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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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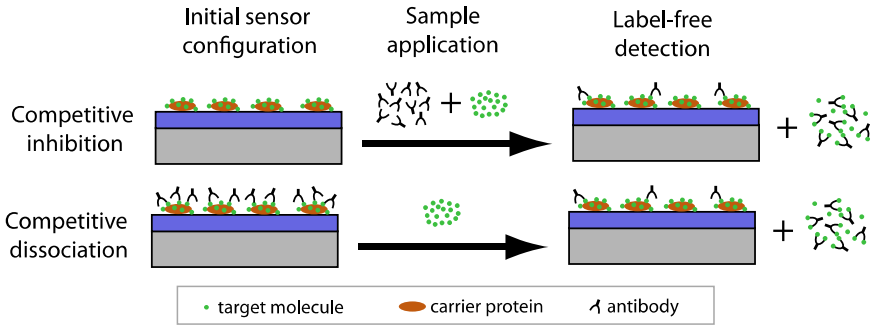

Fig. 1. Possible formats for competitive assays.

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,