. Such instrumental problems have been solved by the novel technique of cantilever arrays. Recent experiments show that this technology works without any radioactive or fluorescent labelling [4], and is easily scalable to allow massive parallelization [5]. A major field to be exploited is the detection of *in vivo* concentrations of bioanalytes. First applications of cantilever arrays as tools for biomolecule detection have already been demonstrated in the field of DNA hybridization detection [3, 4].

For medical applications, especially in an intensive care unit, it is desirable that many physiological parameters are monitored simultaneously and in real time, for example specific protein biomarkers indicating cardiac diseases such as heart infarction. Detailed knowledge of these protein levels would allow life-saving treatments of patients suffering from acute myocardial infarction (AMI). Current methods for pro-

tein detection involve labelling procedures, are time consuming and non-instantaneous [6]. Cantilever arrays, however, will allow label-free investigation of processes and real-time analysis. Currently, the most reliable diagnosis of AMI is based on the detection of a temporary significant increase of a few protein levels in blood [7, 8]. For this, three major markers of cardiac myocyte necrosis (i.e. myocardial infarction) are clinically used: creatin kinase (MB fraction), the troponins, and myoglobin. While the former two are highly specific, they tend to increase with a certain delay after myocardial injury compared with myoglobin, which increases rapidly, but is less specific. Current diagnostic schemes therefore rely on a combination of necrosis markers to yield information as fast as possible and as specific as feasible for a given timepoint [9]. As the development or absence of myocardial necrosis strongly predicts the individual mortality risk of a patient [10] and has immediate therapeutic implications, continuous monitoring of a combination of these markers in real time would be very attractive, although so far this is not technically feasible. For this reason, early, rapid and real-time detection of cardiac-specific plasmatic biomarkers [11, 12] is of large interest. Protein detection experiments using a single cantilever have been demonstrated [13]. Here, we present a multiple antibody cantilever-array approach that holds promise for biosensing applications, because it can detect multiple proteins in parallel. We have used reference cantilevers [14] to exclude false positive detection, as might occur when using single cantilevers. In particular, the label-free detection of two biomarkers in a background environment of unspecific proteins is demonstrated. Temperature changes on injection of liquids in the measurement cell and unspecific binding of proteins to a non-passivated cantilever surface will influence the cantilever deflection signal and will mask a specific sensor response. The setup is based on an array of eight cantilevers functionalized with antibodies and *in situ* thermomechanical and chemical reference cantilevers for deflection calibration and cancellation of undesired competing chemical influences, respectively.

2. Materials and methods

2.1. Instrument

The functionalized cantilever array (see figure 1) is inserted into a liquid chamber (volume: 40 μl) and mounted on a wedge-shaped support aligned at an angle of 11° with respect to the incident laser beam (vertical-cavity surface-emitting laser; wavelength 760 nm, Avalon, Zurich, Switzerland). A Peltier element (Deltron, Kirchberg, Switzerland) is placed below the cell, and the temperature is measured using a thermocouple (Thermocoax, Suresnes, France). The incident laser beam is reflected at the apex of the cantilever and then redirected by a mirror to a position-sensitive detector (SiTek, Partille, Sweden). The cantilever deflection is determined with an accuracy of 0.1 nm, and the data are acquired using a multifunctional data acquisition board (National Instruments, Austin, TX) driven by LabView software. The software further controls the liquid-handling system of the setup, i.e. the syringe pump (GENIE, Kent Scientific Corp, Torrington, CT), and the ten-position valve system (Rheodyne, Rohnert Park, CA), as well as data processing and cell-temperature measurement.

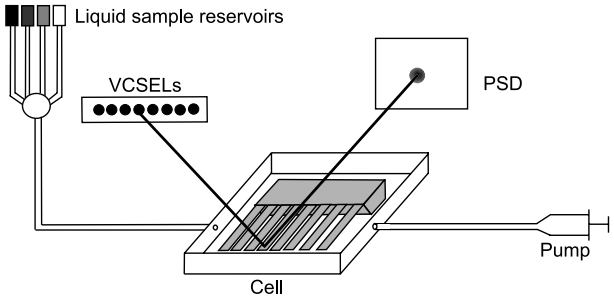

Figure 1. Schematic of the experimental setup with liquid cell, optical readout of cantilever deflections and sample liquid exchange system. VCSEL = vertical cavity surface emitting lasers, PSD = position sensitive detector.

2.2. Functionalization of cantilevers

Eight cantilevers are linearly arranged in an array at a pitch of 250 μm, each of them being 500 μm long, 100 μm wide and 500 nm thick. Cantilever arrays are batch-fabricated on the wafer scale by the Micro-/Nanofabrication Group at the IBM Zurich Research Laboratory. Prior to functionalization, the cantilever arrays are cleaned using Piranha (30% H_2SO_4 conc. in H_2O_2, 1:1) solution for 20 min.

The functionalization of cantilevers involves a metal evaporation process and multi-step chemical coating, which differ for sensor and reference cantilevers. The upper side of the cantilever array is coated using a double electron-beam evaporator (Boc Edwards, Sussex, UK). First, a titanium adhesion layer of 2 nm was deposited at a rate of 0.1 nm s^{-1}, and then a 20 nm-thick gold film (rate: 0.1 nm s^{-1}). This gold surface is coated with a 1 mM di-thio-bis-succinimidylundecanoate (DSU) [15] self-assembled monolayer in waterfree 1, 4-dioxane for 20 min, which provided an amino-reactive surface towards primary amino groups (e.g. lysine). The cantilever array was then rinsed with 20 mM HEPES buffer set to pH 7.5 (HEPES). The functionalization of each cantilever was accomplished in quartz micro-capillaries (inner diameter 150 μm, Garner Glass Inc., Clarmont, CA). The sensor cantilevers were exposed to a 200 μM solution of different antibodies in HEPES with 20 mM NaCl for 1 h (see figures 2–4). The functionalized surfaces of reference and sensor cantilevers are schematically shown in figure 2. For the reference cantilevers, two methods for surface passivation were used:

(1) the DSU-activated upper surface is quenched with ethanolamine (see figures 2(b) and 3(b)),
(2) 1 mg ml^{-1} bovine serum albumin (BSA) was coupled covalently in HEPES (see figure 4(b)).

The lower sides of sensor and reference cantilevers were protected in both cases with a layer of BSA. Important findings were that the hinge region of each individual cantilever must also be coated accordingly, and that the cantilever array has to be kept wet during further processing steps and measurements. After functionalization, the arrays may be stored in HEPES at 4 °C at least for one month. The following types of antibodies were used for biomarker detection: anti-creatin kinase antibody (Biomeda, Los Angeles, CA) and anti-myoglobin (Hytest Co, Helsinki, Finland). The proteins used in the experiments were creatin kinase, BSA (both by Merck Inc, Glattbrugg, Switzerland) and myoglobin (Hytest Co, Helsinki, Finland).

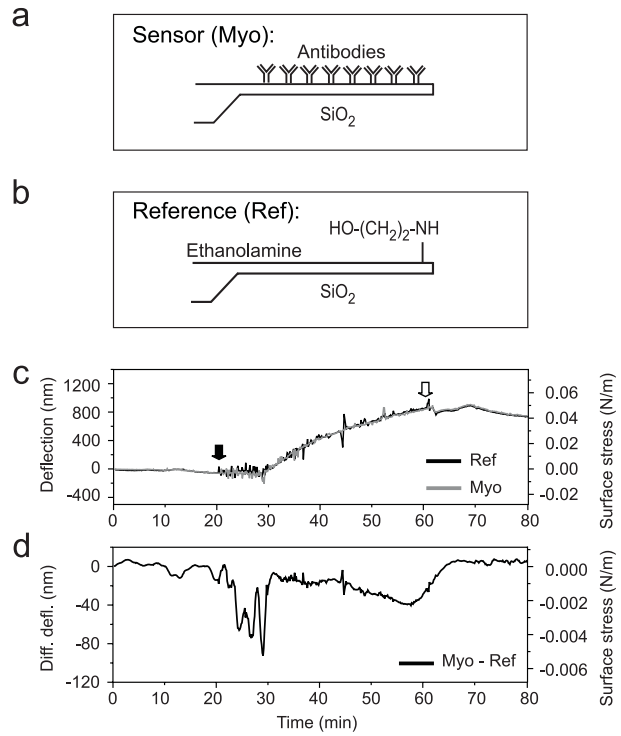

Figure 2. (a) Sensor cantilever functionalized with myoglobin (Myo) antibodies, (b) reference cantilever (Ref) quenched with ethanolamine. The lower surfaces are not passivated. (c) Absolute deflection of cantilevers. Upward bending of the cantilever increases deflection and produces tensile surface stress, whereas downward bending of the cantilever decreases deflection and builds up compressive surface stress. Myo is shown in black, Ref in grey. The black arrow indicates protein injection, white arrows injection of buffer. The injection of myoglobin protein (50 μg ml^{-1}) produces drift of both cantilevers. No conclusive interpretation is possible owing to the competition between unspecific interaction of protein with lower silicon surface of the cantilevers and antigen–antibody interaction on the upper surface. (d) Differential deflection signal (Myo–Ref). The net reaction response is 40 nm.

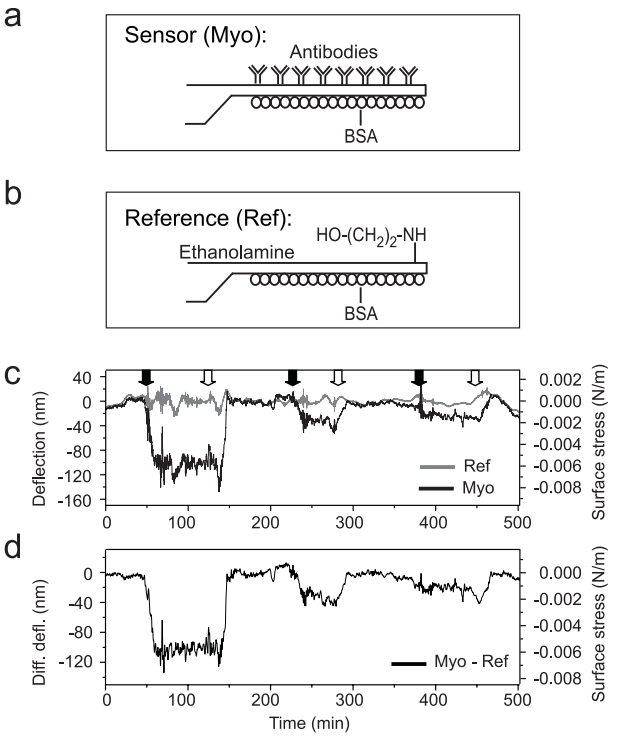

Figure 3. (a) The sensor cantilever is functionalized with myoglobin (Myo) antibodies and the lower surface is passivated with BSA. (b) The reference cantilever is quenched on the upper surface with ethanolamine, and the lower surface is passivated with BSA. (c) Myoglobin (Myo) was detected at three different concentrations (100, 50, 20 μg ml^{-1}). The reference cantilever (Ref) does not show a significant response. (d) Differential deflection signal (Myo–Ref): the response of the sensor cantilever is almost linear with protein concentration in the range investigated. The accuracy of deflection determination is 20%, as derived from the noise in the cantilever deflection divided by deflection magnitude.

3. Results and discussion

3.1. Mechanical requirements for the cantilever array

To measure molecular recognition reactions, cantilevers are required that convert chemical reactions very efficiently into mechanical bending. The cantilevers used here have a thickness of only 500 nm. Because they are sensitive to external influences such as turbulence during fluid exchange and temperature variations, the microfabrication process must be controlled to such an extent that the mechanical properties of the cantilevers are almost identical. Measurement of the cantilever resonance frequencies has revealed a variation of less than 0.5%. This enables the use of cantilever arrays rather than single cantilevers and internal *in situ* reference cantilevers. Myoglobin detection using a single sensor cantilever and a cantilever with a much larger spring constant as a reference cantilever has been reported in [16]. However, it is very important that the reference cantilever is part of the same cantilever array and has, ideally, mechanical properties identical to those of the sensor cantilever. Sequential measurement of a reaction using a single sensor cantilever followed by measurement of the same reaction with a single reference cantilever will lead to an inaccurate differential deflection signal, because the two responses were not acquired at the same time. Reference cantilevers within the same array enable compensation of external influences, such as thermal drifts, or turbulence from injections. To ascertain the uniformity of the array, the cantilevers were subjected to heating and cooling cycles using a Peltier element. All cantilevers are expected to bend by the same extent owing to the bimaterial effect (different thermal expansion of gold and silicon). The absolute deflection due to temperature regulation using the Peltier element is of the order of 100 nm °C^{-1}. The spread in deflection magnitude is only a few per cent (data not shown). Temperature-induced deflection changes are cancelled out in differential deflection measurements with sensor and reference cantilevers [14].

3.2. Chemical reference cantilever

To minimize uncontrolled reactions taking place on unmodified silicon cantilever surfaces, a passivation procedure for the reference cantilever is required to prevent unspecific adsorption of proteins on the reference cantilever. To demonstrate the influence of unspecific protein–silicon interaction, the upper surfaces of two cantilevers were functionalized (see figures 2(a) and (b)). The sensor

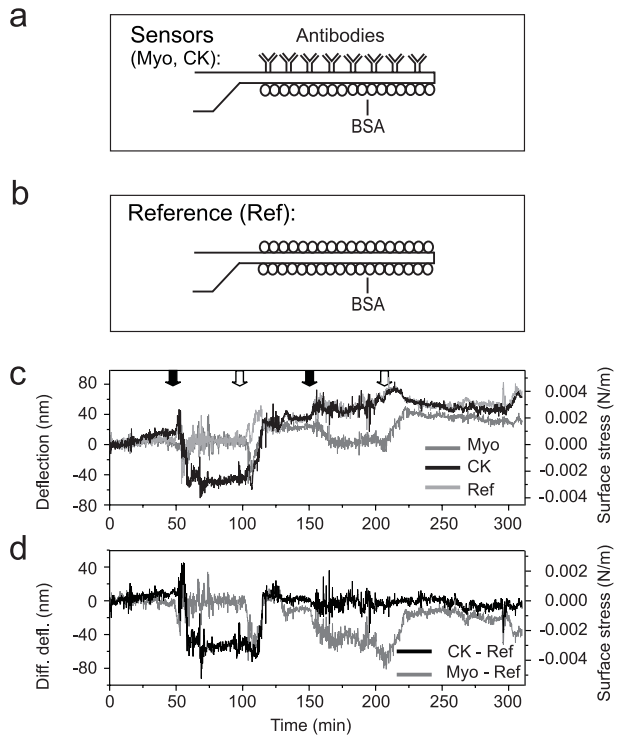

Figure 4. (a) Two cantilevers in the array are coated with myoglobin (Myo) and creatin kinase (CK) antibodies and passivated on their lower surface with BSA. (b) The reference cantilever is quenched on the two surfaces with BSA. (c) Sequential injections of creatin kinase and myoglobin proteins reveal deflection of the corresponding cantilever due to compressive stress. The experiment is performed in a background of unspecific protein (BSA 100 μg ml^{-1}). (d) Differential deflection signals: CK–Ref, Myo–Ref.

cantilever was activated with anti-myoglobin antibodies, and the reactive surface of the reference cantilever was quenched with ethanolamine. The lower surfaces of both cantilevers were not passivated. After injection of myoglobin protein (concentration: 50 μg ml^{-1}), a common drift of both cantilevers was observed (see figure 2(c)). This is interpreted as an unspecific interaction of myoglobin protein with the sensor surfaces. The raw data of the cantilever excursion does not allow conclusive information to be drawn from the specific interaction, because the sensor response was influenced massively by the competition between unspecific interaction of the protein on the lower surface and the specific antibody–antigen interaction on the upper surface. However, it was still possible to visualize the specific signal by using differential deflection measurements. The amplitude of the differential signal is 40 nm (see figure 2(d)). Improved reference cantilevers for the subsequent experiments feature a BSA-passivated lower surface and a quenched upper layer with either ethanolamine (figure 3(b)) or BSA (figure 4(b)).

3.3. Myoglobin detection

A cantilever array was prepared according to the procedure described above. For myoglobin detection, the upper surface of the cantilever was functionalized with anti-myoglobin antibodies and passivated on the lower surface with BSA (see figure 3(a)). The reference cantilever was coated as shown in figure 3(b). Various concentrations (100, 50 and 20 μg ml^{-1}) of myoglobin in HEPES were injected, with intermittent purging of the measurement chamber with HEPES buffer to regenerate the sensor surface. The injection speed in this experiment was set to 30 μl min^{-1} to decrease thermal drifts and turbulences in the measurement chamber. Ten minutes after the injection of 80 μl of myoglobin solution (concentration: 100 μg ml^{-1} in HEPES) the signal reached equilibrium (see figure 3(c)). The direction of cantilever bending implies compressive stress. Injections of lower concentrations (50 and 20 μg ml^{-1}) show that the sensor response is proportional to the amount of analyte protein, i.e. myoglobin. The magnitude of the response measured depends linearly on the concentration in the range investigated. This is supported by calculating the differential deflection signal (figure 3(d)). Note that the absolute thermal drift of the cantilevers during the experiment was less than 2.5 nm h^{-1}.

3.4. Label-free protein assay

Real-world applications require simultaneous detection of multiple analytes. We functionalized two cantilevers of an array using two different types of antibodies (figure 4(a)) and measured the response in a protein background (BSA: 100 μg ml^{-1}), see figure 4(c). The two antibody-functionalized cantilevers (anti-creatin kinase and anti-myoglobin) were passivated on the lower surface with BSA (figure 4(a)), and the reference cantilevers were passivated according to figure 4(b). The biomarker proteins are sequentially injected at a concentration of 50 μg ml^{-1} in a background of unspecifically binding BSA protein (BSA, 100 μg ml^{-1}). First, creatin kinase was injected (see figure 4(c)). The cantilever coated with anti-creatin kinase antibodies deflected immediately upon interaction with the biomarker, whereas the BSA reference cantilever and the anti-myoglobin antibody-coated cantilever did not respond. After purging the measurement chamber with buffer to remove all analytes and to regenerate the sensor surfaces, we injected myoglobin. This time, the anti-myoglobin-functionalized cantilever deflected. During the experiment the drift of the signal was 10 nm h^{-1}. The differential deflection signal with respect to the BSA-coated reference cantilever revealed a deflection of 50 nm for creatin kinase and of 45 nm for myoglobin (see figure 4(d)).

4. Conclusions and outlook

We developed a novel protein assay for a biomedical environment. We demonstrated multiple real-time label-free protein detection. The ability to measure specific signals in a background reflects the reliability and the specificity of cantilever array sensors. These experiments show that multiple biomarker proteins are detected without crosstalk between two antibody-functionalized cantilever sensors in an unspecific background. The cantilever response is quantitative (concentration of the analyte proteins) and qualitative (multiple proteins in parallel). Proteins are detected in real time in less than 10 min. Currently, our device achieves a sensitivity of better than 20 μg ml^{-1} (i.e. $\sim$1 μM). The sensitivity is by several orders of magnitude lower than for the detection of DNA hybridization [4] (data not shown). The method is able

to detect creatin kinase and myoglobin independently, two proteins involved in the diagnosis of myocardial infarction. Continuous monitoring of multiple cardiac myocyte necrosis markers will allow the earliest possible biochemical diagnosis of myocardial infarction. Shortening the time to treatment as well as reducing the time to exclusion of infarction in this deadly disease have the potential of minimizing the risk for the patient.

Functionalization of the cantilevers is very important in the further development of the technique, in particular the orientation of antibodies coupled to the surface must be controlled. The way the covalent coupling of antibodies was performed may limit the sensitivity of the detection. Antibodies are bound covalently in random orientation to the cantilever surface via primary amines. Therefore, the coupling with their active binding pocket facing the cantilever surface may result in a partial inactivation of some of the antibodies. The flexibility of the hinge region of the antibody could also lower the surface stress generated during antibody–antigen interaction. Restricting the antibodies to their active part (antibody single-chain Fv fragment (scFv) [17]) and coupling them oriented via a specific group to the surface will enhance the sensitivity of the assay considerably. This will establish the binding sites closer to the surface in order to increase the stress induced on the surface. Further developments of the cantilever array biosensor will provide real-time monitoring of biomarkers, especially cardiac-specific proteins.

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