

Development of a nanomechanical biosensor for analysis of endocrine disrupting chemicals†

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A nanomechanical transducer is developed to detect and screen endocrine disrupting chemicals (EDCs) combining fluidic sample injection and delivery with bioreceptor protein functionalized microcantilevers (MCs). The adverse effects of EDCs on the endocrine system of humans, livestock, and wildlife provides strong motivation for advances in analytical detection and monitoring techniques. The combination of protein receptors, which include estrogen receptor alpha (ER- α) and estrogen receptor beta (ER- β), as well as monoclonal antibodies (Ab), with MC systems employing modified nanostructured surfaces provides for excellent nanomechanical response sensitivity and the inherent selectivity of biospecific receptor–EDC interactions. The observed ranking of binding interaction of the tested EDCs with ER- β is diethylstilbestrol (DES) > 17- β -estradiol > 17- α -estradiol > 2-OH-estrone > bisphenol A > *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE) with measurements exhibiting intra-day RSDs of about 3%. A comparison of responses of three EDCs, which include 17- β -estradiol, 17- α -estradiol, and 2-OH-estrone, with ER- β and ER- α illustrates which estrogen receptor subtype provides the greatest sensitivity. Antibodies specific to a particular EDC can also be used for analyte specific screening. Calibration plots for a MC functionalized with anti-17- β -estradiol Ab show responses in the range of 1×10^{-11} through 1×10^{-7} M for 17- β -estradiol with a linear portion extending over two orders of magnitude in concentration.

Introduction

EDCs can adversely affect the health of human, domestic, and wildlife species by altering or inhibiting the function of the endocrine system.¹ Due to the extremely wide range of biological processes EDCs can influence, often impairing, it is crucial to screen and detect for them. EDCs include a wide range of naturally occurring and synthetic chemicals. These chemicals and/or their byproducts include but are not limited to pesticides, plasticizers, detergents, pharmaceuticals, and biological compounds excreted by animals and plants.^{2,3} Their interaction with hormone receptors, like estrogen receptors, often disrupt the normal function of the receptor causing chronic, debilitating health problems and disease.^{1,4,5} These contaminants cause undesirable effects to the endocrine system by mimicking or inhibiting a natural hormone.¹ The Endocrine Disruption Screening Program of the Environmental Protection Agency (EPA) has mandated the

screening and testing of chemicals to identify potential EDCs and their toxicity, then determine and manage the risk associated with the compound.¹ It is crucial to identify and manage potential EDCs in their environments, so appropriate action can be taken to lessen or eliminate their effects.

The screening of potential EDC candidates and the monitoring of known ones, as well as mechanistic studies of endocrine processes, requires modern sophisticated analysis methods and innovative, integrated instrumentation. Traditionally, the endpoints used to determine these chemicals and their effects involve relatively complicated bioassays (competitive binding assays, cell growth assays, and cell-based reporter assays) that are time consuming to perform and include the use of complex biological systems.^{4–6} More recently, simpler, non-label sensing methods to monitor protein receptor–EDC interactions have been reported that employ surface plasmon resonance and quartz crystal microbalance techniques.^{7,8} Relevant to this report, biosensing applications have benefited in recent years from the attributes of modern microelectromechanical systems (BioMEMS).⁹ Prominent among MEMS approaches are nanomechanical methods based on microcantilever (MC) transducers. The high sensitivity and widespread availability of inexpensive MCs has generated intense interest in their use as chemical^{10–13} and biological sensors.^{14–19} Additionally, MCs can be used with on-chip circuitry and in microcantilever arrays (MCAs) for high throughput, simultaneous differential assays with a very small sensor footprint that potentially can be employed in the field, advantages not fully realized with more traditional sensors.

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† Electronic supplementary information (ESI) available: Enhanced response of the dealloyed nanostructured surface relative to smooth MC surfaces, as well as with increasing thickness of the dealloyed layer (Fig. SI-1 and SI-2). The reversible response characteristics of the nanostructured surface are also demonstrated; MC deflections for an IgG protein-anti IgG antibody system, demonstrating that proper orientation of bioreceptor proteins on the MC surface does not always show improvements in nanomechanical response (Table SI-1). See DOI: 10.1039/b704723a

A MC suitable for biosensing is modified on one side with a suitable receptor phase that has some degree of affinity for the analyte. Specific interactions of the target analytes with that phase cause an apparent surface stress and nanomechanical bending of the MC. The bending may be conveniently monitored using the beam bending technique commonly used in atomic force microscopy. The static bending (tip deflection, z_{\max}) of the MC varies in selectivity and sensitivity due to preferential binding of analyte molecules on the functionalized, active MC surface and is governed by Stoney's equation²⁰

$$z_{\max} = 3l^2(1 - \nu)\Delta\sigma/Et^2 \quad (1)$$

where ν and E are, respectively, the Poisson ratio and Young's modulus for the cantilever, t is the thickness of the MC, l is the cantilever effective length, and $\Delta\sigma$ is analyte-induced differential surface stress ($\Delta\sigma_{\text{active side}} - \Delta\sigma_{\text{passive side}}$).

We demonstrate that detection and screening for EDCs can be accomplished with bioreceptor functionalized MCs. These sensors provide real-time measurements of surface stress changes in the low-to-sub-nanomolar range.¹⁵ By exploiting the protein receptor–EDC interaction, as well as antibody–antigen/hapten reactions, we are able to screen for potential EDCs and target specific compounds quickly and without extensive, time-consuming labeling techniques.^{2,4,6} Immobilization of EDC receptor proteins on MCs with a non-specific orientation glutaraldehyde protocol does not appear to appreciably denature the protein or otherwise inhibit interactions with known EDCs and, moreover, allows for sufficient surface stress for sensitive detection. The sensitivity and reversibility afforded by MCs with nanostructured active surfaces, as well as the biological interaction, allows impressive limits of detection (LOD) in this work down to $\sim 1 \times 10^{-11}$ M, though the thickness of the nanostructured MC surfaces are not optimized in this work (see figures in the ESI†). The EDCs studied herein includes various steroids, a plastic component, a synthetic estrogen, and the heavy metal cadmium. Versatile screening of EDCs is accomplished by estrogen receptor- α (ER- α) and estrogen receptor- β (ER- β) protein receptor-based MC systems, which can respond to a variety of EDCs. Conversely, it is demonstrated that a specific estrogenic compound (17- β -estradiol) can be targeted by antibody mediated nanomechanics. To our knowledge, this is the first time estrogen responding receptors have been immobilized on a MC surface for nanomechanical-based sensing.

Experimental

Reagents

Experiments were performed using commercially available silicon arrays of MCs having dimensions 400 μm length, 100 μm width, and approximately 1 μm thickness (Mikro Masch Co., Sunnyvale, CA). Chromium, gold, and silver metals deposited on the MCs were obtained from Kurt J. Lesker, Gatewest, and Alfa Aesar Co., respectively, at 99.9% purity. 2-Aminoethanethiolhydrochloride (AET), glutaraldehyde (GA), the salts employed for the preparation of buffer solutions, cadmium chloride, and all other reagents were

purchased from Sigma-Aldrich Chemical Co (St. Louis, MO) or Fisher at highest available purity and used as received. The EDCs, diethylstilbestrol (DES), 17- β -estradiol (17- β -ES or beta-ES or β -ES), 17- α -estradiol (17- α -ES or alfa-ES or α -ES), bisphenol A (BisA), androstenedione, *p,p'*-DDE and protein bovine serum albumin (BSA) were also obtained from Sigma-Aldrich. In addition, 2-OH-estrone was obtained from Steraloids Inc. (Wilton, NH, USA). Monoclonal anti-17- β -estradiol antibody (17- β -ES Ab) (mouse generated to a BSA conjugate of the hapten) was purchased from Biogenesis, Inc. (Kingston, NH, USA). Human recombinant ER- α and ER- β were purchased from Invitrogen Corp. (Carlsbad, CA, USA). Water used to prepare solutions was obtained from a Branstead E-pure water filtration system.

Cantilever modification

The process of creating nanostructured surfaces on MCs is described in detail elsewhere.²¹ The cantilevers were first cleaned in a piranha bath (75% H_2SO_4 , 25% H_2O_2) for 30 min, followed by thorough rinsing in deionized water [Caution: piranha solution reacts violently with organics]. The MCs were then placed into a physical vapor deposition (PVD) chamber (Cooke Vacuum Products, Model CVE 301, South Norwalk, CT) to be coated on one side with the appropriate metallic films using thermal deposition. To create a nanostructured MC, a thin film (~ 5 nm) of chromium was applied to the surface to act as an adhesion layer followed by a thin film of gold (~ 15 nm). Next, a film consisting of gold and silver was co-deposited. Subsequently, the silver was chemically removed *via* oxidation from the film ("dealloying") using an aqueous solution of 5 mg mL^{-1} HAuCl_4 leaving a gold surface with nanosized, colloid-like features. The thickness of the dealloyed gold layer was ~ 50 nm in these studies.

In these studies, nanostructured MCs were chemically modified by immersion in 1 mM aqueous solution of AET (16 h) producing a self-assembled monolayer of AET on the cantilever surface. Following thorough rinsing in deionized water, the amino groups were derivatized with the cross linker by immersing the cantilever in a 2% (w/v) solution of GA in water for 3 h.²² The chemically treated cantilever was allowed to soak in a large volume of water for a few minutes to remove any nonspecifically bound GA on the nanostructured and silicon sides of the cantilevers. Subsequently, immobilization of both the estrogen receptor proteins (α and β) and 17- β -ES Ab was achieved in random orientation by dipping the functionalized cantilevers into 100 mg L^{-1} solutions of proteins or antibody in phosphate buffered saline (PBS, 10 mM, pH = 7) for 4 h. During functionalization with ER- α and ER- β , the MCs dipped into the protein samples were kept at 4 $^\circ\text{C}$ to maintain the ER activity. Both estrogen receptor proteins (α and β) and antibodies were separately immobilized on the functionalized surfaces of different cantilevers from separate arrays. After washing with PBS, the functionalized microcantilevers could be stored in PBS at 4 $^\circ\text{C}$ until used. Although we used an array of MC, in this study we chemically treated all the cantilevers the same and simply recorded the response of a single randomly chosen MC within an array.

Instrumentation

The MC deflection measurements were carried out using the optical beam-deflection technique as depicted in Fig. 1A.

The apparatus included a 5 mW diode laser (Coherent Laser Corp., Auburn, CA) operating at 632 nm, a spatial filtering and focusing system, and an in-house built position sensitive optical detector. The output of the detector was displayed and recorded using a SRS 850 DSP lock-in amplifier as a multichannel digital recorder (Stanford Research Systems, Sunnyvale, CA). The signal output is recorded as volts (approximately 1 nm z_{\max} per mV output). Data was collected at 1 Hz and then a moving averaging algorithm covering 180 data points was used to generate the figures presented herein (*Y*-axis in response plots represents that data). This smoothing did not alter the shape of the true response curves.¹¹

The cantilever system was mounted inside a $\sim 5 \mu\text{L}$ volume flow cell made of a 3 cm diameter by 2 cm long Delrin rod that was machined with 1/16 inch diameter input and output holes that meet at 1 cm distance apart at the face of the rod. Narrow bore tubing of 1/16 inch od was slid into the holes up to the rod face and secured with fittings. A semi transparent silicone gasket, which is slightly thicker than the MC chip ($\sim 500 \mu\text{m}$ versus $400 \mu\text{m}$), was cut with a scalpel tool to form a $\sim 250 \mu\text{m}$ wide flow channel between the input/output holes and to tightly hold the MC chip (Fig. 1B). The silicone gasket is sandwiched between the Delrin rod face and a thin quartz window. A Watec CCD camera (Edmund Industrial Optics, Barrington, NJ) was used to image the MC chip in the flow cell. The camera facilitated aligning the focused laser beam to reflect off the cantilever tip. Analyte solutions were delivered to the flow cell *via* a system of vessels connected to three-way valves allowing for switching between different solutions. The gravity-driven flow was generally adjusted to $30 \mu\text{L min}^{-1}$ by adjusting vessel height.

Most of the EDCs are sparingly soluble in water. Thus, 1×10^{-2} M stock solutions of all EDCs were prepared in pure methanol and then diluted with PBS (10 mM phosphate buffer + 10 mM NaNO_3 , pH = 7.0) to make the desired concentration of each EDC [Caution: because of their potential harmful effects, care must be taken in the handling and disposing of

EDC solutions]. PBS was also used as a background solution. MCs mounted in the flow cell were initially allowed to equilibrate in PBS until the signal was stable. For our purposes, tensile and compressive responses involve contraction and expansion of the active MC surface, respectively.

Results and discussion

Our work addresses three analysis scenarios. Since hundreds to thousands of potential EDC candidates have been targeted, and the effects of these candidates may be seen in various mixed combinations, there is a pressing need for high throughput EDC screening methods. Because a large number of EDCs exert their effects through estrogen receptor (ER) proteins,²³ our studies have focused on the development of MC systems using these proteins as bioreceptor phases to screen for estrogenic compounds. A second scenario involves the targeted detection of known EDCs in environmental samples such as waste treatment streams, feed stocks, *etc.* wherein target specific bioreceptor phases can be employed. We use cantilevers modified with anti-17- β -ES antibody to demonstrate the potential utility of MC sensing in this situation. Finally, in comparison to existing methods, we are developing MC systems that may prove to be a quicker, simpler, and less expensive means to detect EDC actions in support of fundamental endocrinology studies.

Response characteristics of nanostructured (dealloyed) MCs

The promise of our nanomechanical approaches will depend largely on whether adequate levels of sensitivity are reached for EDCs, since they are known to show effects at extremely low concentrations. We have achieved substantial improvements in sensitivity by nanostructuring the active surfaces of MCs by the described dealloying process. In many cases the response enhancement has surpassed the increase in surface area of the active surface.^{10,21,24} The enhancement in bioaffinity response with nanostructuring is discussed and illustrated in the ESI (see Fig. SI-1 and SI-2).†

Conformational changes of MC surface immobilized proteins after binding with analytes may give rise to relatively large cantilever responses.^{14,15} Since conformational changes in the ligand binding region of ER proteins give rise to changes in the DNA binding region of the proteins, this system is a good candidate for MC nanomechanical sensing. However, there can be concern that chemical attachment to the MC surface will make binding sites inaccessible or distort the sites such that the natural affinity for ligands will be substantially altered. The results presented below provide strong evidence that at least an appreciable fraction of the immobilized bioreceptors remain active to ligand binding. It is important to note that unlike spectroscopic or simple mass responding sensors, the MC sensor requires transfer of the energy of ligand binding into surface stress. Thus, in the case of the ER proteins, immobilization at the DNA binding sites which change configuration in response to conformation changes in the ligand binding region, may be desirable. It has been observed that proper orientation of the bioreceptor proteins on the MC surface does not always yield improvements in

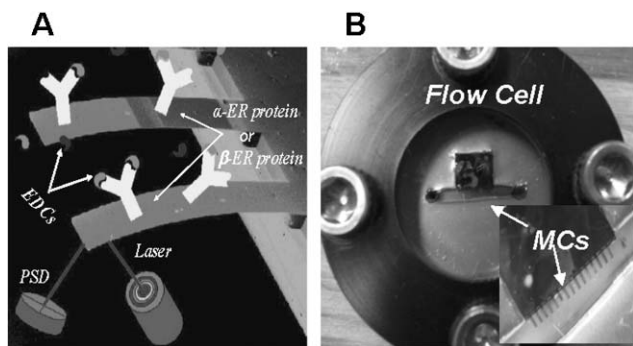


Fig. 1 (A) Micrograph of silicon cantilevers ($100 \mu\text{m} \times 400 \mu\text{m} \times \sim 1 \mu\text{m}$ thick) (a linear 16 cantilever array was used in this work). Schematic depiction of the optical detection system and surface-immobilized receptor proteins and antibodies (Y symbols) are included in the figure. (B) Photograph of the delrin flow cell showing the silicone gasket that defines a $250 \mu\text{m}$ flow channel and holds the MC chip.

response (see Table SI-1 in the ESI†), presumably because of the stress induction response requirement.

Screening and selective detection of EDCs using estrogen receptor protein modified MCs

Fig. 2A shows the comparison of nanomechanical responses of an ER- β functionalized MC on exposure to 1×10^{-7} M solutions of six different potential EDCs in PBS, illustrating the selective interaction of DES and estradiols over the other EDCs. The relatively slow response kinetics is consistent with prior protein bioreceptor MC work^{14,15} and indicates that the small EDC molecule causes conformational changes in the ER- β (see above) which translates into a large apparent surface stress on the cantilever. The very high binding affinity of ER- β protein for DES followed by 17- β -ES and 17- α -ES has been observed by other researchers.^{4,25} Also, we have observed the similar trend in the value of response magnitude for both 1×10^{-7} M and 1×10^{-9} M solutions of these three EDCs. The binding affinity for BisA is much lower than that of ES (both α and β) and the very low binding affinity for *p,p'*-DDE and androstenedione are in agreement with previous studies.^{4,7,25} The inset demonstrated the response behavior of ER- β functionalized MC as a function of time for 1×10^{-7} M of DES and BisA. Also, it is demonstrated in the inset that ER- β functionalized MC shows no response on exposure to 0.01% MeOH prepared in the background PBS, validating that all the observed responses are authentic (not solvent artifacts) and due to the interactions between EDCs and protein receptors. An exposure time of 10 min produced a compressive surface stress (expansion of the active surface) for both the EDCs that reverse when the analyte solution was replaced by background buffer (PBS) solution. The relative magnitude of responses are

consistent with prior reports.^{7,8} In our previous studies, the reversible compressive response is also observed for other bio-receptor functionalized dealloyed surfaces whereas similarly functionalized smooth gold MC shows an irreversible compressive response on exposure to the same concentration of analytes^{14,15} (also, see related discussion and Fig. SI-1 in the ESI†).

Fig. 2B compares the response of specific protein (ER- β) functionalized MC to nonspecific protein (BSA) functionalized MC (blank) on exposure to the same concentration (1×10^{-7} M) of 17- β -ES. A large compressive response was observed due to the binding of 17- β -ES with a MC modified with ER- β protein whereas no response was observed when the same analyte was exposed to the nonbinding protein (BSA) immobilized MC. The fact that our MC system's relative response magnitudes are similar to prior works, and does not show a nonspecific blank response, is critically important and indicates the surface immobilization of the ER- β does not substantially alter its EDC ligand binding function and selectivity. However, it can not be assumed that the surface immobilized receptors will retain the same ligand binding affinity constants as observed in free form.

Differential ligand binding with ER- α & - β proteins and cadmium binding to ER

Studies have shown that there are a number of functional similarities between human ER- α and ER- β , especially in the DNA binding domain.²⁶ However, there are also significant structural differences noted for human ER- α and ER- β , and some of the EDCs have differential binding affinity for human ER- α and ER- β .^{4,7,25} Fig. 3A compares the nanomechanical responses of ER- α against ER- β functionalized MCs on exposure to 1×10^{-9} M of three test EDCs, 2-OH estrone,

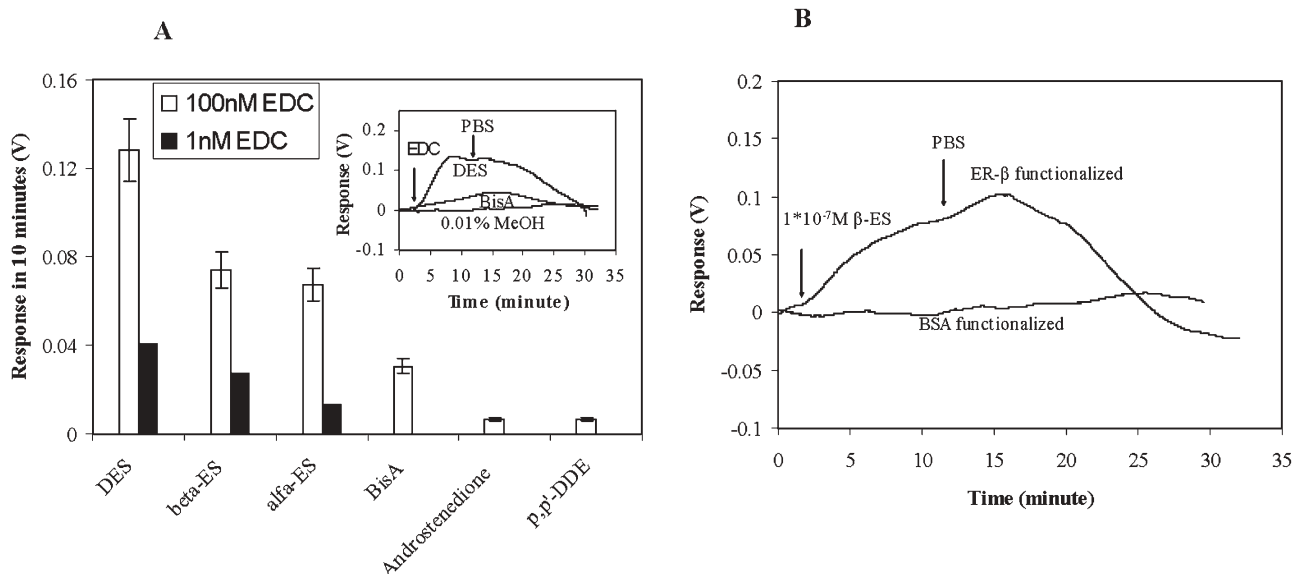


Fig. 2 (A) Comparison of nanomechanical responses of ER- β functionalized MC on exposure to 1×10^{-7} M and 1×10^{-9} M of several different potential EDCs in PBS illustrating selectivity. The error bar indicates the standard deviation (with CV = 10%) for three replicate measurements with three different MC arrays functionalized with ER- β at different times using 1×10^{-7} M of different EDCs. The inset in (A) provides representative time traces of ER- β functionalized MC on exposure to 1×10^{-7} M of two of the EDC analytes, DES and BisA, as well as 0.01% methanol (MeOH) blank prepared in PBS (note: arrows denote points of EDC solution and PBS background in flow cell). (B) Comparison of ER- β to BSA (blank) functionalized MC on exposure to 1×10^{-7} M of 17- β -ES in PBS.

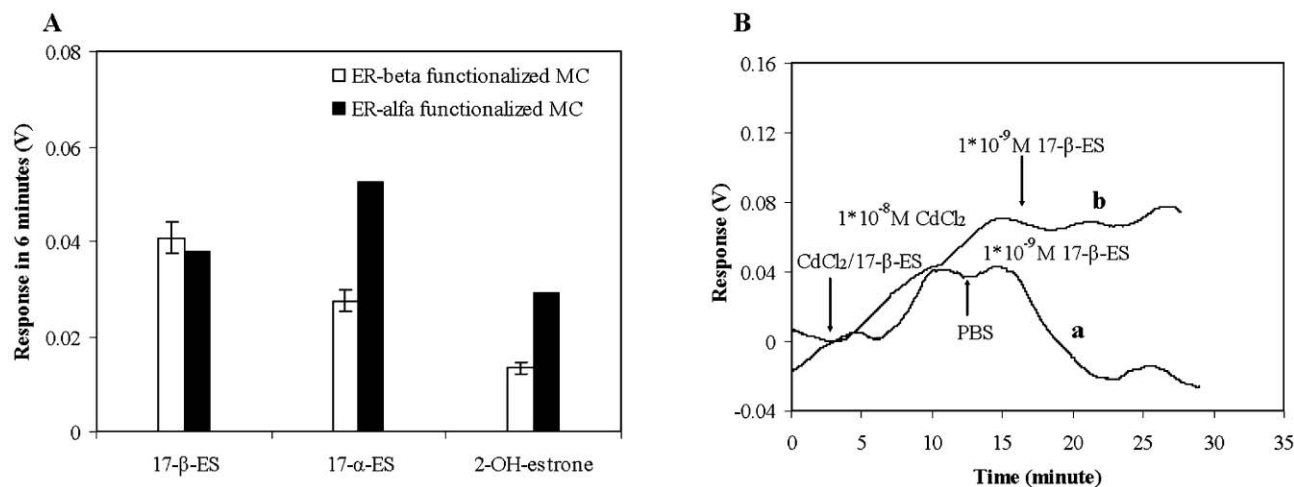


Fig. 3 (A) Comparison of nanomechanical responses of ER- α and ER- β functionalized MCs on exposure to 1×10^{-9} M of 2-OH-estrone, 17- α -ES, and 17- β -ES in PBS. The error bar indicates the standard deviation (with CV = 8%) for three replicate measurements with three different MC arrays functionalized with ER- β at different times using 1×10^{-9} M of different EDCs. (B) Response of ER- β functionalized MC to 1×10^{-9} M of 17- β -ES; (a) absence and (b) presence of 1×10^{-8} M of CdCl₂.

17- α -ES, and 17- β -ES. The results in Fig. 3A show that 17- β -ES produce comparable responses with both ER proteins indicating that it binds with equal relative affinity to both ER subtypes, which was also observed before.^{4,25} The other two EDCs, 2-OH estrone and 17- α -ES preferentially bind with ER- α producing 2-3 fold greater responses than with ER- β . Greater binding for these EDCs with ER- α than ER- β was also noted by others in a prior report, although by a greater factor than in our work.⁴ The optical systems used in our prior reports on MC arrays, that were differentially coated with non-bioaffinity receptor phases and optically probed, should transfer well to arrays employing different protein receptor phases for simultaneous multi-bioaffinity measurements based on MCA nanomechanics.²⁷

Previous studies have shown that heavy metal cadmium activates ER- α through an interaction with the hormone binding domain of the receptor, thereby inhibiting estradiol binding to the receptor.⁵ Mechanistic studies of hormone agonist or antagonist actions are vital for understanding and controlling the impacts of EDCs in the environment. To demonstrate the potential of the MC nanomechanical sensor in such studies, we sought to determine whether cadmium may also block estradiol binding to ER- β . The response was recorded for a ER- β functionalized MC on exposure to 1×10^{-9} M of 17- β -ES in absence and in presence of 1×10^{-8} M of CdCl₂ (Fig. 3B). Initially, in absence of cadmium ion, estradiol showed appreciable compressive response which is reversed upon flushing the cell with background buffer. Subsequently, the same MC upon exposure to 1×10^{-8} M of CdCl₂ for 14 min produced a large compressive response, most likely indicating the binding of cadmium ion with ER- β protein. Martin and coworkers have shown that the interaction of cadmium with ER- α appears to involve several amino acids present in the hormone-binding pocket of the receptor, suggesting that the metal may form a coordination complex with the hormone-binding domain.⁵ Also, it is known that cadmium binds to ER- α with an affinity similar to that of estradiol for the receptor. An injection of 1×10^{-9} M of

17- β -ES for 10 min in presence of CdCl₂ showed no additional compressive response (Fig. 3B), which provides evidence that the metal ion may also inhibit the binding of 17- β -ES to ER- β protein.

Characterization of the MC immunosensor for specific detection of 17- β -ES

High levels of specificity involving molecular recognition, *e.g.*, antibody-antigen/hapten interactions are generally considered desirable in analytical chemistry. However, inherent to this high level specificity can be a lack of versatility and reversibility. Herein, we have developed a MC-based immunosensor using a monoclonal antibody for 17- β -ES for selective and sensitive detection of 17- β -ES in presence of other nonspecific analytes. 17- β -ES has no immunogenicity due to its small molecular size, but antibodies generated to a BSA conjugate are commercially available and we have functionalized dealloyed MCs using this antibody. In our prior work, glutaraldehyde-based immobilization resulted in a cantilever resonance frequency decrease of approximately 30 Hz, indicating less than a femtomole of antibody was immobilized on the functionalized surface of a single MC.¹⁴

Fig. 4 shows the cantilever response as a function of time when exposed to 1×10^{-8} M of 17- β -ES in PBS. The specific interaction of the immobilized antibody with 17- β -ES resulted in a 60 mV compressive response (positive voltage signal), which is likely to occur in similar fashion to the endocrine receptor protein case; *i.e.* a combined effect of hydrogen bonding and hydrophobic interactions with the hapten cause rearrangement of the conformation of the large antibody biomolecule and an apparent surface stress. It is interesting to see that the response increases even after injection of background buffer which may involve the gradual conformational changes of the antibodies to achieve a more stable conformation or wash out issues with our flow cell. Also, it is interesting to observe that the nanomechanical response of the cantilever produced by the antibody-hapten interaction on the

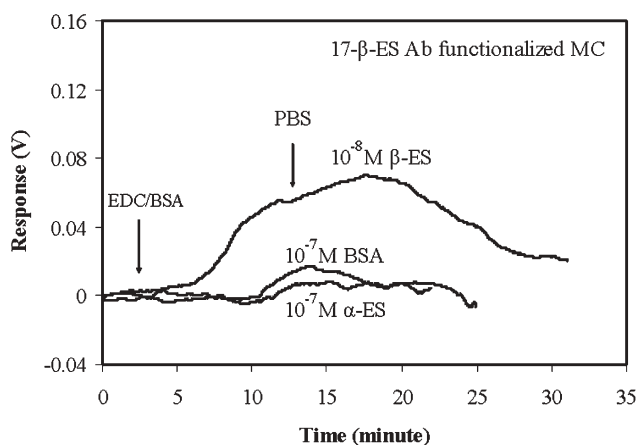


Fig. 4 Nanomechanical responses of an anti-17- β -ES antibody (Ab) functionalized MC to 1×10^{-8} M of 17- β -ES in comparison no responses to non-specific analytes 17- α -ES and BSA at higher concentration of 1×10^{-7} M.

nanostructured surface was easily reversed with the replacement of the 17- β -ES solution by the background buffer (PBS) solution despite the expected very large affinity constants (see “Response characteristics of nanostructured MCs” in the ESI†). In our previous studies,¹⁵ we have observed the similar behavior; *i.e.* antibody functionalized nanostructured surface of MCs are more easily regenerated than similarly functionalized smooth gold surface of MCs. Irreversible response of the smooth gold surface was also observed by other researchers for specific interactions of different antibody–antigen pairs.^{16,17,28} To check for specificity, the same MC was again exposed to one order of magnitude higher concentration (1×10^{-7} M) of BSA (Ab was generated to BSA-hapten conjugate) and 17- α -ES, producing negligible deflection for each potential interferent.

Calibration and reproducibility

Fig. 5A demonstrates nanomechanical response profiles of ER- β protein functionalized MC to different concentrations of

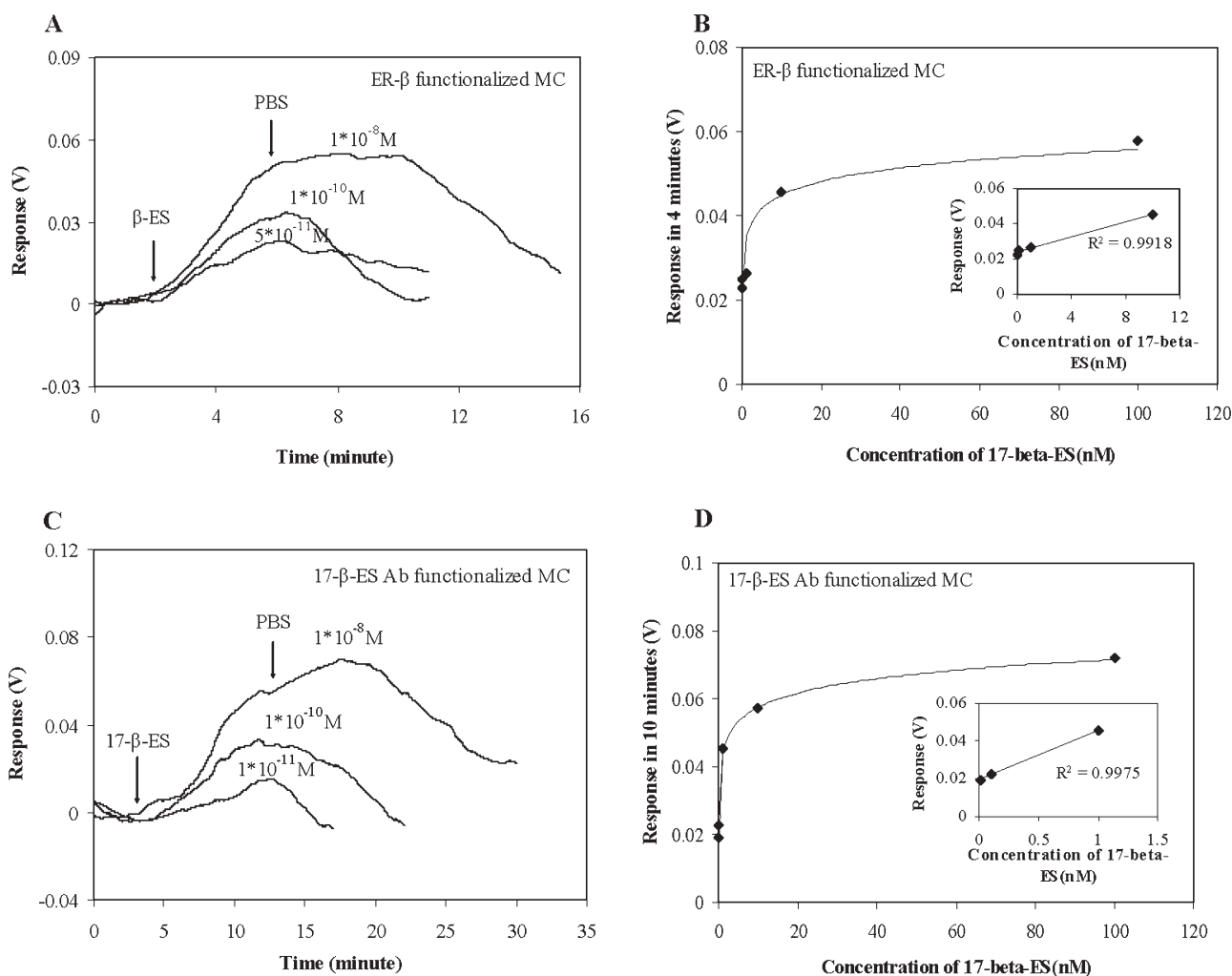


Fig. 5 Concentration-based nanomechanical responses of (A) ER- β protein and (C) anti-17- β -ES Ab functionalized MC to different concentrations of 17- β -ES. Net responses to 17- β -ES after 4 min and 10 min for ER- β protein and anti-17- β -ES Ab, respectively, functionalized MCs are plotted (B & D) over a concentration range from less than 1×10^{-10} M (see lowest concentrations in A & C) to 1×10^{-7} M. The linear portions of these plots extend over two orders of magnitude in concentration (see inserts in B & D). The first data points in insert B and D correspond to 5×10^{-11} M and 1×10^{-11} M of 17- β -ES respectively.

17- β -ES in the range of 50 pM to 10 nM where the response increases with increasing concentration. The kinetic response of the cantilever after 4 min of exposure is plotted against the concentration of 17- β -ES ranging from 50 pM to 100 nM in Fig. 5B. As seen in the figure, the response increased gradually and reached a plateau by 100 nM. The inset in Fig. 5B illustrates a linear dynamic range for more than two orders of magnitude (the first data point corresponds to the lowest concentration of Fig. 5B) in concentration (from an approximate LOD less than 50 pM to 10 nM). Similarly, Fig. 5C shows the response profiles of an anti-17- β -ES antibody functionalized cantilever for different concentrations of 17- β -ES in PBS in the range of 1 pM (approximate LOD is somewhat less than this) to 10 nM. The kinetic response of the cantilever after 10 min exposure of the antibody functionalized MC is plotted against the antigen concentrations over a range of 1 pM to 100 nM (Fig. 5D). Our current experiment involving optimization of the thickness of the dealloyed surface of the MC may improve the LOD lower than pM level (see the ESI and Fig. SI-2†).

Prior work showed that calibration plots are generally linear for two or more orders of magnitude, while coefficients of variation (CVs) for measurements using a given system of MC and molecular-recognition phase are generally 10% or better.^{10,11,14,15} Our experiments in the detection of 17- β -ES using both the receptor protein (ER- β) and antibody (anti-17- β -ES Ab) showed good measurement reproducibility in the same day tested *via* three replicate consecutive measurements of a solution of 1×10^{-10} M of 17- β -ES (see Fig. 6). ER- β functionalized MC arrays prepared in different batches showed 8–10% CV values in the detection of different concentrations of 17- β -ES.

To investigate stability, anti-17- β -ES Ab functionalized MC was exposed to 1×10^{-9} M of 17- β -ES at three different periods after storing in PBS at 4 °C (2nd, 4th, and 8th day after functionalization), an average value of deflection on day 4 and day 8 were 97% and 76% of the initial response, respectively.

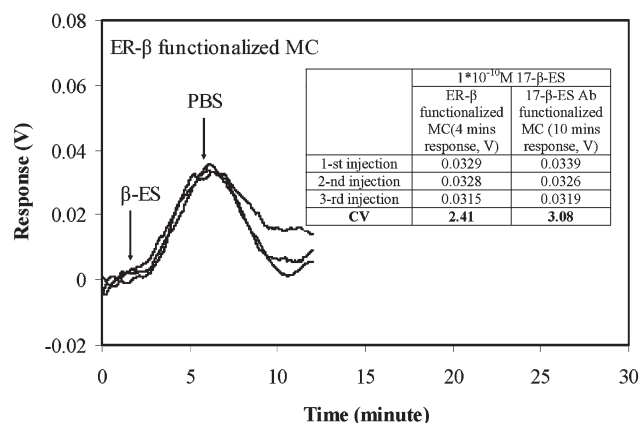


Fig. 6 Nanomechanical responses of ER- β functionalized MC on exposure to 1×10^{-10} M 17- β -ES for three replicate injections demonstrating reproducibility in nanomechanical responses where the inset shows the response and CV for three replicate measurements of this EDC using both the receptor protein and the antibody functionalized MCs.

Similarly, the stability of ER- β functionalized MC was studied over periods of 5 days after storing in PBS at 4 °C wherein it showed poorer stability; exposure to 1×10^{-9} M of 17- β -ES yielded responses after 3 and 5 days that were 78% and 36% of the initial response, respectively. Also, the stability of ER-functionalized MC was tested at 4 °C varying the environment (stored dry *versus* in PBS). The results showed that the immobilized antibody retained its functionality for a longer period of time (7–10 days after functionalization) if stored dry at 4 °C whereas it can be stored in PBS for near immediate use. Surprisingly, in both of the experiments performed, the response actually increased by a factor of two after one week of dry storage at 4 °C.

Conclusions

In summary, a highly sensitive, biospecific, and reusable biosensor for the detection and screening of EDCs has been developed using nanostructured MCs by exploiting protein receptor–EDC and antibody–EDC interactions. Our results indicate that the interaction of ER protein with different ligands produced different cantilever responses showing the maximum response for the synthetic estrogen DES with the ER- β functionalized MCs followed by estradiols and other EDCs. While receptor proteins provide generalized responses to subclasses of EDCs with impressive LODs, antibodies specific to a particular EDC can be used for specific analyte detection with a linear dynamic range over two orders of magnitude in concentration and about 3% of intra-day RSD. Also, measurements exhibited 10% RSD between different MC arrays functionalized at different times.

Since a single analyte ligand can stimulate changes in multiple receptor proteins and thereby synergistically mediate diverse biochemistry in complex living systems, an integrated analysis tool in a small, inexpensive platform is highly desirable. Thus, future research will involve the development of efficient and reproducible methods to differentially functionalize the cantilevers in arrays with different types of receptor proteins, the resulting chip platforms are expected to provide unique capabilities and exhibit significant biomedical and environmental utility.

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