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A label-free, multiplex competitive assay for small molecule pollutants

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ABSTRACT

Understanding the amount of exposure individuals have had to common chemical pollutants critically requires the ability to detect those compounds in a simple, sensitive, and specific manner. Doing so using label-free biosensor technology has proven challenging, however, given the small molecular weight of many pollutants of interest. To address this issue, we report the development of a pollutant microarray based on the label-free arrayed imaging reflectometry (AIR) detection platform. The sensor is able to detect three common environmental contaminants (benzo[a]pyrene, bisphenol A, and acrolein) in human serum via a competitive binding scheme.

1. Introduction

Human health concerns are driving an ever-increasing need for simple and sensitive methods for detecting a broad range of contaminants in the environment. Of particular interest are small molecules known or suspected to have deleterious health effects. While individual tests are available for some of these (Ohkuma et al., 2002; Chung et al., 2013; Li et al., 2004), as far as we are aware there is no system available for detecting environmental pollutants in a label-free, multiplex fashion with high sensitivity and selectivity in human serum. Therefore, we set out to develop such a system using the arrayed imaging reflectometry (AIR) biosensor technology.

Details of the theoretical foundations and operation of AIR have been reported elsewhere (Mace et al., 2006). In brief, the technique relies on the creation of a near-perfect antireflective condition on the surface of a silicon chip. When illuminated with S-polarized laser light at the HeNe wavelength and at an appropriate angle, an array of capture molecules spotted on the chip may be imaged with a CCD, showing minimal reflectivity in the absence of target. Binding of target analytes to the appropriate capture molecule spot causes a predictable, quantitative perturbation in the antireflective condition that may be measured as a change in reflected intensity. Thus far, we have demonstrated that AIR is useful for detecting bacterial cell-surface proteins (Horner et al., 2006), human cytokines in serum (Mace et al., 2008; Carter et al., 2011), and a variety of immune system markers including antibodies to human papilloma virus (Mace et al., 2009) and influenza (Mace et al., 2011). Quantitative analytical performance of AIR is well correlated with theory and reference techniques such as surface plasmon resonance (SPR) and spectroscopic ellipsometry (Sriram et al., 2011). Intense interest in label-free sensing technologies has driven the development of a number of methods capable of multiplex detection, including photonic crystals (Pal et al., 2012), interferometric methods (Cheng et al., 2014; Varma et al., 2004), and surface plasmon resonance imaging (Nelson et al., 2001). While all of these have found utility in various applications, AIR is particularly notable for its simplicity (no moving parts in the imaging system) and insensitivity to thermal effects. Since AIR relies on measuring changes in intensity relative to a near-zero reflectance condition rather than a shift in a non-zero minimum, it is also quite sensitive, as we have demonstrated in previous work.

Although AIR is capable of detecting small molecules directly, we sought to examine the performance benefits of a competitive assay format. This potentially allows for more sensitive detection of very small targets, effectively amplifying the amount of mass change observed in the sensor. Several examples of competitive assays in label-free sensor platforms have been reported; for example, Bonnano and DeLouise described a competitive format porous silicon sensor for urinary metabolites of morphine and related drugs of abuse (Bonanno and DeLouise, 2010; Bonanno et al., 2010). Two formats for such an assay are possible (Fig. 1). In the competitive inhibition assay, a sensor surface is prepared with the target molecule covalently attached. Exposure of this sensor to a solution of the analyte of interest mixed with an appropriate antibody causes a loss of signal relative to that observed when the

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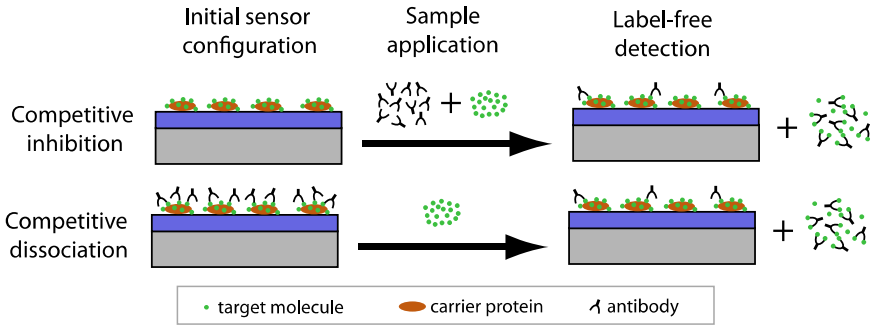

Fig. 1. Possible formats for competitive assays.

antibody alone is mixed with the sensor. Alternatively, in the competitive dissociation format, antibodies are pre-bound to the immobilized analytes on the sensor; the target analyte solution is then added. The competitive dissociation format has the advantage of providing a simpler work flow to the user; however, for this format to be successful the binding affinities of surface-bound and solution-phase analytes must be comparable, and the surface-bound antigen–antibody complex must have a reasonable off-rate. As discussed below, we tested both formats in order to compare relative performance.

2. Methods

2.1. Sources of materials

Irgasan (5-chloro-2(2,4-dichlorophenoxy)phenol), 4,4-bis(4-hydroxyphenyl) valeric acid (BHPVA), bisphenol A (BPA), benzo[a]pyrene, and *N*-hydroxysuccinimide (NHS) were obtained from Sigma-Aldrich (St. Louis, MO). Acrolein was obtained from Ultra Scientific (N. Kingstown, RI), ethyl succinyl chloride and ethylenediaminetetraacetic acid from Acros Organics (Geel, Belgium), 6-chlorohexanoic acid from TCI Chemicals (Portland, OR), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) from CreoSalus Life Sciences (Louisville, KY), Polysorbate 20 (Tween-20) from Avantor Performance Materials (Center Valley, PA), 3,3′,5,5′-tetramethylbenzidine (TMB) from Alfa Aesar (Ward Hill, MA), bovine serum albumin (BSA) and peroxidase-conjugated protein A from Rockland Immunochemicals (Pottstown, PA), keyhole limpet hemocyanin (KLH) from EMD Millipore (Billerica, MA), porcine serum from Lampire Biologicals (Pipersville, PA), and human serum was obtained from Innovative Research (Novi, MI). Antibodies against benzo[a]pyrene (GTX20768) and acrolein (GTX15138) were purchased from GeneTex (Irvine, CA). Anti-bisphenol A (AS132735) was obtained from Agrisera (Vännäs, Sweden).

2.2. Array fabrication

Amine-reactive AIR substrates were spotted with probe solutions using a Scienion SciFlexArrayer S3 equipped with a PDC50 capillary. This provides non-contact, piezoelectric dispensing of 250 pL droplets, producing spots approximately 150 µm in diameter. All array spotting was conducted in a humidity-controlled chamber at 70% relative humidity. Following spotting, chips were immersed in a solution of 0.5% (7.5 mM) BSA in 50 mM NaOAc, pH 5.0 for 1 h to block. Chips to be used in assaying human serum samples underwent a two-step blocking process, being first exposed to 0.5% (7.5 mM) BSA in NaOAc, pH 5.0 for 20 min, followed by exposure to a 1% porcine serum solution in PBS-ET, pH 7.4 for 40 min.

2.3. Conjugation of haptens

A linker was added to benzo[a]pyrene through a Friedel–Crafts acylation. Equimolar quantities of benzo[a]pyrene and ethyl succinyl chloride were combined in the presence of two equivalents of $AlCl_3$ in dry dichloromethane under nitrogen. The reaction was run under reflux and monitored by thin layer chromatography. It was quenched with ice and concentrated HCl, and the product was washed with water, dried over magnesium sulfate, concentrated with rotary evaporation, and stored at 4 °C.

The benzo[a]pyrene-linker product and 4,4-bis(4-hydroxyphenyl) valeric acid (BHPVA) were activated with 1.25 equivalents of EDC and NHS in DMF for (3 h, room temperature, 400 rpm) before conjugation with keyhole limpet hemocyanin (KLH) at 2000-fold excess of small molecule to KLH in 100 mM sodium carbonate/bicarbonate buffer pH 10.0 (20 h, 4 °C, 400 rpm) to form benzo[a]pyrene- and bisphenol A-KLH haptens. Acrolein was allowed to react with KLH under the same conditions to form the acrolein-KLH hapten. The reactions were quenched with 1% lysine and dialyzed against mPBS pH 6.0 with three buffer changes. The conjugations were confirmed through spectrophotometric analysis.

2.4. Competitive binding experiments (AIR platform)

For the competitive inhibition experiments, dilutions of benzo[a]pyrene, bisphenol A, and acrolein were preincubated with the three respective antibodies, each at 1 µg/mL (6.7 nM) in 0.5% (7.5 mM) BSA in PBS-ET for one hour. Following hybridization, AIR substrates (probe content as shown below in Fig. 5) were exposed to each solution for another hour. For the competitive dissociation experiments substrates were exposed to a solution of the three antibodies (1 µg/mL (6.7 nM) each in PBS-ET plus 0.5% (7.5 mM) BSA) for one hour prior to exposure to a solution of 10 µM benzo[a]pyrene, bisphenol A, and acrolein in 0.5% (7.5 mM) BSA PBS-ET for another hour. Following target exposure in each condition, the substrates were washed in PBS-ET, rinsed in nanopure water, dried under a stream of nitrogen, and imaged.

3. Results

We selected four representative environmental toxicants of immediate interest to exposure biology in the US populace representing three classes of persistent organic pollutants, including environmental phenols (bisphenol A (Vom Saal and Hughes, 2005; Calafat et al., 2005; Gray et al., 2004; Tyl et al., 2002; Kolpin et al., 2002) and triclosan (Calafat et al., 2008; Sandborgh-Englund et al., 2006; Veldhoen et al., 2006; Clayton et al., 2011; Stroker et al., 2010), polycyclic aromatic hydrocarbons (benzo[a]pyrene (Calafat et al., 2008; Barhoumi et al., 2000; Benzo, 1994; Faust and Reno,

1994)) and reactive aldehydes (acrolein (Moghe et al., 2015)). These pollutants are currently subject to active monitoring by the CDC and were detected at biologically relevant concentrations in nearly all population substrata as described in the Fourth National Report on Human Exposure to Environmental Chemicals (Fourth National Report on Human Exposure to Environmental Chemicals, 2009).

3.1. Preparation of conjugates and confirmation of activity

Direct attachment of small molecules to a planar sensor surface may result in an inactive device, since the proximity to the surface acts as a steric barrier to antibody binding. This can be addressed by first conjugating the small molecule to a long-chain linker prior to immobilization; an alternative is to conjugate the small molecule to a carrier protein, by analogy to standard methods used for raising antibodies to small molecule targets. This is the approach we pursued. Since conjugate activity can vary depending on carrier protein, we examined two possibilities. Both bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) are common carriers for hapten conjugation in antibody development, and were used here. Literature methods were employed for the preparation of protein conjugated analogs of bisphenol A (Zhao et al., 2002), triclosan (Kugel et al., 2012), acrolein (Uchida et al., 1998), and benzo[a]pyrene (Miura et al., 2003). Initial experiments suggested that KLH conjugates had higher activity in our hands, and were used for all further experiments (Fig. 2). Characterization of KLH conjugates to bisphenol A, acrolein, and benzo[a]pyrene are shown in Fig. 3. As anticipated, differing reactivity of each hapten (toxicant) with the carrier proteins results in differing levels of carrier decoration. We were unable to detect a response to the triclosan–KLH conjugate using several commercial antibodies, and as such, we set this target aside until a separate antibody development effort could be accomplished. The suitability of these constructs for incorporation into a competitive assay format was further tested via a competitive ELISA for bisphenol A as a representative (Fig. 4). The lower limit of detection for bisphenol A in this format was 0.64 ng/mL (2.8 nM). We also verified that each antibody selected for inclusion in the assay was specific for its requisite toxicant. These data (included in Supplemental Information), indicated a high degree of specificity for each antibody to its antigen, yielding $\leq$12% observable cross-reactivity for all commercially sourced antibodies.

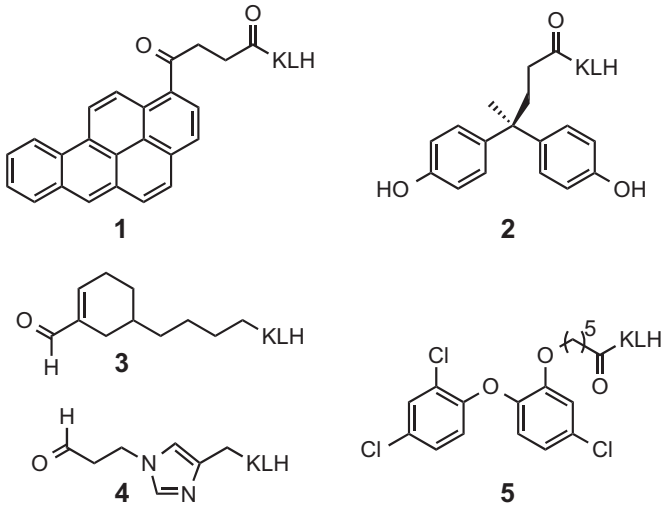

Fig. 2. KLH conjugates of benzo[a]pyrene (**1**), bisphenol A (**2**), acrolein (**3** and **4**), and triclosan (**5**). Note: Two structures are provided for Acrolein conjugates as both are reported as adducts under native condition to serum albumin (Moghe et al., 2015).

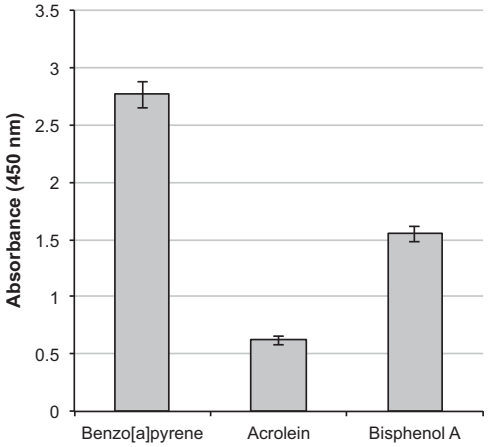

Fig. 3. Verification of KLH-toxicant activity via standard ELISA. Error bars one standard deviation of the mean across duplicate wells.

deviation of the mean across duplicate wells.

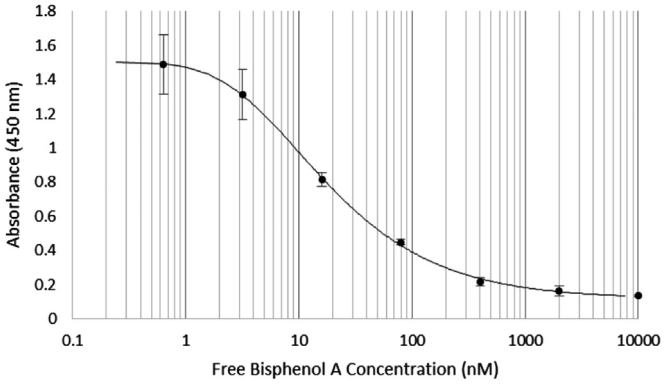

Fig. 4. Competitive ELISA of anti-bisphenol A. Error bars indicate one standard deviation of the mean across duplicate wells. The dose vs. response profile is fit using a standard 5-Parameter Logistic Model ($f(x)=D+(A-D)/((1+(x/C)B)E)$), yielding an R^2 value of 0.9995 and RMSE of 0.0213. Note: Additional methodological details for this assay are provided as Supplemental Information.

3.2. Fabrication of the toxicant array

AIR assays are most sensitive if the starting condition (control spots) are at or near the minimum reflectance condition. Therefore, an essential first step in the creation of the toxicant array was to determine printing conditions for conjugates that would yield uniform spots at the appropriate thickness. To that end, a concentration screening exercise was conducted to determine the optimal print concentration for each KLH-toxicant probe on the array. Additionally, as KLH is the carrier for all toxicants, we used dilutions of KLH, as well as other proteins to serve as nonreactive reference probes on the array to assist in normalization during downstream data analysis. A representative array is shown in Fig. 5 (note that triclosan conjugates were included in the experiment in anticipation of the availability of an effective triclosan antibody). We observed some aggregation in the control (KLH only) probes, as evidenced by speckling in the spots. A result of this study was the inclusion of a non-nucleophilic additive (5% DMSO) to prevent aggregation during spotting, by analogy to previous work (Mace et al., 2008). Passivation of remaining reactive groups on the surface of the chip was accomplished via immersion in a blocking solution of 0.5% (7.5 mM) BSA in 50 mM NaOH (pH 5.0) for 1 h.

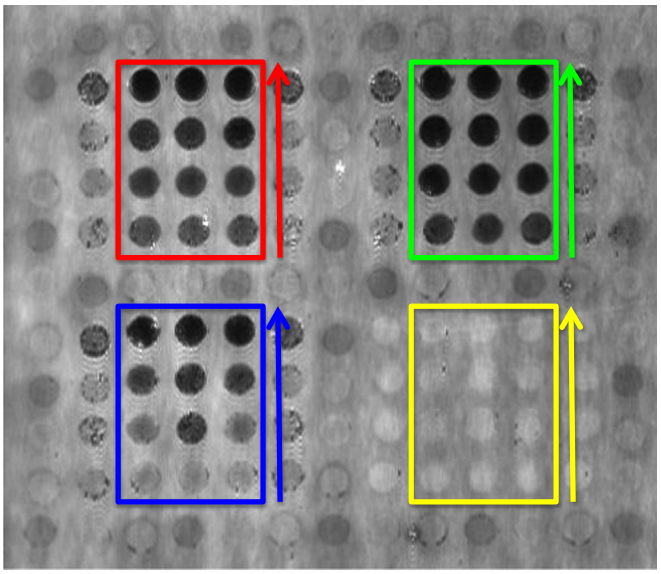

Fig. 5. A representative KLH-toxicant AIR array. Conjugates are indicated by color (red: benzo[a]pyrene, green: acrolein, blue: bisphenol A, yellow: triclosan); each row consists of three replicate spots, and rows increase in concentration of the conjugate spotting solution in the direction of the arrows (0.5, 0.75, 1.0, 1.5 mg/mL). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

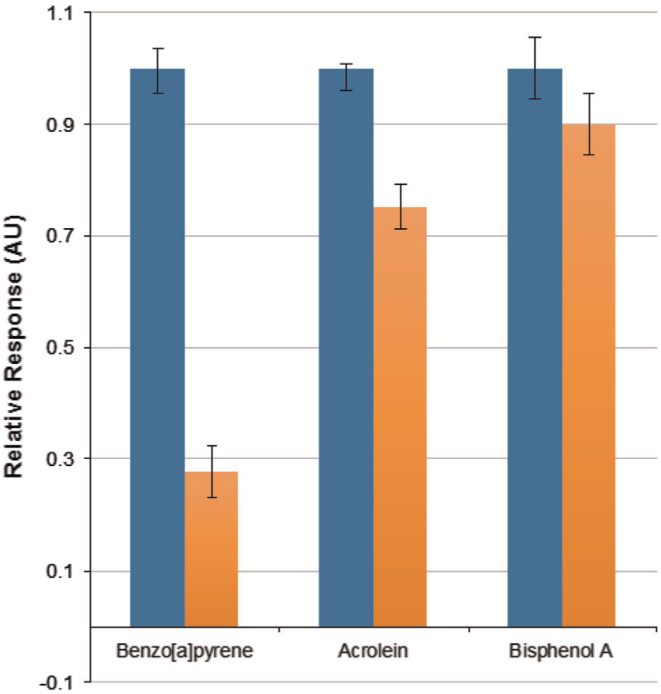

Fig. 6. Relative response (where signal is scaled to greatest responder for each assay) for competitive inhibition (blue) and competitive dissociation (orange) assay formats, as implemented on the toxicant microarray. Error bars represent one standard deviation of the mean probe response across three replicate sensor chips per condition. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Using the screening approach demonstrated in Fig. 5, we selected the most optimal probe formulation for each hapten. After finalizing these parameters, we next examined the performance in the competitive inhibition and competitive dissociation formats. We found that the competitive inhibition format provided substantially greater response as determined by Student's *T*-test at equivalent concentrations for benzo[a]pyrene ($p < 0.0001$) and

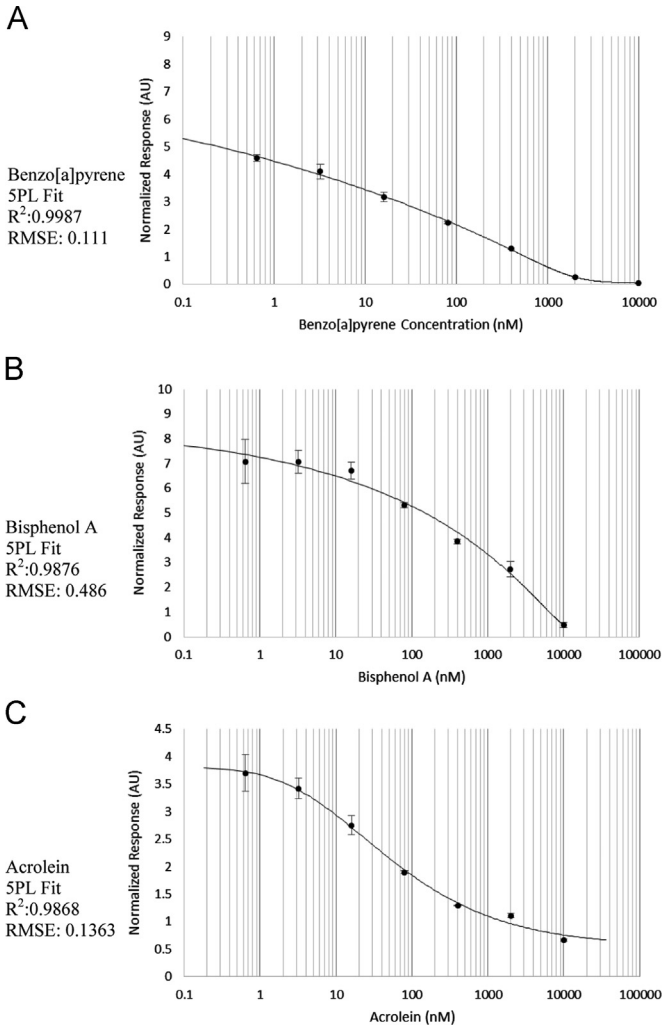

Fig. 7. Titration response profiles for benzo[a]pyrene (A), bisphenol A (B), and acrolein (C) in buffered 0.5% (7.5 mM) BSA. In each case, a constant amount of antibody was incubated with the array, while the concentration of the analyte was varied. Error bars represent one standard deviation of the mean probe response across three replicate sensor chips per condition. All dose vs. response profiles are fit using a standard 5-Parameter Logistic Model ($f(x)=D+(A-D)/((1+(x/C)B)E)$).

acrolein ($p < 0.0001$), while activity in both formats was reasonably comparable for bisphenol A ($p < 0.0013$) (Fig. 6). As such, all remaining experiments were conducted in the competitive inhibition format.

Response profiles for the three toxicants were next determined on the array, using a simple background matrix of 0.5% (7.5 mM) BSA in PBS-ET. In each case, the amount of the appropriate solution-phase antibody was kept constant at 6.7 nM, while the concentration of analyte was varied from 10 μM to 640 pM in 1:4 serial dilutions. All three analytes produced well-behaved response curves (Fig. 7). Notably, we observed an obvious shift in the response profile for Bisphenol A in the AIR assay relative to the ELISA performed earlier (Fig. 4). This effect is likely due to the differing levels of optimization between the two assays, as well as a signal saturation effect at higher concentrations using the labeled reported element in the ELISA assay.

Table 1 provides lower limits of detection and quantitation. For this work we calculated the limit of detection for each assay as the lowest concentration at which a signal was observed that was greater than twice the signal error from the assay baseline. Limits of quantitation were determined as the lowest concentration in the standard curve at which the coefficient of variation is less than

Table 1
Lower limits of detection (LLOD) and quantitation (LLOQ) in nM for each toxicant in the three-plex assay.

Analyte	Reference concentration in plasma	LLOD	LLOQ
Benzo[a]pyrene	0.95–5.2 (Neal et al., 2007; Sherson et al., 1990; Neal et al., 2008)	16	16
Acrolein	14,400–31,200 (Hiroi et al., 2004; Takeuchi and Tsutsumi, 2002)	16	16
Bisphenol A	2.8–12.7 (Tomitori et al., 2005; Sakata et al., 2003)	80	80

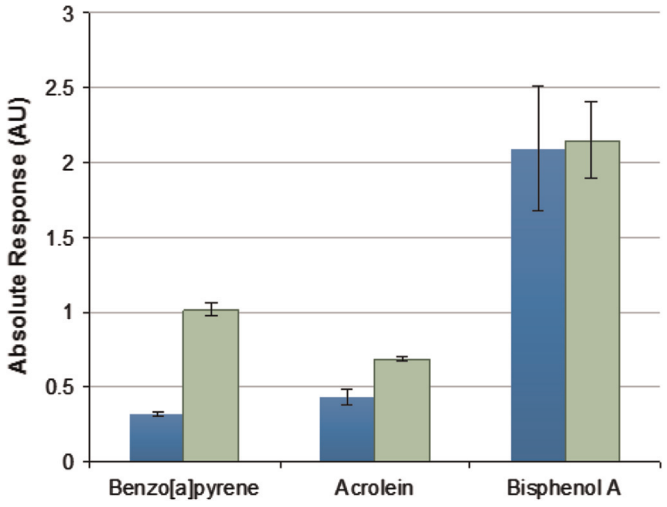

Fig. 8. An AIR toxicant array operating in competitive inhibition mode is able to selectively detect three common toxicants in human serum. Absolute responses are shown for each analyte doped at 10 (blue) or 50 μM (green). Error bars one standard deviation of the mean across triplicate spots on duplicate chips. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

30% of the assay response. The CV is determined by the relationship between the standard deviation of each dose dependent observation and its mean signal intensity, or more specifically $CV = 100^*(\sigma/\mu)$. We were pleased to observe in each case that lower limits of detection were near or lower than concentrations observed in studies of human samples for all three analytes (benzo[a] pyrene, (Neal et al., 2007; Sherson et al., 1990; Neal et al., 2008) bisphenol A (Hiroi et al., 2004; Takeuchi and Tsutsumi, 2002) and acrolein (Tomitori et al., 2005; Sakata et al., 2003)).

Importantly, the array was also able to detect individual analytes doped in commercial pooled normal human serum (PNHS). Both benzo[a]pyrene and acrolein produced concentration-dependent responses at 10 and 50 μM (Fig. 8); bisphenol A produced a maximal response for both concentrations indicating some degree of nonspecific detection in the serum itself.

4. Discussion and conclusions

We have successfully demonstrated a label-free, 3-plex environmental toxicant array based on the arrayed imaging reflectometry platform. This array is able to sensitively and specifically detect benzo[a]pyrene, acrolein, and bisphenol A in relatively simple backgrounds and in human serum using a competitive immunoassay format. The limits of detection and quantitation for acrolein were well below typical concentrations observed in toxicology assays. While detection limits for bento[a]pyrene and bisphenol A were slightly higher than those needed for most human samples, we anticipate that these can be improved via further optimization of the assay. Because we have previously demonstrated that AIR may be used for sensitive and specific detection of cytokines and other inflammatory biomarkers in serum, it should be possible to fabricate a combined array able to simultaneously detect both the toxicant itself, and a cytokine-mediated inflammatory response. Efforts along those lines are currently in progress.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.08.064.

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