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Flow-through microfluidic immunosensors with refractive index-matched silica monoliths as volumetric optical detection elements

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Abstract

A sensitive and rapid absorbance based immunosensor that utilizes ex situ functionalized porous silica monoliths as volumetric optical detection elements is demonstrated in this study. The porous monolith structure facilitates high capture probe density and short diffusion length scales, enabling sensitive and rapid assays. Silica monoliths, synthesized and functionalized with immunocapture probes off-chip before integration into a sealed thermoplastic microfluidic device, serve to capture target antigens during perfusion through the porous structure. Gold nanoparticle immunoconjugates are combined with silver enhancement to create microscale silver clusters, followed by perfusion of an aqueous sucrose solution to limit light scattering and enhance optical signal. Using this approach, detection limits as low as 1 ng/mL are achieved for a sandwich assay, with a dynamic range of at least 4 logs. The results confirm that the combination of on-chip index matching with functionalized porous silica monoliths can enable simple and practical flow-through immunoassays for the sensitive and rapid detection of target antigens.

Keywords

index matching; silver enhancement; volumetric sensing

1. Introduction

Optical detection is a preferred sensing modality for many diagnostic assays for its flexibility, low infrastructure requirements, and potential for high sensitivity measurements. Optical detection is nearly ubiquitous for lateral flow assays that dominate commercial point-of-care diagnostic tests [1,2]. For these tests the biological sample travels by capillary

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action across the substrate and biomarkers within the solution bind to colored or fluorescent antibody-functionalized nanoparticles such as colloidal gold or colored latex beads [3]. These complexes are then captured downstream by immobilized capture probes and accumulate facilitating qualitative analysis by direct optical observation or semi-quantitative analysis using a calibrated colorimetric or fluorescent reader [3]. To improve on lateral flow test performance, microfluidic technology has been widely explored for next-generation diagnostic assay development [2,4]. Microfluidic assays can improve multiple common assay steps including reagent consumption reduction, higher throughput, integration of multiple assay steps, automation, and multiplexing capabilities [4–6]. An important consideration in microfluidic assay design is the material used to construct the device. While previous studies have commonly used glass, silicon, or silicone elastomers as substrate materials [7–10], thermoplastics such as polymethylmethacrylate (PMMA), polycarbonate (PC), polystyrene (PS) and cyclic olefin polymers (COP) are particularly attractive because they are inexpensive, mechanically robust, and scalable for industrial manufacturing by injection molding or hot embossing [7–9]. Thus thermoplastic based lab-on-chip devices show great promise for low-cost diagnostic tests amenable to a diverse array of biological samples [11].

Microfluidic assays can employ various functionalization routes to anchor proteins, peptides, nucleic acids, or other assay-specific capture probes to chemically modified internal microchannel surfaces [4]. The high surface-to-volume ratio inherent to these microfluidic channels reduces the total sample volume and/or incubation time required to deliver a fixed biomarker concentration to capture probes anchored on the channel surface. However, assay sensitivity and dynamic range are constrained by both the geometry and available surface chemistry of the microfluidic channel. The use of planar microfluidic channel surfaces for biomarker capture limits assay performance since each channel wall can be functionalized with, at most, a single layer of probes. For the specific case of thermoplastic substrates, challenging immobilization chemistries limit the effective capture probe density [12,13]. In contrast to planar capture surfaces, porous flow-through biomarker capture elements can significantly increase reaction site density [14,15]. By optimizing porosity and pore size the characteristic diffusive length scale between target biomarkers in solution and capture probes attached to the porous matrix surface is reduced, thereby enhancing assay speed and sensitivity. These porous elements can also improve assay performance by introducing materials with surface chemistries enabling significantly more efficient anchoring of biomolecular capture probes than typical thermoplastic surfaces. A variety of macroporous materials have been explored as matrices for selective biomarker capture in microfluidic systems, including packed bead beds, hydrogels, polymer monoliths, membranes, paper, and porous silicon [4]. However, for optical detection, a common limitation of these materials is that light scattering within the volumetric capture matrix presents an inherent constraint that diminishes sensor performance. Variations in dielectric constant between the porous matrix and fluid within the open pores cause strong coupling with incident light, leading to scattering of photons passing through the matrix [16]. This light scattering can significantly decrease optical transparency, limiting the probed volume and reducing sensitivity for optically based assays. A further limitation is that many macroporous materials such polymer monoliths typically require *in situ* polymerization and functionalization. This

constraint increases fabrication cost and complexity, particularly for the case of multiplexed devices employing multiple sensing elements with different capture probes [17].

Here we demonstrate a microfluidic immunoassay with a simple optical readout that overcomes issues of light scattering and challenging *in situ* synthesis to achieve rapid and sensitive antigen detection. The platform employs off-chip functionalized porous silica monoliths integrated into thermoplastic microchannels as discrete porous detection elements. The rigid silica monoliths are synthesized in a polymer mold using a sol-gel chemistry [18] and then functionalized *ex situ* with antibody capture probes anchored covalently to the monolith surface using silane chemistry. The silica monolith segments are then inserted into solvent softened thermoplastic microchannels to physically embed the outer layer into the channel walls, effectively sealing the monoliths to ensure a uniform flow field through the pores during assay operation [19]. The immunoassay consists of selective capture of immunogold conjugates followed by silver enhancement to create micron scale silver clusters. An aqueous sucrose solution with the same refractive index as the silica is then infused through the monolith to eliminate index gradients and thereby enhance optical signal using a technique previously demonstrated within a microfluidic packed silica bead column [15]. Using this approach, both absorbance and reflectance based measurements were investigated for optical assay readout. For the case of absorbance measurements, a limit of detection (LOD) between 1–10 ng/mL for a direct assay and 10 ng/mL for a sandwich assay were achieved, with linear response over a dynamic range of up to 3 logs. For a reflectance based sandwich immunoassay, an LOD of 1 ng/mL was realized over a 4 log dynamic range after index matching.

2. Materials and Methods

2.1 Materials

Tetramethoxysilane (TMOS), polyethylene glycol (PEG) (10,000 MW), ethanol, acetic acid, phosphoric acid, ammonium hydroxide, citric acid, sodium hydrosulfide, sulfuric acid, sodium citrate, sodium phosphate dibasic, phosphate buffer saline (PBS), bovine serum albumin (BSA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), human IgG, (3-Glycidyloxypropyl)trimethoxysilane (GPTMS), Tween 20, sucrose, tris buffer, and hydroquinone were purchased from Sigma-Aldrich (St. Louis, MO). Decahydronaphthalene (decalin) and N-(γ -maleimidobutyryloxy) sulfosuccinimidyl ester (GMBS), anti-human IgG (rabbit host) were purchased from Thermo Fisher Scientific (Rockford, IL). 5 nm gold nanoparticle (AuNPs) tagged anti-human IgG and anti-rabbit IgG were purchased from Cytodiagnosics (Burlington, ON). Silver acetate was purchased from Carolina Biological Supply Co. (Burlington, NC). Microfluidic fittings were purchased from IDEX Health & Science (Oak Harbor, WA) and needle tubing was purchased from Hamilton Syringe (Reno, NV). Capillary tubing with 360 μ m O.D. and 250 μ m I.D. was purchased from Polymicro Technologies (Phoenix, AZ).

2.2 Silica monolith preparation and functionalization

Silica monoliths were formed in a poly(methyl methacrylate) (PMMA) (US Plastic, Lima, OH) mold using a procedure previously developed for silica monolith production [18]. The

channel side of the mold was milled with a CNC machine 3-axis desktop CNC milling machine (MDX-650, Roland DGA, Irvine, CA) using an endmill with a 45 degree angled tip to a depth of 350 μm to create a negative mold feature with a trapezoidal cross section profile. The milled plaque was sonicated to remove debris and washed with methanol, isopropanol, and deionized water before drying with N_2 and degassing overnight under vacuum at 75 $^{\circ}\text{C}$. The milled mold and a flat PMMA capping layer were exposed to UV ozone treatment for 10 min using a (Novascan PSD-UV, Ames, IA) and pressed together at 75 $^{\circ}\text{C}$ at 2.07 MPa for 10 min using a hot press (AutoFour/15, Carver, Inc., Wabash, IN). Silica monolith precursor solution was prepared by stirring 20 mL of 0.01 M acetic acid in an ice bath for 5 min. Next 1.88 g of PEG was added and stirred for 10 min to fully dissolve before adding 9.28 g TMOS and stirring 30 min in the ice bath. The precursor solution was then introduced into the PMMA mold using capillary action. The channel ends were sealed and the mold was incubated at 40 $^{\circ}\text{C}$ in an oven for 48 h to complete the gelation process. The silica monoliths were then removed from the PMMA mold after removing the weakly bonded capping layer and immersed in 0.01 M ammonium hydroxide overnight at 40 $^{\circ}\text{C}$ to complete solvent exchange. The monoliths were then rinsed three times with deionized water and dried at 120 $^{\circ}\text{C}$ overnight before being heated at 600 $^{\circ}\text{C}$ for 2 h to burn out the PEG, and cooled to 50 $^{\circ}\text{C}$. The monoliths were then cut into 1.2 mm long segments, rinsed three times with ethanol, and then dried.

The silica monoliths segments were functionalized with capture probes as shown in Figure 1a. The monolith segments were immersed in 5% GPTMS in toluene for 18 h, rinsed three times with toluene and ethanol each, and dried. Next the monoliths were immersed in 2 M sodium hydrosulfide in 20% methanol-80% 0.1 M aqueous phosphate dibasic adjusted to a pH of 8.15 with 2 M phosphoric acid for 4 h, rinsed three times with deionized water, and incubated in 0.5 M sulfuric acid overnight at 65 $^{\circ}\text{C}$. The monoliths were then immersed in a solution of 2 mM GMBS in ethanol for 4 h and rinsed three times with 1x PBS. For the direct assay, either the monolith was incubated for 2 h in either serial dilutions of human IgG in 1x PBS ranging between 10 ng/mL to 10 $\mu\text{g/mL}$, or 2 mg/mL BSA in 1x PBS (control) before incubating overnight in 2 mg/mL BSA in 1x PBS at 4 $^{\circ}\text{C}$. For the sandwich assay all silica monoliths were incubated for 2 hours in 10 $\mu\text{g/mL}$ human IgG in 1x PBS before overnight storage in 2 mg/mL BSA in 1x PBS at 4 $^{\circ}\text{C}$.

2.3 Microfluidic chip fabrication

Thermoplastic plates for immunoassay device fabrication were made with Zeonor 1020R cyclic olefin polymer (COP) resin (Zeon Chemicals, Louisville, KY) using a hot press (AutoFour/15). COP chips were cut to the desired size before channels and fluidic access ports were milled with a 3-axis desktop CNC milling machine (MDX-650) and sonicated to remove debris. Channels were 250 μm deep and 780 μm wide at the top and 380 μm wide at the base in the sensing region. The milled microchannel substrate and unmilled capping layer were washed with methanol, isopropanol, and deionized water before N_2 drying and overnight degassing at 75 $^{\circ}\text{C}$. Individual silica monolith segments were then inserted into the device before solvent bonding with decalin as shown in Figure 1a. Briefly, each side was exposed to 30% decalin in ethanol (v/v) for 5.25 min before dipping in ethanol and blow drying excess solvent with N_2 gas. The silica monolith segment was placed into the milled

trapezoidal sensing region using a wetted paintbrush as described [19]. The two COP surfaces were then placed together by hand and pressed at 1.38 MPa for 10 min at room temperature (AutoFour/15) to complete the bonding. Fluidic interfaces for syringe pumps were created by inserting needle tubing segments into the access ports following a previously reported method [20] and were stored at 4 °C until use. All devices were used within 24 h of bonding.

2.4 Direct immunoassay

Syringe pumps were connected to the microfluidic device at room temperature using capillary tubing connected to stainless steel tubing ports press fit into the inlet ports of the chip. First, 2 mg/mL BSA in 10 mM HEPES buffer with 140 mM NaCl was infused for 10 min at 2 μ L/min as a static coating to minimize non-specific protein binding on the monolith surface and channel walls. Next, AuNP-labelled anti-human IgG solution was diluted 1:50 (v/v) in HEPES buffer containing 1% BSA and 0.05% Tween 20 and infused for 15 min at 2 μ L/min, followed by rinsing with 2 mg/mL BSA in HEPES for 10 min at 2 μ L/min to remove unbound sample. Silver enhancement solution was prepared using a two part mixture [21] chosen due to its stability under light exposure, consisting of 1.0 mg silver acetate in 5 mL deionized water and 2.5 mg hydroquinone in 5 mL citrate buffer at pH 3.8. Deionized water was infused for 5 min followed by a 5 min infusion with silver enhancement solution combined in an off chip junction, and a 10 min rinse with deionized water at 2 μ L/min for each solution. Finally an aqueous index matching solution of 68% sucrose (w/w) was infused for 5 min at 3 μ L/min. The refractive index to the sucrose solution was $\sim$ 1.46, closely matched to the refractive index of silica for optimal light transmission through the monolith [15]. The experimental setup is shown in Figure 2.

2.5 Sandwich immunoassay

For sandwich immunoassays, each device was treated in the same manner as the direct assay case. The device was infused (2 μ L/min) sequentially with 2 mg/mL BSA in HEPES buffer (10 min) and anti-human IgG (rabbit host) at concentrations from 0.001–10 μ g/mL in 1x PBS (15 min). This was followed by infusion with 2 mg/mL BSA (10 min) in HEPES buffer, AuNP anti-rabbit IgG conjugate solution (15 min) diluted 1:50 (v/v) in HEPES buffer containing 1% BSA and 0.05% Tween 20, and 2 mg/mL BSA in HEPES buffer at 2 μ L/min for 10 min. Silver enhancement was carried out as described previously with 5 min deionized water rinsing, 5 min silver enhancement, and 10 min deionized water rinsing. Finally a 68% aqueous sucrose solution (w/w) was infused for 10 min (1 μ L/min) through the device.

2.6 Imaging and data analysis

Images of the chips before and after each assay step were captured using a color CCD camera (DFK 41AU02, The Imaging Source, Charlotte, NC). For transmittance imaging, the chip was positioned on an AZ100 Multizoom microscope (Nikon) with diascope lighting provided by a halogen lamp without filtering (Figure 2). Transmittance (T) was determined as the average light intensity of the region of interest (ROI), divided by the intensity of a nearby featureless area, and the absorbance (A) was defined as 1-T. Reflectance images were captured by illuminating the chips with a 150 W ring illuminator light source (Fiber-Lite 180

Illuminator, Dolan-Jenner Industries, Boxborough, MA) attached to a stereoscope (S8 APO, Leica Microsystems, Buffalo Grove, IL). Each chip was positioned with its lower surface 2 mm above a cleanroom cloth (Texwipe TechniCloth Nonwoven Dry Wiper, Fisher Scientific, Pittsburgh, PA), which served to provide a uniform white background with low specular reflection for imaging. Images were captured using the color CCD camera in an episcopic configuration. For both transmission and reflection measurements, images were analysed using ImageJ software. The ROI used to determine the intensity of the silica monolith was 300 μm by 600 μm , and positioned approximately 200 μm downstream from the entrance into the silica monolith. The ROI excluded the sloped sidewalls to ensure consistent optical measurements across samples. In instances where bubbles were observed to form during sucrose infusion, regions containing bubbles were omitted from analysis.

3. Results and Discussion

3.1 Device fabrication

Obtaining reproducible optical signals for the immunoassay requires that fluids flow uniformly through the porous silica monolith structure, rather than entering gaps between the monolith and microchannel sidewalls. During the sol-gel curing process the silica monoliths were observed to shrink, resulting in final monolith elements that were approximately 65–70% the width and height of the original PMMA mold dimensions. Accordingly, the COP microchannels into which the monoliths were integrated were designed to be approximately 10% smaller than the final monoliths to ensure a tight seal [19]. By exposing the mating COP sides to the decalin/ethanol mixture, a solvent softened surface layer formed that, under pressing, filled the outer layers of the silica monolith, creating a tight seal with the microchannel walls as shown in Figure 1c [19]. Visual inspection of fluid flow through the monolith during operation, together with destructive testing of selected devices followed by electron microscopy, was used to confirm that good sealing of the monoliths within the thermoplastic microchannels was achieved. During assay operation, no flow irregularities or changes in fluidic resistance were observed at the flow rates used. This approach contrasts with the use of silica beads to create a packed bed as a porous matrix, which results in a loose structure that can be disrupted during fluid infusion. The sloped sidewalls served to encourage the monolith to settle into the channel after manual placement, and generate a normal force component during sealing that ensured reliable bonding between the monolith and microchannel surfaces without gaps or cracks. The technique was attempted using monoliths with rectangular cross section, but this geometry was found to result in gaps between the channel sidewalls and the monolith, or fracture of the monolith due to stress concentrations during the sealing process. In contrast with *in situ* polymerization or packed bead bed methods used for volumetric capture element creation, there was excellent anchoring of the silica monolith to the COP caused by interlocking of the materials.

A further motivation for employing the *ex situ* integration method was to improve reproducibility of the porous monolith morphology. For *in situ* polymerization, maintaining uniform pore dimensions at the head and tail of the patterned monolith is challenging, since diffusion of reactants at these boundaries during synthesis can result in significant variations

in the resulting morphology. In the ex situ method, this issue is avoided entirely through synthesis of a long monolith segment which is later cleaved into smaller pieces, ensuring that the termini of the final elements exhibit the same properties as the bulk monolith. Once functionalized with IgG the monoliths can be stored up to one week in buffer and 48 hours dry in the fabricated device at 4° C without any discernible loss of reactivity to the target antigens. Longer storage times were not explored in this work, but storage performance is expected to be similar to established immunoassays employing immobilized antibody probes. Furthermore, it may be possible to employ lyophilization and preservation of the on-chip probes with sugars to extend the shelf life for real-world point of care applications.

Several properties of the silica monolith inserts were considered prior to fabrication of the devices. The silica monoliths were somewhat brittle, limiting the length to width ratio (L/W) of the inserted elements. For longer segments ($L/W > 5$), the monoliths often fractured during handling, while shorter segments ($L/W < 0.25$) were difficult to align. For consistent results, monoliths 1.2 mm long and 270 μm tall, with lower and upper widths of 380 μm and 780 μm , were used here. The brittle nature of the monoliths also resulted in small debris breaking off during the placement process that would become trapped in the solvent softened polymer during bonding. Similarly, because the channels are 10% smaller in cross-sectional dimensions than the monolith elements, the upper edges of the monoliths experience high forces during sealing that can lead to surface fracturing and the formation of debris. These particles of debris appear as darkened regions surrounding the edges of the channel, but are fully encased in COP after bonding and therefore do not affect the fluid flow.

3.2 Transmission immunoassay

Microfluidic assays employing silver enhancement with planar capture surfaces require long incubation times to achieve the lowest LOD. However, the porous volumetric capture elements explored here, combined with index matching fluids to limit light scattering within the porous matrix, offer potential to achieve low LOD with shorter incubation times by taking advantage of the high surface area and small diffusion length scale associated with the porous monolith structures. Diffusion within the porous silica matrix is characterized by a length scale of half the average pore diameter, or approximately 1.3 μm . This diffusion length is between 10–100 times smaller than a typical microfluidic immunoassay employing an open microchannel. Since diffusion time scales with the square of the characteristic diffusion length, exceptionally rapid interactions may be expected between perfused analyte molecules and the functionalized monolith surface. At the same time, the volumetric probe density is also greatly enhanced by the use of the porous silica support. Based on the reported porosity of similar silica monoliths [18], the exposed surface area of the 250 μm thick monolith is estimated to be 180 times greater than the area of the underlying channel surface. Thus the use of silica monoliths as porous detection elements provides a significant enhancement to both assay time and volumetric probe density.

Optical absorbance detection was first explored based on the transmission of light from a source positioned on one side of the monolith to a detector located on the opposite side. Most absorbance-based immunoassays utilize some form of amplification to enhance the optical signal, such as enzymatic amplification or the use of a secondary probe to increase

the absorbance signal per binding event. To increase optical signal in the present work, a secondary gold nanoparticle probe was used in combination with a silver amplification step, followed by refractive index matching to decrease the LOD and increase the dynamic range of the immunoassay. The silver amplification step utilized stable, inexpensive reagents [22] to enhance optical detection as previously demonstrated in a variety of microfluidic immunoassays [12,23–25] using colorimetric readers [26], scanometric methods [23,27,28], CCD cameras [24,25], or direct visual inspection with the naked eye [12] to detect silver aggregate formation [22].

A direct assay was first demonstrated to validate the performance of *ex situ* functionalized silica monoliths integrated into thermoplastic microchannels. Serial dilutions of human IgG or BSA (control) were covalently bound to the silica monoliths *ex situ* and then integrated into a sealed microchannel. AuNP-conjugated anti-human IgG was infused into the fabricated device for selective capture on the functionalized silica monolith surface. At high IgG concentrations (>10 µg/mL) the white silica monolith was observed to turn pink to the naked eye, while at lower concentrations no change was observed. Next, optical signal from the captured AuNPs was enhanced by silver amplification to create micrometer scale silver aggregates that turn the monolith a reddish-brown color. Finally, aqueous sucrose solution was infused to match the refractive index of the porous monolith, amplifying optical signal by minimizing light scattering (Figure 3). No removal or displacement of silver aggregates due to flow-induced shear forces during infusion of the viscous aqueous sucrose was observed at the flow rates used in these experiments. The back pressure observed during aqueous sucrose infusion did not result in excessive back-pressure at the flow rates used due to the short length (1 mm) of the monolith insert. Example transmission images of the porous silica monolith capture region after each assay step including index matching are shown in Figure 3a. As the concentration of immobilized human IgG increases, more AuNP's are captured and more silver aggregates form. As the human IgG concentration increases the detection region becomes darker due to absorption of the incident light. The ratio of absorbance measured after index matching (A) to the initial absorbance after silver amplification (A_0) was used to define the relative absorbance (A/A_0). The relative absorbance of the control sample was defined as background and subtracted from the relative absorbance at each concentration to compare performance across assays. By employing background corrected relative absorbance, the impact of variations caused by changes in ambient lighting, lamp output, and variations in chip geometry were minimized to reduce signal variance across different trials.

Relative absorbance measurements from the direct immunoassay covered a range of human IgG concentrations of 0.001–10 µg/mL (Figure 3b.) Defining the noise floor as the average plus 3 standard deviations of the negative control signal, an LOD between 1–10 ng/mL was achieved, with an overall dynamic range of 4 logs. These results verify that capture antibodies immobilized on the silica monolith surface prior to integration into the microfluidic device remain functional throughout the full set of chip fabrication steps.

Relative absorbance measurements from the sandwich immunoassay covering a range of anti-human IgG concentrations (0.001–10 µg/mL) are presented in Figure 3c. As with the direct assay, an LOD between 1–10 ng/mL was achieved, with an overall dynamic range of 3

logs. The LOD is similar to the results of a previously-reported sandwich immunoassay using a planar functionalized thermoplastic surface requiring multiple pipetting steps and 60 min IgG and AuNP conjugate incubation steps to complete [13]. In comparison, the assay presented here requires only 15 min IgG and AuNP conjugate incubation steps by using a simple flow-through protocol that is readily amenable to automation.

3.3 Reflectance immunoassay

A sandwich assay was also evaluated using reflectance imaging of the porous silica capture region. For point of care applications, reflectance imaging offers several advantages over transmission imaging. Illumination and imaging are both done on the same side of the device minimizing the need for a custom optics set-up and facilitating the use of portable cell phone cameras for imaging and analysis. The optical path length examined can be increased up to 2x using reflectance based imaging to enhance sensitivity. Finally the effect of the volumetric capture element depth on variance between devices is reduced since the light will penetrate and reflect back at the same depth and at multiple angles independent of the element size to help average the optical signal. For the completed assay the measurement resolution was much lower for reflectance imaging compared to transmission imaging. In this case, images were captured after anti-human IgG infusion, immunogold conjugate capture, silver enhancement, and index matching solution infusion as shown in Figure 4a. The intensity of the ROI, imaged using an 8 bit color CCD camera, was determined as a function of IgG concentration (Figure 4b). The intensity was then converted to absorbance (A), defined as $A = 1 - I/I_0$, where I is the measured light intensity and I_0 is the maximum signal for the 8-bit camera. Next, the absorbance of the control was defined as background and subtracted from the absorbance measured at each concentration (Figure 4c). After the initial IgG infusion, all silica monolith test regions remained white to the naked eye, with no significant difference in absorbance across different dilutions. Following the introduction of gold immunoconjugates, detectable darkening of the monoliths was observed at the highest antibody concentrations, and an LOD of 1 $\mu\text{g/mL}$ was determined using the control absorbance plus 3 standard deviations as the noise floor, with a dynamic range of 2 logs. After silver enhancement, the LOD was reduced to 100 ng/mL with a commensurate increase in dynamic range to 3 logs. The formation of silver clusters was found to significantly increase the contrast in absorbance between the tested dilutions. Finally, the infusion of index matching solution was successful in significantly reducing the LOD to 1 ng/mL, with a dynamic range of 4 logs. The observed increase in sensitivity and dynamic range was attributed to the ability of the index matching solution to reduce optical scattering within the porous monoliths, thereby allowing a greater portion of the porous silica volume to be accessible during optical detection. In addition, index matching resulted in a significant improvement in absorbance signal uniformity at low target concentrations, compared with silver enhancement alone, thus reducing data variance and further improving the detection limit. The results also confirmed that the assay is quantitative, with optical signal for each IgG concentration tested found to be significantly different ($p < 0.05$, Student's t-test) from other tested concentrations. Following optical index matching (Fig. 4c), the measured data exhibited an average relative standard deviation of 12.7% over the 4-log dynamic range, with a resulting standard curve having a form consistent with a nonlinear dependence

between optical signal and antibody concentration based on Langmuir binding kinetics [29,30].

We note that for each datum presented in Figures 3 and 4, at least 3 replicates were run to assess sensor reproducibility. As noted previously, overall repeatability was found to be relatively poor for the transmission-based assay, while the reflectance-based assay provided significantly improved reproducibility and lower LOD. Various aspects of the fabrication methods implemented in this work, such as uniformity of the monoliths and reproducibility of the monolith functionalization and integration processes, may contribute to unwanted experimental variance, and future efforts to address these issues through more reliable manufacturing methods may enhance sensor performance.

The detection limit and assay speed for the monolith immunosensor compare favorably with similar microfluidic assays employing planar capture surfaces. Performance of the silver-amplified reflectance-based immunosensor before and after index matching is presented in Figure 5, together with other reported microfluidic sandwich immunoassays employing metal nanoparticle amplification to enhance sensitivity. In all cases the reported assay times assume the use of a purified target sample and do not include time required for sample preparation. With the exception of the monolith results, the data in this figure come from microfluidic platforms using planar immobilization surfaces for target capture, with detection performed by either optical absorption [12,13,23,31,32] or electrical readout through the use of interdigitated microelectrodes to detect impedance changes resulting from the growth of metal nanoparticles or aggregates [23,33–38]. As revealed by this figure, the use of volumetric target capture and transmittance-based detection within the silica monoliths provides enhanced performance compared with the planar devices. Evaluation of the relative performance of different immunoassay platforms is challenging due to the inverse relationship between assay time and detection limit. To avoid this constraint, a useful figure of merit for comparing the performance of different immunoassays is the inverse of the product between detection limit and assay time. Using this metric, the reflectance-based monolith immunosensor provides significantly enhanced performance compared to the surveyed planar optical immunosensors, with a figure of merit over 80% higher than the nearest comparison from the cited literature.

The results presented here emphasize the utility of volumetric target capture and detection using a combination of silver enhancement and index matching to improve performance of simple optical assays. The achieved detection limit of 1 ng/mL suggests the platform is suitable for a range of practical assays, for example the detection of common foodborne pathogens such as Norwalk virus, where the detection of serum IgA concentrations on the order of 1 µg/mL is sufficient for diagnosis [39], or measurement of cardiac troponin, a clinically important biomarker of heart attack severity, that requires detection limits on the order of 10 ng/mL [40]. Furthermore, the materials explored in this work are compatible with disposable point-of-care diagnostic devices due to the inherently low material and fabrication costs associated with thermoplastic microfluidics. Although direct machining of the microchannels was employed for rapid prototyping of the devices, thermoplastics are amenable to high throughput replication methods including injection molding and roll-to-roll imprinting, making these materials ideal for applications demanding exceptionally low

unit cost. For point-of-care applications, long term storage of the chips will be necessary, introducing the potential for degradation of the monolith-bound capture proteins. To prevent loss of capture probe functionality over time, it may be necessary to dry or lyophilize the antibodies in a sucrose or trehalose matrix before integrating the monolith elements into the final devices. Lyophilization of antibodies in the presence of sugars stabilizes proteins by maintaining their native conformation during long term storage [41], while removal of the sugar layer may be rapidly achieved during sample introduction.

Finally, we emphasize that *ex situ* functionalization of porous silica monoliths enables the parallel functionalization of large numbers of detection elements in an off-chip batch process. This stands in contrast to conventional microfluidic immunoassays that require serial functionalization of microchannel surfaces using attachment reactions that typically require lengthy perfusion and incubation steps. Thus the presented method shifts the time-limiting aspect of device preparation from a serial process to a scalable parallel process suitable for high throughput manufacture. Additionally, batch-scale functionalization also offers future potential for multiplexed assay development by enabling a facile approach to preparing and inserting multiple monoliths targeting different proteins into a single microfluidic device.

4. Conclusion

This work demonstrates the use of *ex situ* functionalized porous silica monoliths integrated within thermoplastic microchannels as a flexible method for sensitive immunoassays using a straightforward interrogation scheme based on optical absorbance measurements. The use of silica as the monolith material facilitates the use of efficient silane attachment strategies for biomolecule functionalization. The functionalized monolith structures were synthesized and conjugated to immunocapture probes in a batch process, offering economical fabrication compared with *in situ* detection element preparation. The subsequent infusion of refractive index matching fluid facilitates the key advantage of porous sensing elements, namely volumetric biomarker capture and detection, by minimizing light scattering that would otherwise compromise optical measurement sensitivity to image the sensing element volume and not just its surface. Significantly, despite the additional assay step required to implement index matching, relatively short assay times were achieved with good sensitivity in the flow-through devices. Transmission immunoassays were completed in 50 min for a direct assay and 75 min for a sandwich assay, with an LOD between 1–10 ng/mL and a 3 log dynamic range, while the reflectance sandwich immunoassay achieved an LOD of 1 ng/mL and 4 logs of dynamic range for the same assay time. Because the monolith detection elements are fabricated and functionalized off-chip, large numbers of inserts may be prepared in a manufacturable and scalable batch process, while multiple inserts functionalized for different targets may be integrated for multiplexed analysis within a single flow-through chip. The resulting chips offer promise for low cost point-of-care devices for applications including environmental monitoring and medical diagnostics.

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Highlights

- On-chip silica monoliths are functionalized with proteins for use as porous supports in microfluidic immunoassays.
- Index matching enhances sensitivity and dynamic range through volumetric detection.
- Off-chip monolith functionalization enables highly scalable microfabrication.
- Sub-pM (ng/mL) detection limits are achieved in a flow-through sandwich assay.

Figure 1.

The fabrication of the microfluidic device is outlined here (A). Porous silica monolith segments created within a mold with a trapezoidal cross section are functionalized with antibodies using the surface chemistry shown. The functionalized inserts are then placed into an open microchannel with a trapezoidal cross section and sealed using solvent bonded cyclic olefin polymer (COP). A picture of the constructed microfluidic device is shown (B) with an SEM image of the detection zone cross section (C) demonstrating a fluidic seal with the channel walls (scale bar 100 μm wide). A magnified SEM image of the porous silica monolith structure reveals ~2 μm globules and an average pore size of approximately 2.5 μm (D).

Figure 2.

Direct immunoassay process using transmittance and reflectance based detection. (a) A silica monolith with human IgG is (b) infused with anti-human IgG attached to gold nanoparticles (AuNP) that are (c) silver enhanced to create large aggregates prior to (d) introduction of index matching sucrose solution to render the volumetric matrix transparent for optical absorbance measurements. Using transmission based imaging the negative control without silver aggregates is more transparent than a high concentration positive sample with silver aggregates. Using reflectance based imaging the negative control test is white while a high concentration test results in a reddish-brown silica monolith due to silver aggregate formation. The dimensions of the various system components depicted in this figure are as follows: $\sim 2.5\ \mu\text{m}$ silica monolith pores, 10–15 nm long antibodies, 5 nm diameter gold nanoparticles, and $\sim 1\ \mu\text{m}$ diameter silver aggregates.”

Figure 3.

(A) Grayscale images of the silica monolith detection region are shown for a direct assay before and after index matching with different concentrations of IgG used during monolith functionalization. Background corrected relative absorbance (A/Ao) plots for (B) a direct immunoassay and (C) a sandwich immunoassay for each concentration of human IgG and anti-human IgG, respectively. For each datum, $n = 3$ and error bars represent $\pm 1\sigma$. All data were collected using the same batch of silica monolith integrated within different fabricated devices.

Figure 4.

(A) Images of silica monolith detection elements before the assay and following each assay step, including AuNP conjugate capture, silver enhancement, and refractive index matching with aqueous sucrose. (B) Measured intensity (arbitrary units) of the detection region for different IgG concentrations is presented for reflectance based images after each assay step. (C) Background corrected absorbance for different IgG concentrations after each step. For each datum, $n = 3$ with error bars $\pm 1\sigma$. All data were collected using the same batch of silica monolith on different fabricated devices.

Figure 5. Detection limit vs. total assay time the reflectance-based monolith platform, and for reported microfluidic sandwich immunoassays utilizing planar capture surfaces and metal nanoparticle amplification, with either optical or electrical interrogation used for assay readout.