Impedimetric Immunosensing in a Porous Volumetric Microfluidic Detector

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Abstract

A sensitive and rapid impedimetric immunosensor is demonstrated utilizing porous volumetric microfluidic detection elements and silver enhanced gold nanoparticle probes. The porous detection elements significantly increase capture probe density and decrease diffusion length scales compared to conventional planar sensors to improve target capture efficiency and enhance impedance signal. In this work, a packed bed of silica beads functionalized with antibody probes serves as a porous sensor element within a thermoplastic microchannel, with an interdigitated gold electrode microarray used to measure impedance changes caused by the concentration dependent formation of silver aggregates. The measured impedance change is independent of electrode spacing, enabling a device with low resolution electrodes to achieve a sandwich immunoassay detection limit between 1-10 ng/mL with a 4-log dynamic range, with a total assay time of 75 min.

Keywords: impedance sensing; conductometric; immunoassay; nanoparticles; silver enhancement

1. Introduction

Conventional impedimetric sensors employ macroscale electrodes immersed into a sample solution to measure solution impedance as a function of biomolecule concentration [1,2]. More recently, miniature impedimetric biosensors have employed interdigitated electrode array (IDA) topologies that increase electrode surface area and reduce inter-electrode spacing [3–6], thereby reducing assay times, detection limits, and detection volume compared to macroscale electrodes [5]. With these miniature sensors, the impedance between IDA pairs varies as biomarkers are captured on the microchannel surface between the electrodes or on the electrode surface itself. The capture probe density is thus constrained by capture probe functionalization efficiency and microchannel geometry [7]. In particular, thermoplastic surfaces that enable low-cost disposable assays tend to offer low functionalization efficiency due to inefficient immobilization chemistries [7,8]. In addition, high resolution electrodes are required to concentrate the electric field near the surface where biorecognition events occur to maximize signal [4,6,9]. However, even when using sub-micrometer electrodes for impedimetric immunoassays, relatively high detection limits on the order of tens of $\mu\text{g/mL}$ are common [4].

To increase sensitivity, gold nanoparticles (AuNPs) have been employed as active components in microwell-based impedimetric immunoassays. With secondary antibodies attached to AuNPs, the gold

immunoconjugates bind with target captured on a functionalized surface, thereby changing the impedance measured across the underlying electrodes. For example, a detection limit around 50 ng/mL and 1 log dynamic range was demonstrated for methamphetamine using this approach [10]. Other work has combined AuNPs with silver enhancement. In the presence of gold and a reducing agent, silver ions nucleate on the AuNPs to create large conductive aggregates [11]. Using this approach, various microwell sandwich assays have been described in which a direct silver conduction path is formed between adjacent electrode pairs [12–14]. However, these assays require multiple silver growth steps for quantitation, offer limited dynamic range, and require long silver growth times using high resolution (6 μm) electrodes to yield low detection limits [13]. Furthermore, the microwell format prohibits automation without the use of robotic handling to execute all assay steps. While various semi-automated microfluidic optical biosensors have been demonstrated using silver enhancement [15–18], the use of AuNPs and silver growth to enhance the performance of microfluidic impedimetric biosensors remains unexplored.

Here we report a flow-through microfluidic impedimetric immunosensor employing AuNP immunocomplexes that is capable of quantitative readout with high sensitivity and dynamic range. A key advance in the present work lies in the use of a porous matrix for the capture of target molecules in a three-dimensional volume. This strategy was originally developed to increase reaction site density and reduce diffusion length scales for optical biosensing [18,19], thereby improving both assay time and sensitivity. By moving from a planar system (Figure 1a) to a volumetric format, the fluidic volume within which biosensing occurs is greatly increased. Additionally, the use of volumetric sensing can relax constraints on electrode resolution, potentially allowing the use of non-photolithographic techniques amenable to high throughput fabrication. Here we explore this concept through the fabrication and characterization of thermoplastic microfluidic chips containing low-resolution IDAs with 100 μm spacing and functionalized silica beads as a porous matrix for volumetric detection (Figure 1b). The silica beads are functionalized off-

Figure 1. Idealized views of a (a) planar and (b) volumetric impedimetric immunosensor. (c) Fabricated thermoplastic device, and (d) magnified view of the detection zone including thin film gold IDA and packed bed of functionalized silica beads in a 150 μm deep channel.

chip, and introduced as a packed bed prior to assay execution. Using this approach, a sandwich immunoassay is demonstrated with a detection limit between 1-10 ng/mL and dynamic range of 4 logs.

2. Materials and Methods

Materials

Sodium citrate, ethanol, hydroquinone, phosphate buffer saline (PBS), bovine serum albumin (BSA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Tween 20, (3-Aminopropyl) triethoxysilane (APTES), and human IgG were purchased from Sigma-Aldrich (St. Louis, MO). Bis (sulfosuccinimidyl) suberate (BS3), and decahydronaphthalene (decalin) were purchased from Thermo Fisher Scientific (Rockford, IL). NR1-3000PY negative photoresist and RD-6 developer were purchased from Futurrex Inc. (Franklin, NJ). Silver enhancement solution, anti-human IgG (rabbit host), and 40 nm AuNPs tagged with anti-rabbit IgG were purchased from Cytodiagnostics (Burlington, ON). Silver acetate was purchased from Carolina Biological Supply Co. (Burlington, NC). Silica beads (20 μm diameter) were purchased from Corpuscular Inc. (Cold Spring, NY). Microfluidic fittings were purchased from IDEX Health & Science (Oak Harbor, WA) and needle tubing was purchased from Hamilton Syringe (Reno, NV).

Device fabrication

Thermoplastic plaques made with cyclic olefin polymer (COP) resin (Zeonor 1020R, Zeon Chemicals, Louisville, KY) were cut into square pieces before washing with methanol, isopropanol, and deionized water (DI), followed by N_2 drying and overnight degassing at 75 $^{\circ}\text{C}$. Interdigitated electrodes were formed using a liftoff process, with a 200 nm Cr/Au layer deposited by e-beam evaporation on patterned negative photoresist, followed by removal of photoresist in acetone to yield interdigitated electrode arrays consisting of 10 μm wide fingers with 10-200 μm spacing between adjacent fingers. A mating COP substrate was then milled to define microchannels 350 μm wide and 150 μm deep using a CNC milling machine (MDX-650, Roland DGA, Irvine, CA). Both substrates were washed with methanol, isopropanol, and DI before N_2 drying and overnight degassing at 75 $^{\circ}\text{C}$, and bonded using a liquid-phase decalin solvent bonding process [20]. After exposure to a decalin/ethanol mixture, the substrates were rinsed with ethanol and nitrogen gas, and manually aligned before pressing with the mating side by hand. The multilayer chip was then pressed at 200 psi at 30 $^{\circ}\text{C}$ in a hot press (AutoFour/15, Carver, Wabash, IN) to complete the bonding. Fluidic interfaces for off chip syringe pumps were created by inserting needle tubing segments into the access ports following a previously reported method [21] and attaching fluidic fittings after the packed bed was formed.

Immunoassay procedure

Silica beads in a 5% wt. aqueous solution were functionalized with amine groups based on a previously reported method [22]. The beads were washed in piranha solution for 1 hr and rinsed 3 times with DI and ethanol. Amine groups were introduced by incubating the beads in a solution of 5% APTES, 5% DI, and 90% ethanol (v/v) under agitation for 1 hr at room temperature and rinsed 3 times with ethanol and PBS. Human IgG was attached to the beads using BS3, a homobifunctional amine-reactive crosslinker, by combining 900 μL of 50 $\mu\text{g/mL}$ human IgG in PBS, 100 μL of bead solution, and 35 μL of 7.19 mM

BS3 in DI and incubating 30 min under agitation at room temperature. Control beads were functionalized by substituting human IgG with 2 mg/mL BSA in PBS. The reaction was quenched by adding 100 μ L of 1 M tris buffer before agitating for 15 min at room temperature. The resulting mixture was washed 3 times with PBS and suspended in 2 mg/mL BSA in PBS overnight at 4 °C. The functionalized beads were packed into a microfluidic device using vacuum through the side access channel into the restricted sensing region using a syringe and needle in the fluidic outlet port. All chips were stored at 4 °C with tests conducted within 24 hr of fabrication.

Devices were flushed with 2 mg/mL BSA in PBS for 15 min before infusion of serial dilutions of anti-human rabbit IgG (0.01-10 μ g/mL) and 2 mg/mL BSA (control) in PBS for 15 min followed by a 15 min PBS rinse. Next 40 nm gold nanoparticles functionalized with anti-rabbit IgG diluted 1:50 (v/v) in 1% BSA and 0.05% Tween 20 in HEPES buffer were infused for 15 min followed by a 15 min PBS rinse. Silver enhancement solution was prepared using a two part mixture [23] chosen due to its stability under light exposure, consisting of 5.0 mg silver acetate in 5 mL DI and 6.25 mg hydroquinone in citrate buffer at pH 3.8. The silver enhancement solutions were combined in an off chip junction and infused for 15 min followed by a 15 min PBS rinse.

Electrical connections were made using 1 mm diameter wells formed in the chip capping layer before bonding, providing access to the metallization layer using external probes. During testing, the probes were connected to an LCR meter, and impedance measurements were performed using a 50 mV amplitude sinusoidal input at 1 kHz. The impedance magnitude was measured during active PBS rinsing before (Z_0) and after (Z) silver enhancement. Assay output was determined from the normalized impedance change, $(Z_0 - Z)/Z_0$, with the noise floor defined using a negative control (BSA only) as 3σ above the control mean. For all devices used during sandwich assay evaluation, the IDA consisted of 6 electrodes, each 10 μ m wide and spaced 100 μ m apart.

3. Results and Discussion

A fabricated device including inlet and outlet needle ports for fluid control and probe tips for electrical connection to the on-chip electrodes is shown in Figure 1c,d. The electrodes remained undamaged during bonding, avoiding issues of electrode discontinuities that have been reported in other thermoplastic microfluidic bonding processes [24]. Based on the chip design, an equivalent circuit model for the volumetric sensor was developed (Figure 2). The total volume probed by the electric field is defined by the geometry of the interdigitated electrode array. Approximately 95% of the total current that passes between the electrodes travels within a height equivalent to the center-to-center spacing between each electrode pair [9]. Thus, for the present case of an array of 10 μ m wide electrodes spaced 100 μ m apart, nearly all of the current passes through the 150 μ m channel height directly above the electrode array. The contributions to the impedance within this sampling volume consist of the double layer capacitance at the interface between the electrodes and buffer solution (C_e), volumetric capacitance (C_s) associated with the electrical double layer formation on the silver cluster surface, charge transfer resistance (R_s) associated with the movement of

Figure 2. (a) Equivalent circuit model for the porous volumetric sensing platform. (b) Comparison of model and experimental impedance spectra for before and after silver amplification at 100 ng/mL sample concentration.

charge carriers between the ionic solution and the silver cluster surface, series resistance of the buffer solution between discrete silver clusters (R_{sb}) and an additional resistance reflecting the parallel current path through the buffer associated with direct ion transport between the electrodes without silver cluster interaction (R_b). The equivalent circuit model results in a system with two zeros and two poles. At low frequencies the impedance is dominated by the electrode double layer capacitance (C_e), while the bulk buffer resistance (R_b) dominates at high frequencies. A pole/zero pair is introduced at intermediate frequencies, with locations that are functions of all system parameters. In general, silver growth results in decreased impedance associated with the double layer capacitance (C_s) and charge transfer resistance (R_s) of the silver clusters. As silver clusters grow on the AuNP-antibody conjugates during the silver enhancement step, the corner frequency associated with the intermediate pole is reduced, resulting in lower impedance at frequencies between the pole and the higher frequency zero. Because the density of silver aggregates is proportional to the concentration of captured AuNP-antibody conjugates, the total decrease in impedance at intermediate frequencies correlates with target concentration. This behavior can be seen in Figure 2, which presents modeled impedance spectra with different values of C_s reflecting silver cluster growth, together with experimental impedance curves generated during a sandwich immunoassay with 100 ng/mL target concentration.

A sandwich immunoassay was conducted to evaluate the performance of the volumetric impedimetric detection technique, using a chip containing a packed bed of 20 μm silica microspheres functionalized off-chip with human IgG as a capture probe. Before finalizing the electrode spacing for the immunoassay chips, the effect of electrode geometry on sensor performance was first evaluated using interdigitated arrays of 10 μm wide electrodes spaced 15, 40, and 100 μm apart within a 150 μm deep channel, with silica beads functionalized directly with anti-human rabbit IgG. Solutions of 13 fM AuNP-IgG was infused for 15 min before silver enhancement. The background corrected relative impedance change was found to be insensitive to electrode spacing (data not shown), confirming that the impedance change is independent of electrode configuration provided that the microchannel is sufficiently tall to contain the generated electric field. Thus the greatest spacing of 100 μm was selected for further experiments.

For the immunoassay experiments, after infusion of anti-human rabbit IgG at various dilution levels, AuNP-anti-rabbit conjugates were introduced. Following silver enhancement, impedance measurements

were performed using a sinusoidal input with 50 mV amplitude at a frequency of 1 kHz. This frequency was found to lie between the intermediate pole/zero pair described by the lumped parameter model, but well below the high frequency zero defining the upper corner frequency of approximately 50 kHz for the tested devices (Fig. 2). The voltage amplitude was sufficiently low to prevent the formation of bubbles by electrolysis. No change in visible appearance or impedance signal was observed after AuNP capture alone. After silver enhancement, the porous silica bead bed changed color from white to a red-brown color for all positive test samples as expected based on results from prior optical detection studies [25].

The resulting measurements of background-corrected impedance change after 15 min of silver enhancement are presented in Figure 3. Based on these data, a detection limit between 1-10 ng/mL was achieved, with at least a 4 log dynamic range, based on a conservative definition of noise floor based on the mean impedance change for a control test (BSA only) plus 3σ . Measurements at 1 ng/mL remained distinct from the control mean. The demonstrated detection limit is significantly lower than previously reported impedimetric biosensors utilizing planar capture surfaces without silver amplification, which are commonly in the range of $\mu\text{g/mL}$ [4], and compares favorably to microwell-based impedimetric sensors employing silver amplification offering non-quantitative readout [12,14]. The full assay, including all incubation and rinsing steps, was completed in less than 75 min by limiting silver growth time to 15 min.

While lift-off processing was used to pattern the electrodes in this work, it is notable that the volumetric detection elements enable the use of low resolution electrodes that may be economically fabricated using non-photolithographic processes. For example, direct printing of electrodes with 100 μm features can be readily achieved using high throughput roll-to-roll direct deposition, screen printing, and similar large-area processing methods [26,27]. Furthermore, because the silica beads are functionalized off-chip and packed into the device after bonding, there is no need to perform serial functionalization of individual devices as required for planar biosensors. This aspect of device fabrication offers further potential to improve manufacturability of impedimetric biosensors, while also making it possible to realize

Figure 3. Sandwich assay using a silica bead bed with 100 μm electrode gaps. The normalized and background-corrected impedance change was measured at 1 kHz for each concentration of IgG target ($n \geq 3$, error bars $\pm 1\sigma$). Background is defined as the mean control signal plus 3σ .

multiplexed chips by simply packing multiple bead beds with different target probes.

4. Conclusions

This work describes the development of flow-through impedimetric immunosensors that take advantage of selective AuNP capture and silver amplification within porous microfluidic elements, enabling rapid, sensitive, and quantifiable measurements of target analyte within volumetric detection zones. A sandwich assay used to characterize sensor performance achieved a conservative detection limit of 10 ng/mL for an IgG model system. By leveraging the short diffusion length scales inherent in the porous detection elements, the sandwich assay required only 15 min silver enhancement, for a total assay time of approximately 75 min, comparing favorably with conventional planar immunoassays requiring 2~3 hr assay times. The use of low resolution electrodes enabled by the volumetric detection scheme makes the technique amenable to device fabrication using low cost manufacturing techniques. Furthermore, the thermoplastic devices are amenable to use in a portable system requiring only a low-frequency ohmmeter for measurement, offering strong potential for applications in point-of-care testing and beyond.

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