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Electrophoretic build-up of multi nanoparticle array for a highly sensitive immunoassay

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ABSTRACT

One of the challenges in shrinking immunoassays to smaller sizes is to immobilize the biological molecules to nanometer-scaled spots. To overcome this complication, we have employed a particle-based immunoassay to create a nanostructured platform with a regular array of sensing elements. The technique makes use of an electrophoretic particle entrapment system (EPES) to immobilize nanoparticles that are coated with biological reagents into wells using a very small trapping potential. To provide useful information for controlling the trapping force and optimal design of the nanoarray, electrophoretic trapping of a nanoparticle was modeled numerically. The trapping efficiency, defined as the fraction of wells occupied by a single particle, was 91%. The performance of the array was demonstrated with a competitive immunoassay for a small molecule analyte, 3-phenoxybenzoic acid (214.2 g mole^{-1}). The limit of detection determined with a basic fluorescence microscope was 0.006 μg l^{-1} (30 pM); this represented a sixteen-fold improvement in sensitivity compared to a standard 96-well plate-based ELISA; the improvement was attributed to the small size of the sample volume and the presence of light diffraction among factors unique to this structure. The EPES/nanoarray system promises to offer a new standard in applications that require portable, point-of-care and real-time monitoring with high sensitivity.

1. Introduction

Microarrays have facilitated many breakthroughs in the life sciences by identifying specific gene sequences or protein analytes. The multiplexed technology used in microarrays allows for simultaneous detection of different analytes on a single chip with lower detection limits than conventional laboratory assays (i.e. polymerase chain reaction or enzyme-linked immunosorbent assay) (Chan et al., 2004). Chip-type sensing has also shown potential for use as a point-of-care or real-time assay. These successes have opened the door for research into clinical diagnosis or therapeutic treatment of numerous diseases. To build on these successes, recent research has been moving to the much smaller, or "nano" world. In the cases of screening of diseases or cancer biomarkers using proteomic based approaches, the sample sizes can be extremely limited. This requires that the level of detection be as low as possible. However, nanoarray technology still has some technical difficulties to overcome. Among the most difficult challenges is the need to immobilize small biomolecules on specific nano-sized spots for detecting targets. It is almost impossible to use conventional methods for immobilizing antibodies or proteins onto nanoarrays by using surface modification techniques (e.g. self assembly monolayers) (Schwartz, 2001), although there has been some success building nanoarrays that consist of self-assembled DNA blocks (Liu et al., 2005).

Currently, dip-pen nanolithography has been the most useful method that can directly immobilize biological molecules to nanospots with 100 nm minimum resolutions (Lee et al., 2004; Ginger et al., 2004). However, it requires considerable amounts of time because the solution moves from a tip to the surface mostly by diffusion (Salaita et al., 2005). The method also needs to carefully control environmental conditions such as humidity (Sanedrin et al., 2010). A small number of studies have demonstrated immobilization of biological molecules in an array using particles for immunoassays. Particle-based immunoassays have steadily gained popularity as a solid-phase for antibody immobilization owing to the ease of immobilization of antibodies—the surface area, surface charge, chemical groups and choice of signal transduction can be readily controlled, allowing increased possibilities for antibody immobilization, characterization and detection (Wilson et al., 2006; Haukanes and Kvam, 1993). Many of these features are essential ingredients in increasing sensitivity and lowering the limit of detection (Kusnezow et al., 2006).

Chang et al. (2009) demonstrated electrophoretic trapping of a single microparticle coated with alkaline phosphatase for electrochemical measurement. However, their techniques required complicated

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and time-consuming fabrication procedures for loading electrodes into micro-scale-patterns. Juan et al. (2009) demonstrated non-invasive optical trapping of a single 53 nm-nanoparticle into a nanowell using a reduced laser intensity. Their method may be useful in avoiding damage to biological reagents coated on particles due to low optical intensity. However, optical trapping is still complicated and expensive. In addition, it may not be suitable for array-type biosensors, which can include dozens or hundreds, or even many thousands, of trapping spots. Powell and Yoon (2006) demonstrated the method of trapping nanoparticles coated with biological samples into nanowells patterned on a p-doped silicon wafer using electrostatic interactions between the surface charge of the particle and the silicon wafer. This method may lead to easier fabrication and trapping—unfortunately it is impossible to control the trapping force in order to manipulate variously charged particles due to the fixed surface charge at the silicon wafer. Furthermore, considerable numbers of particles may be lost during hydrodynamic rinsing.

In this study, our primary objective was to develop a new method that effectively trapped nanoparticles conjugated with biological reagents into specific locations using electrophoresis. To obtain useful information about optimal trapping forces and the design of the nanoarray, the electrophoresis of nanoparticles into nanowells was simulated by a numerical method. The second objective was to demonstrate the performance of the nanoarray as an immuno-platform by quantifying the metabolic product of some pyrethroid insecticides using a competitive immunoassay. Several compounds in the pyrethroid class of insecticides have transitioned from expensive insecticides to commodity chemicals that are the most widely used insecticides worldwide, thus raising the need for environmental markers of their use (Shan et al., 2004; Laffin et al., 2010). Pyrethroids are also widely used in bed nets to control insect vectors of diseases such as malaria (Zaim et al., 2000; Sharma et al., 2009). These uses also raise the possibility of human exposure. Fortunately, 3-phenoxybenzoic acid (3-PBA) is a marker of both environmental presence and human exposure to the major pyrethroids used in the world (Ueyama et al., 2009). Therefore, developing a highly effective analytical technique to detect this analyte is important for environmental monitoring and human health risk assessment.

2. Experimental

2.1. Chip fabrication

Indium tin oxide (ITO) coated glass wafer (catalog no. CG-81N-1515; Resistance: 30–60 Ω; Delta Technologies, Stillwater, MN, USA) was selected for its electrical and optical properties; ITO is a solid material that exhibits excellent electrical conductivity and optical transparency. Before coating the resist, the wafer was washed with acetone (Sigma-Aldrich, St. Louis, MO, USA) and fully spin-dried. LOL-2000 (Microchem, Newton, MA, USA) was spin-coated on the wafer at 6500 rpm for 45 s followed by being baked at 180 °C for 300 s. After cooling the wafer, 2% 950 PMMA A2 (Microchem) was spin-coated on the LOL–ITO–glass wafer at 500 rpm for 5 s followed by 3000 rpm for 45 s. The wafer was then baked on a hot plate at 180 °C for 80 s. Eventually the bi-layer coating procedure made a total 240 nm thickness coating (85 nm PMMA and 155 nm LOL-2000). The thickness was measured by an ellipsometer (Auto EL-2, Rudolph Research Analytical, Hackettstown, NJ, USA). The coated wafer was cut into 37.5 mm × 25 mm chips. The chip was patterned using a scanning electron microscope (SEM) equipped with a nanometer pattern generation system (NPGS, FEI 430 NanoSEM electron beam lithography system, FEI, Hillsboro, OR, USA) at 30 KeV, 33 pA beam current and 1.2 spot size. The chip was then developed using 1:3 methyl isobutyl ketone (MIBK, Sigma)/isopropyl alcohol (IPA, Mallinckrodt Baker, Phillipsburg, NJ, USA) for 90 s followed by being rinsed with IPA for 60 s. To fully eliminate LOL-2000 residue that remained on the ITO surface, additional developing was performed by sonicating the chip in 1:5:5 CD-26 (tetramethylammonium hydroxide, Microchem):H_2O:IPA for 15 s. The chip was then rinsed with deionized (DI) water and dried. Finally, 12 × 12 arrays with 230 nm-wells and 4 μm spacing were patterned on a 50 μm × 50 μm square

2.2. Immunoreagents and buffers

The detailed synthesis of the antibody specific to 3-PBA and competing hapten were previously described by Shan et al. (2004). Briefly, 3-PBA–BSA was used as the hapten and the antibody (polyclonal; Ab 294) was produced by conjugating 3-((2-oxoethoxy)ethoxy) phenoxybenzoic acid and thyroglobulin (Ahn et al., 2007). For labeling the antibody with fluorescein isothiocyanate (FITC), 1 mg of the antibody in 1 ml of 0.05 M borate buffer (pH 8.5) was gently mixed with 12 μl of 1 mg ml^{-1} N-hydroxysulfosuccinimide fluorescein isothiocyanate (NHS-FITC) for 1 h at room temperature. Free fluorescein was removed by using a desalting column (PD-10, GE Health Care, Uppsala, Sweden). Phosphate buffered saline (PBS) buffer (1 × PBS; 8 g l^{-1} NaCl, 0.2 g l^{-1} KH_2PO_4, 1.2 g l^{-1} Na_2HPO_4, and 0.2 g l^{-1} KCl, pH 7.5) was used for the immunoassay.

2.3. Nanoparticles

Two hundred nanometer-fluorescent-carboxylated-polystyrene (PS)-nanoparticles (FC02F/8251, excitation: 360 nm, emission: 420 nm, Bangs Laboratories, Fishers, IN, USA) or 53 nm-fluorescent-carboxylated-PS-nanoparticles (FC02F/8684, excitation: 480 nm, emission: 520 nm, Bangs Laboratories) were used for testing the EPES or performing competitive immunoassays. The size of particles was measured by a dynamic light scattering (90Plus, Brookhaven Instruments, Holtsville, NY). The zeta potential of particles was measured by light scattering using a zeta potential analyzer (ZetaPlus, Brookhaven Instruments).

2.4. Competitive immunoassay for 3-PBA

A schematic of the 3-PBA competitive immunoassay is illustrated in Fig. S1. Two hundred nanometer-fluorescent-carboxylated-polystyrene (PS)-nanoparticles were washed two times with DI water to remove surfactant on the surface of particles. For passive adsorption of the 3-PBA–BSA to the nanoparticles, 160 μl of 2.6 mg ml^{-1} 3-PBA–BSA dissolved in PBS was mixed with 1 ml of 0.05% (w/v) fluorescent-carboxylated-PS-nanoparticles suspended in DI water and 840 μl of PBS. The mixing time proceeded for 2 h at room temperature, followed by overnight incubation at 4 °C. The mixed solution was then washed three times and finally suspended in DI water. To determine the minimum concentration of 3-PBA fluorescein labeled antibody needed for the competitive assay, the fluorescein labeled antibody was serially diluted with PBS by 10^{-3}–10^{-4} and tested on the 3-PBA–BSA coated fluorescent-carboxylated-PS-nanoparticles trapped into nanowells without free 3-PBA to compete. The results were then compared to the negative control using mouse-IgG conjugated with TRITC diluted by 10^{-3}. The 3-PBA analyte solutions mixed with the 3-PBA fluorescein labeled antibody at a 1:1 volume ratio (total 25 μl) was dropped onto the nanoarray where 200 nm-fluorescent-carboxylated-PS-nanoparticles–3-PBA–BSA were already accommodated. The nanoarray was then incubated for 30 min at room temperature followed by removal of the solution with the same method that was used to remove untrapped nanoparticles. The solution is removed by the Couette-flow that is established by the relative motion between the moving top plate and