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Rapid and ultrasensitive detection of endocrine disrupting chemicals using a nanosensor-enabled cell-based platform

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Abstract

Endocrine disrupting chemicals (EDCs) interact with estrogen receptors (ERs), causing a range of adverse health effects. Current assays for EDC activity are slow and often lack sensitivity. We report here an ultra-sensitive nanosensor that can detect estrogenic cellular changes in ER(+) MCF-7 cells rapidly (minutes) at levels orders of magnitude lower than generally used assays. Notably, the sensor responses at these ultra-low EDC levels correlate with an increased synthesis phase (S-phase) cell population of EDC-treated cells. The nanosensor was also able to detect binary EDC mixture effects, with synergism observed for bisphenol A (BPA) - 17 β -Estradiol (E2) and antagonism for Dicyclohexylphthalate (DCHP) - E2, and Benzo(a)pyrene (BaP) - E2.

Graphical Abstract

A high throughput cell-based nanosensor provides highly sensitive identification of estrogenic agents.

Endocrine disrupting chemicals (EDCs) are structurally diverse compounds that interfere with the endocrine system, cause a broad range of adverse effects.^{1,2,3,4} EDCs featuring estrogenic activity are of particular importance, posing a significant threat to reproduction and developmental processes in human and wildlife.⁵ In common with other toxicological threats, there are tens of thousands of chemicals in use whose estrogenic effects are unknown.

Proliferative assays that rely on increased cell reproduction upon exposure to EDCs are by far the most widely used test for estrogenic activity. The widely used E-screen proliferative assay requires a six-day exposure period. The inability of this protocol to detect low-dose effects in the pico- and femtomolar range causes many false negatives.^{6,7,8} Recently, flow cytometry assay has been used to detect estrogenic responses arising from lower EDC concentration exposures by detecting the percentage of cells in the S-phase.⁶ This assay, however, requires substantial sample preparation and specialized instrumentation, limiting its ability to address the high throughput demands of environmental toxicology. Recently, engineered bacterial sensor was developed to screen estrogenic EDCs at low concentrations. However, while this technique is quite rapid and convenient, it does not measure the direct phenotypic changes after EDC exposures that are relevant at a cellular level. Therefore, this design is more susceptible to false positives.⁹

The wide range of health issues generated by EDCs suggests that there is likewise a range of phenotypic consequences of EDC exposure at the cellular level. Predicting these changes on the molecular level is, however, quite challenging.^{10,11} To address this issue, we report here the use of a hypothesis-free nanosensor to detect estrogenic EDC response of cells at pico- and femtomolar levels. This sensor system uses a gold nanoparticle (AuNP) as a recognition element and green fluorescence protein (GFP) as a transducer.¹² This sensor platform can capture the overall chemicophysical changes of the EDC-treated cells in minutes, avoiding false negatives due to the limitation of the typical single endpoint readout of current assays.¹³ The simplicity of this method makes it likewise a practical tool for addressing the real-world challenge arising from mixtures of EDCs.

We used a sensor system composed of non-covalent complexes of AuNPs and GFPs. We chose the benzyl nanoparticle (BenzNP) due to its sensitivity to differences in cell surfaces.¹⁴ The fluorescence of the GFP is quenched when bound to BenzNP, with fluorescence restored upon displacement by cell surface functionality (Scheme 1).^{15,16,17} Human breast cancer MCF-7 cells were used in our study. This cell line is widely employed in EDC studies^{5,6,8} due to their high level sensitivity to estrogenic agents.^{18,19}

Our initial experiments focused on establishing the response of our sensor to E2 as a positive control. MCF-7 cells were plated in 96-well plate overnight before being treated with E2, using 10,000 cells/well (Figure S1). After 24 hour treatment, cells were washed with Phosphate Buffer Saline (PBS), followed by the addition of the nanosensor BenzNP-GFP. The sensor detected significant cellular changes of E2-treated cells at femtomolar concentration (5×10^{-15} M). In contrast, there was no proliferative effect observed using Hoechst dye at even five orders of magnitude higher in E2 concentration. Significantly, co-incubation of cells with E2 and the anti-estrogen ICI 182,780 generated a response identical

to that of control (untreated) cells, verifying that the sensor was responding to estrogenic changes in cell phenotype (Figure 1a). Our nanosensor was able to detect cellular changes induced by femtomolar concentration of E2, a four order of magnitude more sensitive than the conventional E-screen assay.^{6,19}

We next tested our system on bisphenol A (BPA), an EDC that has generated considerable controversy.^{20,21} As above, the cells were treated with BPA for 24 hours prior to sensing. A significant increase in fluorescence signal from the sensor is observed at picomolar range (5×10^{-11} M, Figure 1b), while no significant response from Hoechst dye. Two other reported EDC agents, Dicyclohexylphthalate (DCHP), and Benzo(a)pyrene (BaP), likewise showed a positive response (Figure 1c).^{5,6,22} Significantly, BaP was successfully detected by our nanosensor at the concentration of 1×10^{-11} M, even though no significant proliferative effect was observed with this EDC using a standard E-screen assay.⁶

In reality, when we are being exposed to EDCs, it is usually a mixture of compounds and not a single agent. Therefore, it motivates us to investigate the low dose effect of EDC mixtures, starting with the binary mixtures of each tested compound described previously with a non-significant concentration of E2 at 1 fM. We used a sub-threshold dosing of E2 with the purpose of making the effects of binary mixtures more apparent by eliminating the potential affect of E2 alone in higher concentrations. Series of BPA, DCHP and BaP concentrations were prepared with and without co-incubation of 1 fM E2 to treat the cells for 24 hours before the detection by the nanosensor. To allow comparison across different compounds, equipotent concentrations (PC_{50}) need to be calculated. PC_{50} indicates the concentration of compound x that evokes 50% activity of the positive control, E2. This approach is more suitable for comparison than EC_{50} due to the fact that not all compounds reach a relative proliferation effect of 100%.^{5,23} Interestingly, BPA-E2 mixture shows a drastic increase in fluorescence signal compared to just single agent BPA. The PC_{50} of BPA-E2 mixture is much lower than that of BPA alone (4.77×10^{-14} M and 1.57×10^{-10} M, respectively). This reduction in PC_{50} makes the mixture of BPA-E2 even as potent as compared to E2 alone. This result is consistent with previously published work using the traditional E-screen method.²⁴ Other tested binary mixtures of DCHP-E2 and BaP-E2 show an opposite trend with BPA-E2 mixture. While BPA-E2 mixture indicates a highly synergistic effect, DCHP-E2 and BaP-E2 show antagonistic effects, where their PC_{50} values could not be determined (Figure 2). The binary mixture of E2 and another phthalate derivative, butylbenzyl phthalate, was shown to be antagonistic in previous work.²⁴ However, to the best of our knowledge, the mixture behaviors of DCHP-E2 and BaP-E2 are not previously reported in the literatures and are new findings enabled by our technology.

Our sensor can rapidly detect cellular responses from ultra-low levels of estrogenic agents, raising the question of what phenotypic change was being detected. Estrogenic EDCs trigger cells to proliferate, which should result in an elevated population of S-phase cells. The S-phase cell population can be measured by flow cytometry. This method has been previously described as flow cytometric E-screen assay, and was validated using a range of estrogenic compounds.⁶ In our study, cells were treated with different concentrations of E2 and BPA for 24 hours, then trypsinized and washed with PBS. Ethanol was used to stabilize these cells at 4°C. After 2 hours, cells were stained with Propidium Iodine/RNase solution before

running flow cytometry. As observed in sensor response of E2 and BPA-treated cells, higher concentrations of E2 and BPA induced more S-phase cell population, which become significant at 2.5×10^{-14} M and 1×10^{-11} M, respectively (Figure 3a). We observed a similar trend in the increased S-phase population as seen with the nanosensor BenzNP-GFP response. This increase in S-phase is eliminated when cells were treated with the co-incubation of E2 or BPA with the anti-estrogen ICI 182,780 (Figure 3b). The strong correlation between our sensing studies and the cytometric data (Figure S2) provides solid evidence that our sensor is responsive to cell cycle changes.

We have demonstrated the usefulness of our simple nanosensor, BenzNP-GFP, for detecting low dose effects of estrogenic EDCs. This technique is rapid, versatile and only involves one step process of adding the nanosensor solution into the EDC-treated cells. We have successfully detected the estrogenic activity of an endogenous (E2) and other xenogenous agents (BPA, DCHP and BaP) on MCF-7 cells at ultra low concentrations (femto and picomolar ranges). By diminishing the E2 sensor response and S-phase population with the anti-estrogen compound, it is strongly supported that the detected sensor response is indeed due to the estrogenic effect caused by E2 exposure. The ease of performing this cell-based assay using BenzNP-GFP complex has made it possible to test the effects of EDCs at a broader range of concentrations. Whereas, a sensitive flow cytometry method still limits number of samples one can perform due to multistep processing procedure. Significantly, this nanosensor can also be used to detect EDC mixture effect, eliminating the lengthy processing time comes with infinite number of possible combinatorial EDC mixtures. Studying the combination behaviors is a step forward to better reflect the effects of EDCs in a more complex system, such as *in vivo*. Such complexity can be complicated by the pre-existence of endogenous estrogen, which when mixed with other xenogenous substances, might be drastically different compared to single agent itself as observed in our study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Fluorescence response from nanosensor BenzNP-GFP and Hoechst 33342 with and without co-incubation of estrogen receptor inhibitor ICI 182,780 with a) 17 β -Estradiol (E2) and b) Bisphenol A (BPA). Fluorescence response of BenzNP-GFP sensor is significantly increased in the absence of ER inhibitor ICI 182,780 in both E2 and BPA treated cells at 10–15 M and 10–11 M respectively. c) Fluorescence response from nanosensor BenzNP-GFP for all tested compounds: E2, BPA, Dicyclohexylphthalate (DCHP) and Benzo(a)pyrene (BaP). Each data point is the mean value of four replicates per treatment (n=4).

Figure 2. Fluorescence response from nanosensor BenzNP-GFP for binary mixture effects of 1 fM 17β-Estradiol (E2) with (a) Bis-Phenol A, (b) Dicyclohexylphthalate, (c) Benzo(a)pyrene. Each data point is the mean value of four replicates per treatment (n=4). d) PC₅₀ values for individual compounds and binary mixtures with 1 fM E2. PC₅₀ value is the concentration of compound x with 50% activity of the positive control (17β-Estradiol, E2).

Figure 3.

(a) E2 and BPA effects on S-phase cell population of MCF-7 measured by flow cytometry. The S-phase percentage of MCF-7 cells increases as the concentration of E2 or BPA increases. (b) Co-incubation effect of E2 and BPA with ICI 182,780, an estrogen receptor antagonist, on S-phase cell population. The proliferation effect of MCF-7 cells when treated with E2 or BPA is inhibited in the presence of 10 nM of ICI 182,780. Each data point is the mean value of three replicates per treatment (n=3).

Scheme 1.

Schematic illustration of the nanosensor. a) The sensor consists of BenzNP and green fluorescence proteins (GFPs). The fluorescence of GFP is quenched when the BenzNP–GFPs complexes are formed. b) When nanosensor is added to cells with and without EDC treatment, due to the different phenotypes of untreated and treated cells, BenzNP interacts differently with the cell surface and releases different amount of GFP, generating signal output.