Expert Opinion

Cantilever biosensors in drug discovery

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Importance of the field: There is a growing need for inexpensive and highly sensitive methods in high-throughput format for measuring drug–target interactions. Cantilever biosensors are ultra-high sensitive electromechanical sensors that have been successfully used for label-free detection of a large number of biological entities. They are emerging as a technology that appears attractive for high-throughput drug discovery applications. Therefore, a brief description of this technology is provided here.

Areas covered in this review: The objective of this review is to present an overview of the development in cantilever biosensor field reported to date and examine applications of cantilever biosensors for biomolecular interaction characterization and drug discovery. The subareas included in the review are: cantilever measurement principles, various interactions measured such as DNA–DNA, protein–protein, membrane protein–ligand and DNA–protein. Discussion on biomarker detection is also included.

***What the reader will gain*:** We analyze the recent publications on cantilever biosensors with an emphasis on drug discovery and biomarker detection. The reader will become informed on the detection sensitivity and limit of detection achieved to date. A description of ongoing commercial activity that is likely to result in a practical instrument for researchers is also included. Technical issues that concern current researchers in the field of cantilever biosensors are also summarized.

***Take home message*:** The cantilever biosensors provide an extraordinarily sensitive method for binding affinity measurement using label-free reagents. They can be fabricated in array format for high-throughput applications using semiconductor manufacturing techniques. When they become commercially available in future, it is anticipated that they will be cost-competitive and easy to use in a research bioanalytical laboratory.

Keywords: biomarker screening, biomolecular interaction, high sensitivity, high-throughput, label-free

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1. Introduction

Microarrays and biochips allow simultaneous interaction analysis of thousands of chemical entities in a single experimental step and have become an essential platform in drug discovery and life science research [1]. Although high-throughput microarrays such as DNA [2] and protein microarrays [3] are powerful tools, they require tedious and expensive fluorescent labeling procedures. The high cost of reagents and time-consuming assays are the reasons for researchers to investigate alternate methods that provide easier and less expensive high-throughput screening methods.

Recent developments of highly sensitive and inexpensive biosensors show promise for applications in the drug discovery field [4-9]. Typical biosensors contain three main parts: a biorecognition element, a transduction component and output

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electronics [10]. The biosensors reported in the literature can be broadly divided based on transduction mechanisms used, namely, optical, electrochemical, electromechanical and others. In a typical biosensor, the biorecognition element is a receptor, antibody, ssDNA or another entity that provides molecular recognition. It is immobilized on the sensor surface and its interaction with the target analyte (drug candidate in the context of drug discovery) is transduced into a measureable signal. In general, the transduced signal is proportional to the strength of interaction, often interpreted as binding affinity. Optical sensors measure changes in light properties as a result of drug interaction with the immobilized receptor on the sensor. Surface plasmon resonance (SPR), which measures changes in refractive index, is the most widely studied biosensor for drug discovery. Electrochemical sensors measure changes in current, voltage or electrical impedance as a result of receptor–drug interaction. Electromechanical sensors, such as quartz crystal microbalance (QCM) and cantilever sensors, measure change in resonant frequency or surface stress change when the analyte binds. Electromechanical methods do not require a label as they measure mass change associated with binding to the biorecognition element. Thus, they are attractive in drug screening applications because labeling introduces variability. Label-free methods are inherently superior due to uncertainty caused by labeling on conformation and potential hindrance of binding epitopes. Because labeling occurs at different sites, the response of each labeled biomolecule may not be equal, and this leads to variability and uncertainty in the results. Label-free detection reduces both time and effort required in assay development while at the same time practical issues related to shelf life and photo-quenching are avoided.

Although a large number of sensor designs and methods for label-free detection have been described in the literature, only a few are commercially available. Among them, SPR and QCM are attractive techniques and have been applied in drug discovery studies. However, their extension to high-throughput assays have been limited due to scaling issues, even though attempts to build multi-channel devices have been reported [11,12]. In the long term, miniaturization of these sensors in a chip format may emerge that will permit high-throughput screening.

Cantilever biosensor is another label-free method suitable for drug screening and holds great promise for rapid high-throughput screening due to its extraordinarily high sensitivity and scalability [13-16]. Recent research has demonstrated feasibility of detecting biological entities in liquid in the picogram (10^{-12} g) to femtogram (10^{-15} g) range, and sensitivity at concentrations of nM (10^{-9} M) to fM (10^{-15} M) [17,18]. Detection of oligonucleotides [19,20], proteins [21-23], pathogens [24-27] and other biological entities [28] have been widely investigated using both bending- and resonant-mode cantilever sensors. The ability to detect biomarkers directly in body fluids [29,30] and pathogens or toxins in complex matrixes [31-33] have been demonstrated. Cantilever sensors on a chip containing hundreds of cantilevers are relatively easy to fabricate, and have been successfully designed and fabricated for oligonucleotides and proteins. These chips when available in a robust sensing platform would find applications in genomics, proteomics and drug discovery.

2. Cantilever biosensor technology

Cantilever-based biosensors evolved from the scientific experience of cantilever tips used in atomic force microscopy (AFM). They are operated in different modes, namely, bending mode, in which binding-induced deflection is measured and resonant mode, in which binding-induced resonant frequency shift is measured. Schematic representation of these two types of cantilever sensor designs that have been reported for biological experiments is shown in **Figure 1**. They range in size from microns to millimeters and the operating resonant frequency ranges from several kHz to ~ 100 MHz.

2.1 Bending-mode cantilevers

Bending-mode cantilever biosensors were the first to be applied for molecular interactions studies (**Figure 1A**). Bending is the result of surface stress change arising from specific interaction between immobilized receptor on the cantilever surface and the analyte in the liquid sample. It is believed that surface stress is induced due to analyte binding, and causes the thin cantilever to bend, whose deflection (Δz) is given by [34]:

$$\Delta z = \frac{3l^2(1-\upsilon)}{Et^2}\Delta\sigma \quad (1)$$

where l is the cantilever length, t is the cantilever thickness, E is the Young's modulus, $\Delta\sigma$ is the differential surface stress across the cantilever surface caused by analyte binding and υ is the Poisson's ratio. The above shows that deflection is proportional to the differential surface stress, which in turn is a function of concentration of analyte and binding affinity. Deflection of tens of nanometers is conveniently measured using various techniques [15].

2.2 Resonant-mode cantilevers

Resonant-mode cantilever sensors measure resonant frequency change due to mass-change that occurs as a result of analyte binding (**Figure 1B**). The nth resonant mode frequency of a uniform cross section cantilever is:

$$f_n = \frac{1}{2\pi}\sqrt{\frac{\kappa}{m_{e,n}}} \quad (2)$$

where k is the effective spring constant, and is given as $k = 3EI_z/L^3$ where E is effective Young's modulus, I_z is the moment of inertia and L is the cantilever length. And $m_{e,n} = 3m/\lambda_n^4$ is the equivalent mass of the cantilever sensor at nth resonant mode, m is the mass of the sensor and λ_n is the

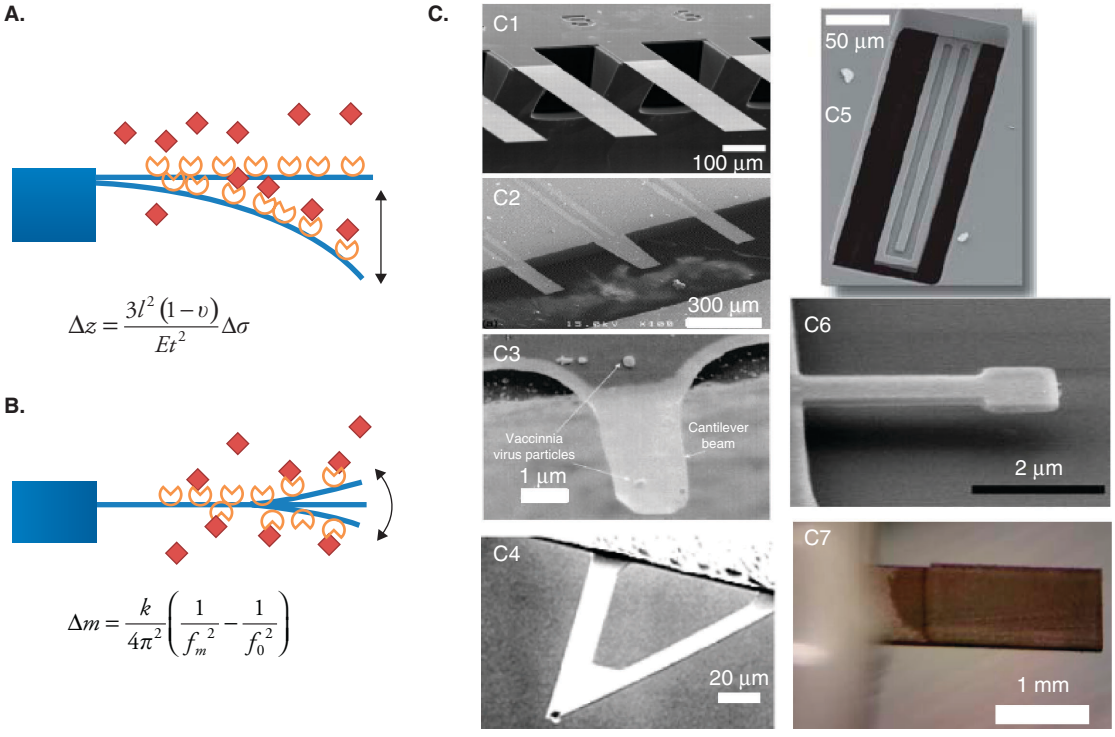

Figure 1. Schematic representation of cantilever biosensors. Bending-mode (**A**) or resonant-mode (**B**) cantilevers. Various cantilever designs reported (**C**). The operation frequencies of resonant-mode cantilevers are: f_0(C1) = 686 kHz (bending mode could also be used), f_0(C2) = 16 kHz, f_0(C3) = 1.27 MHz, f_0(C5) = 220 kHz, f_0(C6) = 13.3 MHz and f_0(C7) = 1 MHz.
Reproduced with permission from [19,23,27,36,58,79].

*n*th eigenvalue of the associated dynamic equation. As the mode number increases, the effective mass decreases. When an analyte or ligand attaches, the relationship between frequency change and mass change is given by [35]:

(3)

$$\Delta m = \frac{k}{4\pi^2}\left(\frac{1}{f_m^2} - \frac{1}{f_0^2}\right)$$

where f_0 and f_m are the resonant frequency values of pre- and post-mass attachments. From Equation 3, quantitative measurement of mass change on the sensor surface is obtained.

2.3 Measurement in liquids

Resonant-mode micro- and nanocantilevers actuated in vacuum have shown mass-change sensitivity of attograms or lower [36,37]. Such a level of sensitivity is far higher than what has been reported for bending-mode sensors. However, some of the resonant-mode cantilevers can be used only in vacuum or in air due to viscous damping of the surrounding air or liquid. Frequency response of resonating cantilever sensors in liquid have been examined by a number of researchers [38-41]. The parameter that defines the usefulness of a resonating cantilever in liquid is the Reynolds number (Re) at the operating frequency and is given as $(2\pi f b^2 \rho/\eta)$; b is cantilever width, f is resonant frequency, ρ and η are density and viscosity of fluid [38,39]. When a sensor is operated at high Re numbers, the dominant forces on the sensor are inertial forces related to mass of the sensor and attached mass. On the other hand, when Re number is low, the dominant forces on the sensor are the viscous forces exerted by the surrounding fluid. For sensors that have a width of ~ 10 µm and operate at 1 MHz, the Re number is ~ 10^2 indicating that the response is partially damped by the surrounding viscous medium. The situation becomes worse when one further reduces the cantilever width to nanometers. The quality factor of nanocantilevers decreases to 10^2 in air from a value of 10^4 in vacuum, and to 1 – 10 in liquids [42,43]. Re number of such sensors is significantly lower than unity in liquids. Viscous damping not only reduces the quality factor, but also significantly reduces sensitivity to changes in mass [44]. On the other hand, when cantilever width is increased to 1 mm and a high order resonance mode near 1 MHz is used, the sensors do not suffer from viscous damping effects, because the Re number is far greater than 10^6 [17]. As a result, they are responsive to mass change and can be monitored continuously both in static and flow conditions. It should be pointed out that the bending-mode cantilever sensors are not affected by the liquid environment because they are static devices that bend in response to surface stress. Thus, the bending-mode microcantilever sensors provide fairly sensitive response to surface binding in liquid environments [19,45].

2.4 Specificity of binding

The cantilever sensor is immobilized with an antibody or a ssDNA as the recognition molecule. Various methods of immobilization have been reported in the literature and immobilization kits are available commercially [46]. The reproducibility of cantilever response to a large extent depends on the uniformity of the immobilized recognition molecule, which was emphasized in [47]. If the sample contains various extraneous biological matter, their weak binding to the sensor surface can result in false signals. To address this issue, reference sensors prepared in various forms were included within the same array and the response obtained was corrected using the signal from the reference sensor [19,22,48,49]. Such an approach has been quite successful in extracting the true signal due to target binding. When the cantilever sensors do not have a reference sensor, various strategies have been utilized to reduce nonspecific binding. These include modification of sensor surface with spacers or blocking agents such as polyethylene glycol [50,51], bovine serum albumin (BSA) [52], casein [53], inert self-assembled monolayer [54] and 1-mercaptohexanol [55]. Blocking reagents have been found to be quite effective in diminishing nonspecific binding. A third approach for reducing nonspecific binding is to use a second specific binding antibody to the target after a primary detection response is obtained. This approach was successfully implemented for food toxin [33] and pathogen [31] detection.

3. Biomolecular interaction measurement

Specific interaction between therapeutic targets and potential drug candidates is fundamental to drug discovery. Therefore, we describe below the various biomolecular interactions that have been reported using cantilever biosensors.

3.1 DNA–DNA interaction

Cantilever biosensors have shown the capability of measuring DNA–DNA hybridization as well as identifying single base mismatch of short oligonucleotides at femtomolar [55,56] to nanomolar level [19,57]. Fritz *et al.* used an eight cantilever array that exhibited deflections only when complimentary 12-mer or 16-mer target sequence was exposed to the immobilized probe, while no response was seen to non-complementary sequence (**Figure 2**). The deflection response was proportional to target concentration. Reference cantilever guaranteed a true signal despite large nonspecific responses of individual cantilevers. In a follow-up study, the same cantilever array format detected multiple unlabeled DNA sequences with different probes immobilized on each of the cantilevers [20]. In the above examples, the measurement of molecular recognition is translated into the mechanical phenomenon of bending, the extent of which is proportional to concentration. Thus, one can calculate equilibrium values for binding from the measured responses. Hansen *et al.* used a microcantilever to discriminate single nucleotide mismatches, where the location of mismatch in target was profiled [58]. Using 20-mer or 25-mer probe, hybridization of 10-mer complementary target resulted in net positive deflection, while reaction with targets containing one or two internal mismatches resulted in net negative deflection.

Rijal and Mutharasan used a piezoelectric-excited millimeter-sized cantilever (PEMC) sensor for detecting DNA hybridization at 1 fM in a background of human serum [55]. In this study, resonant frequency of the cantilever was measured continuously in a flow cell, where the sample containing the target sequence was flown across the sensor immobilized with a 15-mer probe. The cantilever surface was continuously vibrated due to piezoelectric excitation. The authors attributed the absence of nonspecific binding to simultaneous vibration of the sensor and the flow field in which the sensor was placed. The sensor showed no detection compromise in human serum (**Figure 3**). SNP detection at 1 fM and real-time monitoring of DNA replication using PEMC sensor were successfully carried out by the same research group [59].

3.2 Protein–protein interaction

Measurement and characterization of protein–protein interactions are important for the identification of potential drugs as we rely largely on measuring affinity of drug candidates to target. Adhesion force between proteins has been widely investigated using AFM. Recently, cantilever biosensors have also been used for investigating such interactions. Binding interactions such as biotin–streptavidin [45,60], liposome–protein [61] and antibody–antigen reactions such as anti-BSA and BSA [62,63] have been successfully characterized using cantilever biosensors. These studies show the applicability of using deflection or resonant frequency response for characterizing binding and quantifying target protein. Recently, Lam *et al.* used a bending-mode cantilever sensor for screening of specific antibody bound to HIV-1 envelope glycoprotein gp120 [64]. The deflection upon gp120 binding to immobilized monoclonal antibodies (mAbs) A32 or T8 was measured. Subsequent incubation with mAb 17b (known to bind an A32-induced epitope on gp120) further increased deflection of A32- but not T8-presenting cantilevers. The results showed the capability of microcantilever sensors for measuring induced-fit interaction in a very short time period of 45 min at concentrations of 8 μg/ml for gp120 and 0.17 mg/ml for 17b.

Adsorption of proteins on various surfaces is an important concern when designing biocompatible surface. Adsorption of human serum albumin (HSA) on CH_3-, COOH- and OH-terminated self-assembled monolayers of C_{11}-alkanethiols was measured using a resonant-mode cantilever sensor [41]. The sensor was immobilized with thiols containing different end groups and then was exposed to HSA. The results showed strength of albumin interaction decreased in the order as CH_3 > COOH > OH, confirming earlier studies using chronopotentiometry techniques [65]. In this example, we note the sensor response was due to interaction between HSA and the functional groups, and the response was proportional to the strength of interaction.

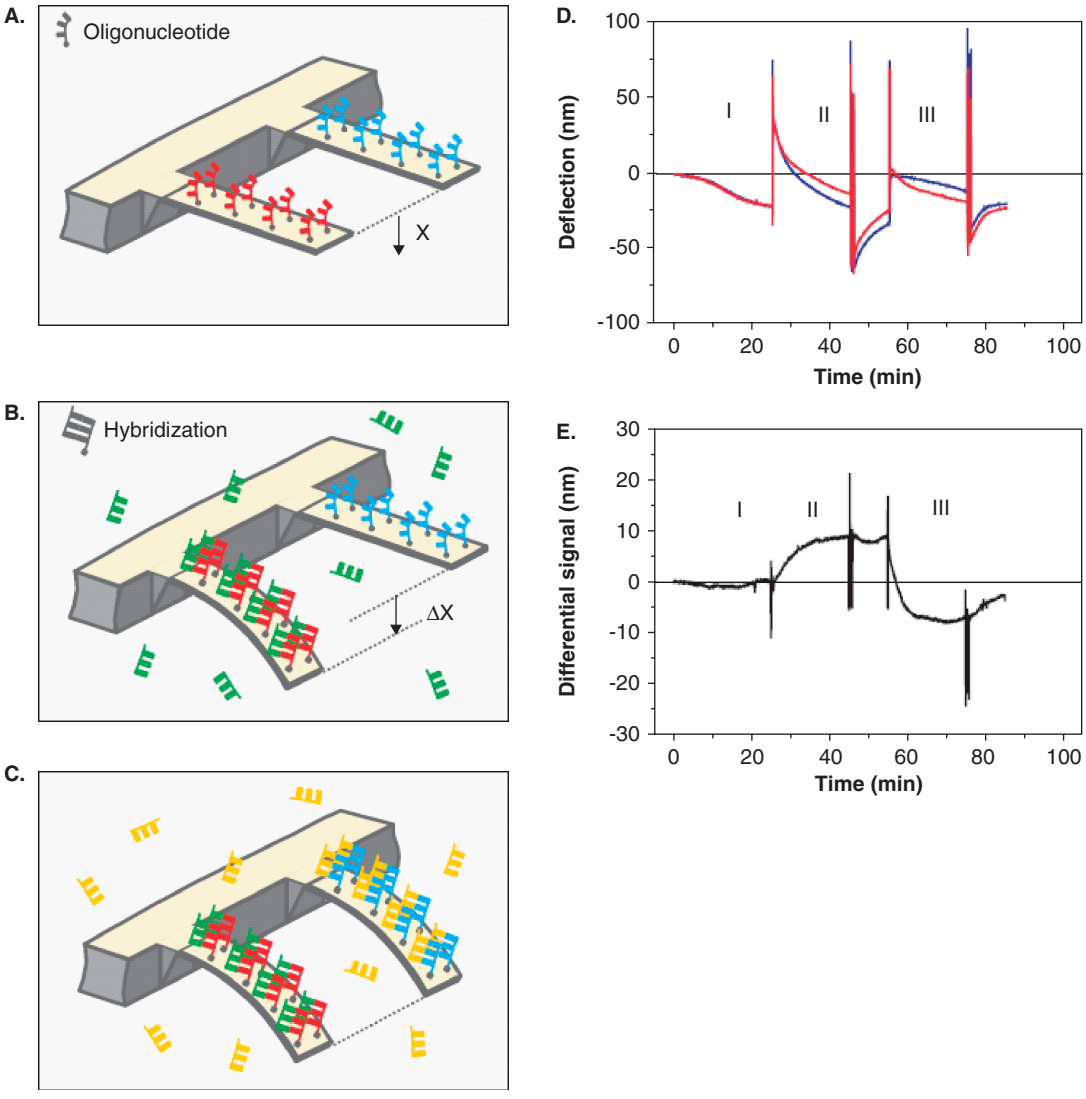

Figure 2. Illustration of the DNA hybridization on cantilever surface. Each cantilever is functionalized on one side with a different oligonucleotide sequence (red or blue). **A.** The differential signal is set to zero. **B.** After injection of the first 16-mer complementary oligonucleotide solution (green), hybridization occurs on the cantilever that provides the matching sequence (red) which induces the increase of the differential signal Δx. **C.** Injection of the second 12-mer complementary oligonucleotide solution (yellow) causes the cantilever functionalized with the second oligonucleotide (blue) to bend. **D.** Absolute deflection values of hybridization experiment using two cantilevers functionalized with the sequences 5′-TGCACTAGACCT-3′, and 5′-TAGCCGATATGCGCAT-3′. After taking a baseline (interval I), the complementary 16-mer oligonucleotide (1 ml, 400 nM in HB) was injected (interval II). The liquid cell was purged 20 min later with 3 ml of HB. Then, the complementary 12-mer oligonucleotide (1 ml, 400 nM in HB) was injected (interval III). The liquid cell was again purged 20 min later with 3 ml of HB. **E.** Corresponding differential signal.
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It has been proposed that protein enzymes which are implicated in diseases are potential therapeutic targets. Several studies have shown that cantilever biosensors can detect not only enzyme–substrate interactions but also measure enzyme activities [66-69]. In a recent study, Raorane *et al.* used cantilever arrays to measure both protease activity and its inhibition [66]. Cleavage of substrate by protease was measured. Bending-mode cantilever array was used as a platform for quantitatively screening inhibitors of a known protease. Dauksaite *et al.* used a glutathione-S-transferase (GST)-antibody immobilized cantilever arrays for detecting GST at 1 ng/µl [67]. Hwang *et al.* used RNA aptamer as a receptor molecule for HCV helicase and the binding was measured by surface stress changes [68]. In another study, Kwon *et al.* showed the detection of activated cAMP-dependent protein kinase via PKI-(5-24) peptide interaction, and the activity assay was used for determining binding affinity [69]. Shu *et al.* used peptide aptamer modified cantilever sensor for detecting less than 80 nM CDK2 protein

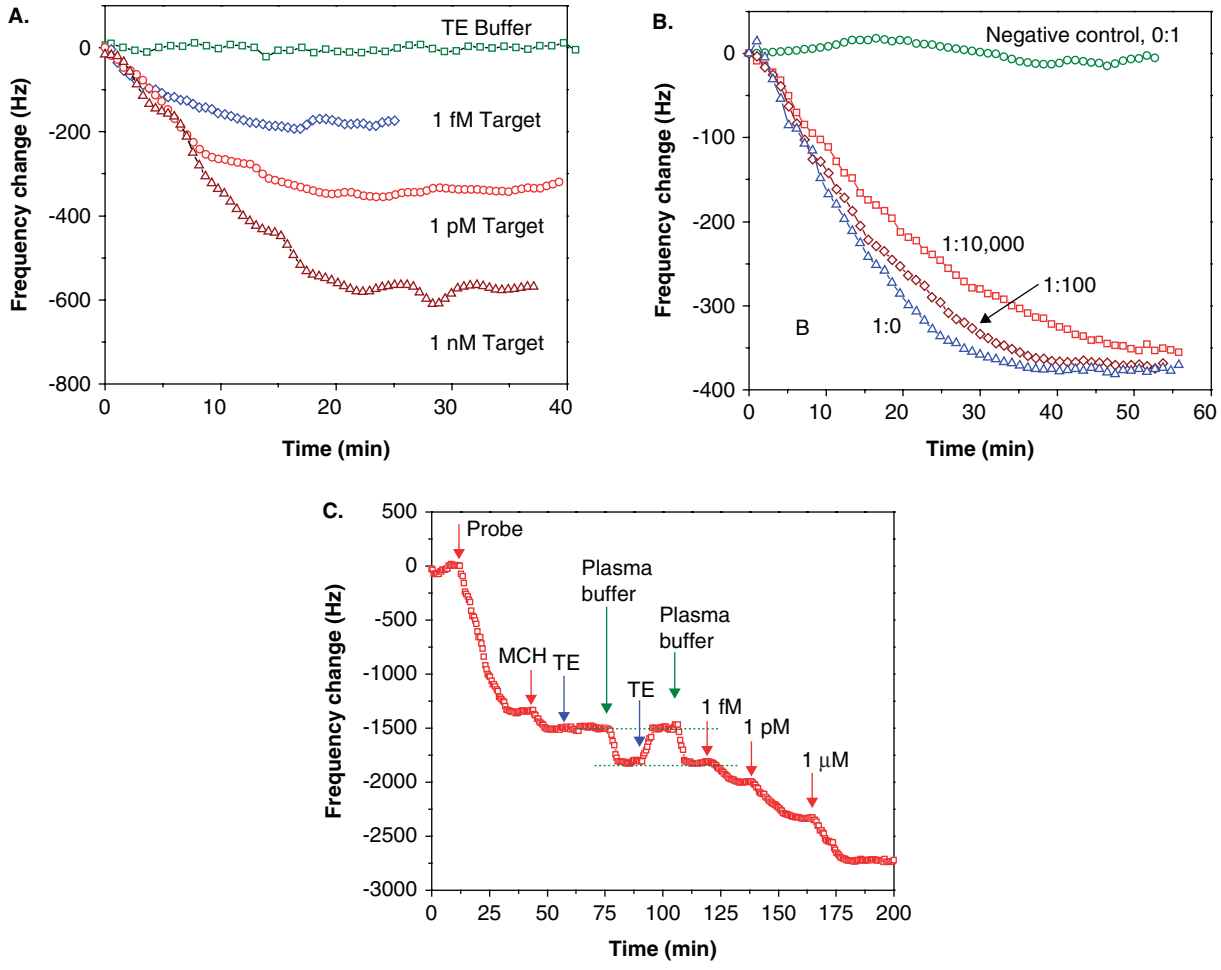

Figure 3. DNA hybridization on PEMC sensor. A. PEMC sensor response to 30 ml of 1 fM, 1 pM and 1 nM ssDNA in a once-through flow arrangement is shown. Flow rate was constant at 0.6 ml/min, and temperature was maintained at 32.1 ± 0.1°C, which is 5°C lower than hybridization temperature. Sensor was immobilized with probe (HS-C_6H_{12}-5′-GGAAGAAGCTTGCTT-3′) and 1-mercaptohexanol (not shown). Response to TE buffer shows zero response. **B.** PEMC sensor response to samples containing complementary and non-complementary ssDNA in mole ratios of 1:0, 1:100, 1:10000 and 0:1 were introduced with each sample containing 5 fmol of complementary stand at 167 fM concentration. The relative responses of the four cases are shown. Note that all samples containing complementary strand give essentially the same overall frequency change at steady-state. Kinetics of hybridization was slower in the presence of copious amounts of non-complementary strands. **C.** DNA hybridization in human plasma buffer. Plasma buffer was made consisting of 50% human plasma and 50% TE buffer with salt concentration adjusted for dilution. Introduction of plasma buffer, followed by TE buffer, gave a reversible response suggesting that plasma proteins did not attach to the sensor surface. The plasma buffer was reintroduced, and complementary ssDNA prepared in plasma buffer was then allowed to flow in sequentially at the three concentrations indicated, allowing for steady-state to be reached between each sample. No reduction in hybridization extent in plasma buffer was seen in comparison to results in TE buffer.

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PEMC: Piezoelectric-excited millimeter-sized cantilever.

from yeast cell lysate [49]. From the foregoing examples, one draws the conclusion that cantilever-based assays can be used for label-free enzyme detection in a single step format. Further, activity and inhibition properties can also be measured.

3.3 Membrane protein–ligand interaction

Membrane protein receptor such as GPCRs is a common drug target [70]. Hence, the study of drug candidate interaction with membrane protein is critical for evaluating potential drugs. Cantilever biosensors have been investigated for measurement of protein–ligand interactions using both bending and resonant mode. Zhang *et al.* modified a microcantilever with membrane containing 5-HT_{3AS} receptor to investigate ligand interaction [71]. Binding affinities of naturally occurring agonist serotonin and antagonist MDL-72222 were then measured by cantilever bending response. Such an approach yielded K_d (dissociation constant) values for serotonin and MDL-72222 that were identical to the values obtained from

radio-ligand binding assays. In another recent study, Braun *et al.* used a resonant-mode microcantilever array to measure membrane protein–ligand interaction quantitatively under physiological conditions (**Figure 4**) [72]. The array was modified with transmembrane FhuA receptor from *Escherichia coli* by ink-jet spotting. The 14th eigenmode of the cantilever was used for detection purpose. From **Figure 4B** we see the mass change was proportional to the resonant frequency change. Their study demonstrated the ability to measure bacterial virus T5 at sub-picomolar concentration.

Cantilever biosensors not only detect the mass increase by ligand-receptor binding, but can also provide information on conformational change. Using bacteriorhodopsin as a model system, Braun *et al.* quantitatively detected the prosthetic retinal removal from the bacteriorhodopsin protein by measuring the induced surface stress change [48]. Cantilever biosensors can distinguish receptors in free and ligand-bound forms [73]. The ligand-binding domain of the human estrogen receptor (ERα-LBD) was used as the model system and ERα-LBD with or without oestradiol (E2) was determined using conformation-specific peptides which recognize E2-bound ER or E2-free ER. In another study, binding of bee venom peptide melittin to the lipid vesicle was measured with cantilever sensor that measured both surface stress and mass changes [74]. The ability to measure the mass and structural changes simultaneously provides useful information on molecular interactions, which is not readily measured by other techniques.

Drug–target binding interaction measurement has also been reported [75]. Antibiotic (vancomycin) binding to bacterial cell wall precursor analogues (mucopeptides) was measured using a cantilever array in blood serum and the response enabled quantification of vancomycin-sensitive and vancomycin-resistant mucopeptide analogues. The sensitivity of vancomycin binding to mucopeptides was 10 nM.

In summary, cantilever biosensors have displayed the capability for measuring various membrane protein–ligand interactions including conformation changes. With the development of high density cantilever arrays, rapid screening of drug candidates that bind favorably with membrane proteins immobilized on cantilever sensors appears feasible.

3.4 Protein–DNA interaction

DNA-binding proteins play a key role in many cellular processes, including transcriptional regulation, replication and repair. Transcription factors are vital for initiation or regulation of gene expression by binding to sequence-specific sites in the promoter region of genes. Their interactions with double-stranded DNA oligonucleotides using microcantilever array were recently reported [76]. Double-stranded DNA containing the binding sites for transcription factors (SP1 and NF-κB) were immobilized on the cantilever biosensor and then were exposed to the transcription factors. Responses were positive to target and showed the feasibility of detecting binding of SP1 at 80 nM and NF-κB at 100 nM.

There is a growing trend of using aptamers (short DNA or RNA sequences) as a recognition element due to their robust properties and ease of production. To date, very few studies have been reported using them in conjunction with a cantilever biosensor. We include one example reported to date as they could potentially reduce cost of analysis. Interaction of DNA aptamer with *Taq* DNA polymerase was reported by Savran *et al.* [77]. The specificity of the aptamer–protein binding was investigated by exposing the sensor to two protein solutions: thrombin and *Taq* DNA polymerase, where only the *Taq* DNA polymerase sample showed a deflection response. The sensor showed approximately picomolar sensitivity.

4. Biomarker detection and mining

Biomarker detection at very low concentration is important for early stage disease diagnosis. Detection of biomarkers and gene expression directly and rapidly in body fluid samples would significantly decrease healthcare cost and possibly provide new targets for drug development. Detection of biomarkers relies largely on the protein–antibody and glycan–antibody interactions because most of the important biomarkers are proteins or glycoproteins. Because some biomarker concentration thresholds are below the detection limit of current diagnostic technologies, the development of an ultrasensitive method for biomarkers is vital for early diagnosis. Here, we describe biomarkers that have been measured using cantilever sensors and cantilever arrays.

4.1 Cancer biomarkers

Specific proteins are released from cells or organs into circulation when cancerous tumors develop, and the level of proteins in serum is used as an indicator of the tumor presence. Normal levels for tumor biomarkers are listed in **Table 1**. Normally, a combination of several biomarkers provides good identification of cancer and its stage of growth. A sensitive, selective, inexpensive, rapid and multi-target measurement system is needed for early diagnosis and monitoring of cancers for which biomarkers are known. Recently, cantilever biosensors were investigated intensely for detecting biomarkers due to their high sensitivity. Detection of cancer biomarkers, such as prostate specific antigen (PSA) [21,29,50,51,78-80], α-methylacyl-CoA racemase (AMACR) [30], CA125 [81], GST [67], carcinoembryonic antigen (CEA) [82], human epidermal growth factor receptor 2 (HER2 or ErbB-2) [83], Vimentin [84] and α-fetoprotein (AFP) [85,86] have been reported. Various cantilever designs used in biomarker detection experiments and limit of detection (LOD) achieved are summarized in **Table 2**. Comparing LOD achieved by the cantilever biosensors and the threshold values of the tumor biomarkers, we see that the sensitivities of the cantilever biosensors are better or at the clinical threshold level, suggesting that they have the sensitivity required for clinical application.

One of the most investigated biomarkers by cantilever biosensors is the prostate cancer biomarker, PSA, which has a clinical threshold of 4 ng/ml. In 2001, Wu *et al.* used a

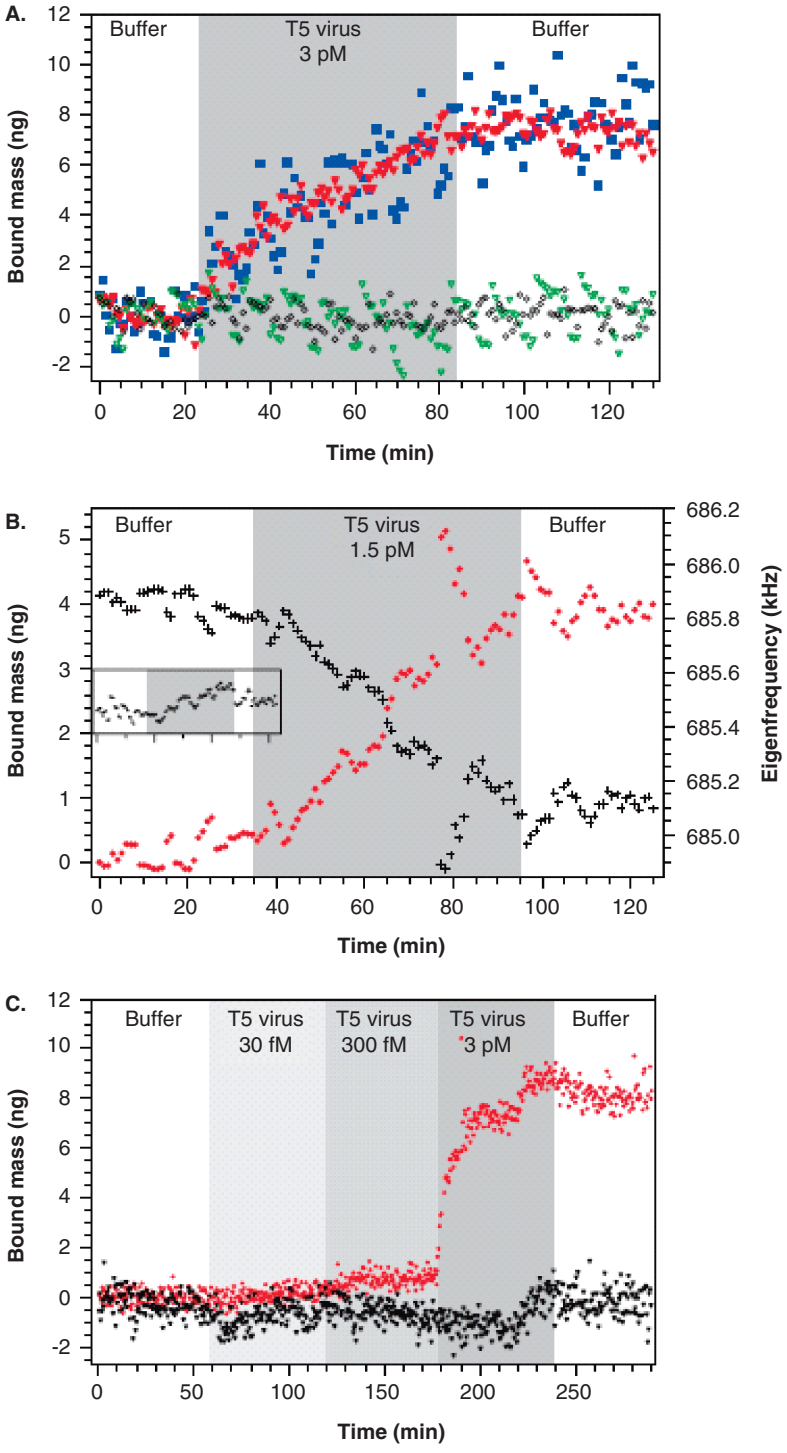

Figure 4. T5 phages interaction with FhuA-functionalized cantilevers. The solutions were injected at a rate of 10 μl min^{-1}. **A.** T5 phage solution (3 pM) was injected for 1 h. The uptake mass was measured simultaneously on four different cantilevers in one single array: two positive controls (FhuA-coated cantilevers, blue squares and red triangles) and two negative controls (casein-coated cantilevers, black open circles and green open triangles). Upon the second injection of buffer (~ 80 min), the mass uptake remains constant, demonstrating the irreversible binding of T5 phages on FhuA. **B.** The eigenfrequency (14^{th} eigenmode) of an FhuA-functionalized cantilever was measured when 1.5 pM T5 phages (black crosses) were injected. Also shown is the corresponding adsorbed mass (red circles). In the inset is shown the response (adsorbed mass) of a negative control cantilever (casein-coated, open circles). **C.** A concentration series experiment, showing the typical sensitivity of the instrument. Red circles and black open triangles indicate the response of the positive (FhuA-coated) and negative (casein-coated) control cantilevers, respectively. At each concentration, a total of 600 μl (containing the T5 phages) was injected.

Table 1. Normal levels of basic tumor markers.

Tumor marker	Threshold	Tumor marker	Threshold
NSE	12.5 mg/l	CEA	3 μg/l
PSA	4 μg/l	CA125	35 kU/l
GST	3.2 U/l	CA153	25 kU/l
ALP	~ 0	CA27-29	36.4 kU/l
CT	100 ng/l	CA549	11 kU/l
SCCA	1.5 μg/l	CA19-9	37 kU/l
Ferritin	250 μg/l	CA50	14 to ~ 20 kU/l
hCG	5.0 IU/l	CA242	20 kU/l
AFP	10 μg/l	CA72-4	6 kU/l

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bending-mode microcantilever to quantitatively detect PSA for the first time [21]. The microcantilever was shown to be able to detect from 0.2 ng/ml to 60 μg/ml of PSA in a background containing HSA and human plasminogen. In another study, Hwang *et al.* used a resonant-mode microcantilever for *in situ* detection and quantification of PSA with a 1 ng/ml detection limit [78]. In a follow-up study, the same group reported detecting PSA directly in undiluted human serum at a concentration range of 0.1 – 100 ng/ml [29]. Maraldo *et al.* used a PEMC sensor for detecting another prostate cancer biomarker, AMACR, at a detection limit of 10 fg/ml directly in patient urine without a sample preparation step [30]. The above results suggest that cantilever biosensors are extremely attractive in a clinical setting because they are not only rapid (< 30 min) but also do not require labeled reagents, or any preparation of body fluid samples.

4.2 Other biomarkers

Besides cancer biomarkers, a number of clinical biomarkers are often monitored as health indicators. There is a great need for rapid detection of specific protein levels for diagnosis of acute diseases such as acute myocardial infarction (AMI), where temporary and significant increase of a few protein indicators in blood is still the most reliable measurement. Three major markers of myocardial infarction are clinically used: creatin kinase and the troponins, which are highly specific but with a certain time delay, and myoglobin that increase rapidly with less specificity after AMI [87]. Cantilever biosensors have been used for rapid detection of these three markers successfully. The feasibility of detecting two biomarkers simultaneously has also been demonstrated [22].

Normal serum myoglobin level ranges from 0 to 85 ng/ml and increases immediately following AMI. Grogan *et al.* used anti-myoglobin coated bending-mode cantilevers and found LOD as 85 ng/ml [53]. On the other hand, a self-actuating piezoelectric microcantilever exhibited superior LOD (1 ng/ml) in resonant mode [88,89]. Arntz *et al.* developed a cantilever array for simultaneous detection of myoglobin and creatin kinase [22]. Their results showed detection feasibility at 20 μg/ml for both creatin kinase and myoglobin in an artificial background of BSA. Their report did not determine LOD. Antibody-immobilized cantilever array holds the promise for simultaneous detection of a panel of biomarkers in a single and short time step.

C-reactive protein (CRP) is a widely used biomarker for inflammation and cardiovascular disease diagnosis. Wee *et al.* designed a piezoresistive self-sensing microcantilever for detecting CRP in buffer and found the LOD as 100 ng/ml [80].

Protein aggregation and the resulting amyloid growth is an effective indicator for Alzheimer's and Parkinson's diseases. Detection of protein aggregation and amyloid growth has been shown possible on a bending-mode cantilever biosensor [90]. Human growth hormone interaction with its antibody was also investigated using a bending-mode polymeric microcantilever [91].

Microcantilever sensors have been used to identify specific mRNA biomarker candidates in total cellular RNA [92]. Differential gene expression of the gene 1-8U was observed on the microcantilever array in a genomic background. The feasibility of measuring gene expression in body fluids without an amplification step is attractive in a clinical setting where such measurement is essential for differential diagnosis. A recent study showed the detection feasibility of protein and DNA on the same cantilever array [93]. This approach may lead to the development of a combinated DNA/protein sensor, in which analysis of transcription and translation could be carried out simultaneously.

5. Conclusions

Cantilever biosensors have been successfully demonstrated for measuring DNA hybridization, protein–protein interaction and antibody–antigen interaction at various concentrations and in various complex backgrounds. For detection purpose, a number of biomarkers, bacteria and proteins have been assayed with cantilever biosensors. The ability of cantilever biosensors to measure biomolecular interactions allows it to be used for affinity-based measurement. They have also been shown to be able to discern conformation changes of proteins. With the development of cantilever arrays, rapid detection of multiple analytes on the same platform appears feasible. The scalability of the cantilever biosensors renders them attractive in high-throughput formats.

6. Expert opinion

Commercial cantilever biosensors are available from only a few vendors (Concentris www.concentris.ch, Cantion www.cantion.com and Veeco www.veeco.com), even though the cantilever sensors have been extensively investigated in research laboratories over the past 15 years. For any biosensor to be a drug discovery platform, high-throughput capability is an absolute must. Most of the cantilever arrays investigated to date contained 8 – 30 cantilevers on a single chip [20,94,95].

Table 2. Biomarker detection using cantilever biosensors.

Biomarkers	Platform	Operation mode	Measurement environment	LOD	Ref.
PSA	Microcantilever	Bending	Buffer w/HSA	0.2 ng/ml	[21]
	PZT microcantilever	Resonant	Air	10 pg/ml	[79]
	PZT microcantilever	Resonant	PBS	1 ng/ml	[78]
	Microcantilever	Resonant	Air	0.1 ng/ml	[29]
	Piezoresistive microcantilever	Bending	PBS	10 ng/ml	[80]
	Microcantilever	Resonant	Air	1 pg/ml	[50]
	Microcantilever array	Bending	PBS	1 ng/ml	[51]
AMACR	Piezoelectric excited millimeter cantilever	Resonant	Urine	10 fg/ml	[30]
AFP	Microcantilever	Resonant	Air	60 ng/ml	[85]
	Microcantilever	Resonant	Air	2 ng/ml	[86]
CEA	PZT microcantilever	Resonant	Air	5 ng/ml	[82]
Vimentin	Cantilever array	Bending	Buffer	90 ng/ml	[84]
HER2	Piezoelectric microcantilever	Resonant	PBS w/BSA	5 ng/ml	[83]
GST	Piezoresistive cantilever array	Bending	PBS	1 ng/μl	[67]
CRP	PZT microcantilever	Resonant	Air	N/A	[52]
	Piezoresistive microcantilever	Bending	PBS	100 ng/ml	[80]
	Microcantilever	Bending	PBS	1 μg/ml	[105]
Myoglobin	Cantilever array	Bending	HEPES buffer	20 μg/ml	[22]
	Microcantilever	Bending	PBS	85 ng/ml	[53]
	PZT microcantilever	Resonant	Air	1 ng/ml	[88]
	PZT microcantilever	Resonant	Air	1 ng/ml	[89]
Creatine kinase	Cantilever array	Bending	HEPES buffer	20 μg/ml	[22]
Troponin C	Microcantilever beam	Bending	Buffer	1 mg/ml	[106]
Amyloid	Microcantilever/array	Bending	10 mM HCl	N/A	[90]
hGH	Microcantilever	Bending	Buffer	N/A	[91]

BSA: Bovine serum albumin; HSA: Human serum albumin; LOD: Limit of detection; N/A: Not available; PBS: Phosphate buffered saline; PZT: Lead zirconate titanate ($Pb[Zr_xTi_{1-x}]O_3$ $0 < x < 1$).

IBM has developed a fully-integrated microcantilever array of 1024 tips on a single chip for data storage, which could potentially be used for biological assays [96]. Several research institutes and organizations have formed a consortium called Alliance for Nanosystems VLSI for the fabrication of nanoelectromechanical system (NEMS) wafers that contain over 2 million NEMS on an 8" wafer. Such highly dense designs will significantly reduce production cost when commercialized (www.nanovlsi.org). However, closely adjacent cantilevers may interact with each other, causing signal crosstalk leading to difficulty in interpreting sensor response. Majumdar's group at Berkeley has developed a set of multiplexed bending-mode cantilever array consisting of 320 – 960 bending-mode cantilevers on a single chip (**Figure 5**) [51,97,98]. These cantilever arrays have already shown the feasibility of detecting DNA hybridization and protein interactions using optical deflection method. With the high density cantilever chips and the advantage of label-free assay, rapid screening of drug candidates becomes highly feasible. Successful production of cantilever chips could eliminate the disadvantages of the current high-throughput assay cost and data reliability. Because cantilever array can be manufactured using existing semiconductor production processes, their unit production cost is likely to be low. With the added benefit of label-free measurement and automation, the cantilever array approach is likely to be well received by pharmaceutical research community.

The high sensitivity of the cantilever biosensors is the most attractive feature for drug discovery. The ability to rapidly detect at a very low concentration opens the door to examining drug chemical structures that bind at a very low concentration. Because the biosensors are sensitive, a basic chemical structure that has affinity at low concentration can be selected and further mutated for higher binding properties. This distinguishing feature is not part of the current drug discovery research because of the unavailability of ultra-sensitive methods. Similarly, biomarkers that are expressed at early stages of a disease could be measured by the cantilever biosensors. For example, analysis of circulating tumor cells which contain rich information on the status of cancer and are difficult to analyze accurately due to their low concentration could be measured using cantilever biosensors. The sensors can potentially be

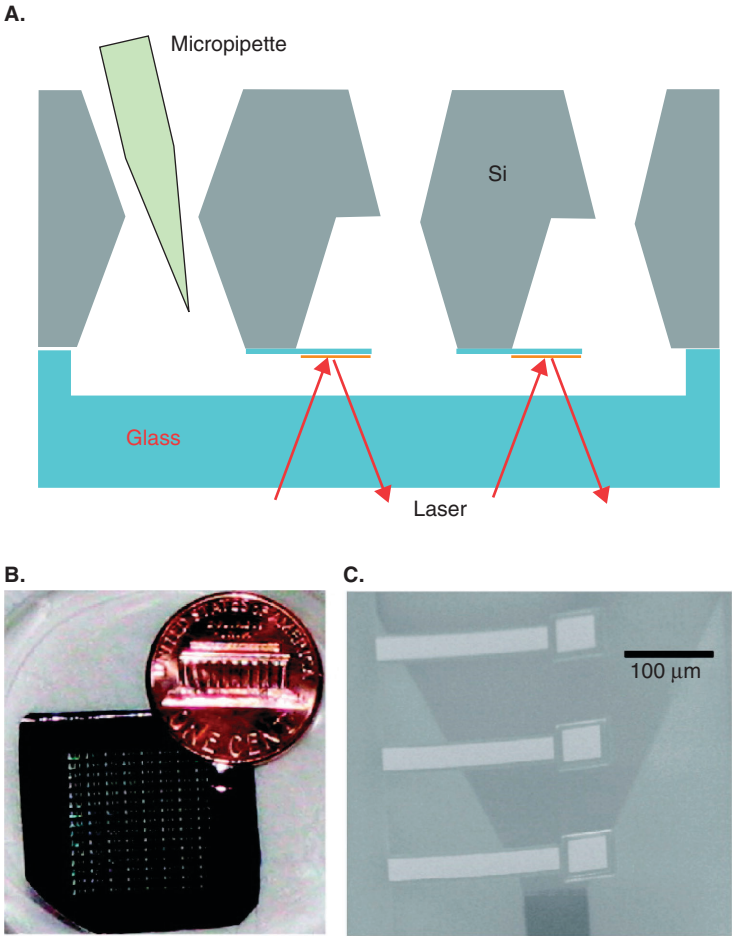

Figure 5. Microcantilever array chip. A. Schematic of a reaction well. One chip contained 80 – 120 reaction wells. Each well consisted of a microfluidic chamber containing 4 - 8 independent cantilevers. Laser light reflected off a cantilever end pad was used to monitor the deflection of cantilevers. **B.** A chip soaked in DI water. **C.** A scanning electron micrograph of three cantilevers in a reaction well.
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used in personalized medicine applications due to the low cost and ease of use.

In spite of the advantages noted above, two technical issues plague cantilever biosensors when used in liquids that contain extraneous biological entities. These are: obtaining uniform density of surface immobilized recognition molecules and false responses dues to nonspecific binding. Uniform density of recognition molecules on the sensor is required for obtaining reproducible binding response. Although several methods of immobilization are available, very few publications have addressed robustness of sensor performance. Because physical dimensions of the sensor can be tightly controlled in a manufacturing process, we attribute the variation in response to surface chemistry. Surface immobilization techniques that ensure uniform surface density of recognition molecules and those which do not alter their binding activity are yet to be developed for cantilever sensor arrays. To address nonspecific binding, several reports have emerged in the last few years that indicate increasing the oscillation magnitude of shear resonators (QCM and SAW) causes reduced binding of extraneous materials [99,100]. For example, by increasing the input power to a QCM from 3.5 to 14 W, the authors showed removal of nonspecific adsorption on sensor surface [99]. By further increasing the excitation voltage to 10 V, several studies found that ligand–receptor bond can be disrupted [101-103]. Flexural oscillation of a cantilever is normal to sensor surface while that of a QCM is in shear mode and thus parallel to sensor surface. Consequently, disruption of binding is expected to be achieved at a much lower excitation voltage with a cantilever sensor. The authors have experimented with PEMC sensors at various excitation voltages and the results obtained thus far suggest that analyte binding affinity can be measured in a single experiment by modulating excitation intensity. If this result is confirmed through extensive experimentations, it will be an attractive feature

in reducing the time required for assessing the binding properties of drug candidates. Using resonant-mode cantilever biosensors, one could excite the cantilever at the appropriate voltage level suitable for reduction of nonspecific binding or disrupting ligand–receptor bond. Binding energies of various drug candidates to receptors differ from one structure to another. This difference could be measured by sensor response to target drug candidates at various excitation voltages. This approach of measurement when fully developed may achieve the goal of screening drug candidates based on the strength of binding. Binding energy assay using cantilever biosensors could provide a more rapid and direct measure of binding affinity. The assay design developed in an array format would allow measurement of 100 – 1000 interactions simultaneously. These attractive and time-saving characteristics could potentially accelerate the drug discovery process.

Declaration of interest

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