Use of Polymer Micropillar Arrays as Templates for Solid-Phase Immunoassays

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ABSTRACT: We investigate the use of periodic micropillar arrays produced by high-fidelity microfabrication with cyclic olefin polymers for solid-phase immunoassays. These three-dimensional (3D) templates offer higher surface-to-volume ratios than two-dimensional substrates, making it possible to attach more antibodies and so increase the signal obtained by the assay. Micropillar arrays also provide the capacity to induce wicking, which is used to distribute and confine antibodies on the surface with spatial control. Micropillar array substrates are modified by using oxygen plasma treatment, followed by grafting of (3-aminopropyl)trimethoxysilane for binding proteins covalently using glutaraldehyde as a cross-linker. The relationship between microstructure and fluorescence signal was investigated through variation of pitch (10–50 μm), pillar diameter (5–40 μm), and pillar height (5–57 μm). Our findings suggest that signal intensity scales proportionally with the 3D surface area available for performing solid-phase immunoassays. A linear relationship between fluorescence intensity and microscale structure can be maintained even when the aspect ratio and pillar density both become very high, opening the possibility of tuning assay response by design such that desired signal intensity is obtained over a wide dynamic range compatible with different assays, analyte concentrations, and readout instruments. We demonstrate the versatility of the approach by performing the most common immunoassay formats—direct, indirect, and sandwich—in a qualitative fashion by using colorimetric and fluorescence-based detection for a number of clinically relevant protein markers, such as tumor necrosis factor alpha, interferon gamma (IFN-γ), and spike protein of severe acute respiratory syndrome coronavirus 2. We also show quantitative detection of IFN-γ in serum using a fluorescence-based sandwich immunoassay and calibrated samples with spike-in concentrations ranging from 50 pg/mL to 5 μg/mL, yielding an estimated limit of detection of ~1 pg/mL for arrays with high micropillar density (11561 per mm²) and aspect ratio (1:11.35).

KEYWORDS: cyclic olefin polymer, microfabrication, polymer micropillar arrays, solid-phase immunoassays, surface modification, wicking

INTRODUCTION

The question related to size and shape of the reaction site in solid-phase immunoassays is a reoccurring one and is being asked whenever novel avenues for conducting such assays outside the standard microtiter plate (MTP) format are investigated. After all, the surface available for immobilizing and detecting target protein is one of the determining factors for the sensitivity that can be obtained from an assay. The three-dimensional (3D) environment provided by the wells has several undisputed advantages for performing enzyme-linked immunosorbent assays (ELISA) or other bioanalytical tests that rely on antibody (Ab)–antigen (Ag) interactions. Each well can be addressed individually through manual or automated pipetting, making it possible to screen ligands against each other in different combinations. The surface area of a microwell that is in contact with 100 μL of Ab solution is ~1 cm². With a hydrodynamic radius of ~5 nm for an immunoglobulin G (IgG) protein, the maximum number of Abs that can be immobilized on the surface of the well accounts for 1.3 × 10¹², while in practical terms, the number is about half that amount. Determining assay response also benefits from depth provided by the well: For a microplate reader, the optical pass length corresponds to the depth of the liquid inside the well, which is ~3.5 mm. Wells in MTPs are typically arranged in a rectangular 2:3 matrix configuration; the most common format is the 96-well plate which allows for handling volumes in the range 100–300 μL, but variants...
incorporating a larger number of wells (e.g., 3456 with a capacity of \( \sim 1 \mu L \)) are also available.\(^3\)

Further miniaturization of sample and reagent volumes as well as reduction in the spacing between reaction zones often necessitates the transition from a 3D reaction environment to a two-dimensional (2D) array format where proteins are immobilized on a planar support.\(^4,5\) Protein microarrays are typically prepared by robotic printing, and the surface of the supporting slides is functionalized with aldehyde or epoxide for covalent binding through amine groups. By use of microarray spotting, several thousand protein samples can be accommodated on an area of few square centimeters in size. However, assays typically involve a large number of spots since addressing protein samples individually is difficult at this size regime. In addition, the amount of protein that can be attached on a planar surface is limited compared to a 3D environment, which reduces assay sensitivity. These shortcomings also apply when combining 2D protein microarrays with microfluidics.\(^6\)

Flow within a confined environment renders supply of assay components independent of diffusion and so enables reaction volumes and incubation times both to be reduced,\(^9\) which provides inherent advantages for conducting immunoassays.\(^11,12\) While combinations of microfluidics and well plate architectures have been demonstrated successfully,\(^13\) many chip-based ELISA implementations lack the third dimension, that is, depth. To generate depth, topography or physical objects are required so that a 3D surface becomes available inside the channel. Depth can be achieved by using particles, typically implemented in the form of a close-packed column, as a 3D solid-phase reaction matrix to perform ELISA. The increased surface-to-volume ratio provided by the particle matrix inside the channel has been shown to provide enhanced assay sensitivity.\(^16\) An example of a commercial bead-based immunoassay is the fully automated, CD-based Gyrolab xPlore platform.\(^17\) Immunoassays performed in a lateral flow format benefit from depth in a similar way.\(^18\) Lateral flow devices

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**Figure 1.** Implementation of solid-phase immunoassays on polymer micropillar arrays. (a) Photograph of a COP substrate comprising a set of 10 micropillar arrays (of identical configuration) in the form of strips with rounded ends. (b) SEM image of an array segment at higher magnification (\( p = 40 \mu m, d = 18 \mu m, h = 48 \mu m \)). Scale bar: 200 \( \mu m \). (c) Schematic of the different steps used for covalent attachment of proteins on the COP surface. See text for details. (d) Fluorescence micrograph and intensity profile taken at the long edge of a micropillar array (\( p = 40 \mu m, d = 18 \mu m, h = 48 \mu m \)). Scale bar: 50 \( \mu m \). Fluorescence intensities denoted in the graph are as follows: \( I_v \) = intensity along the vertical dimension, \( I_t \) = intensity of the top, \( I_f \) = intensity of the floor, and \( I_0 \) = intensity outside the spotted area. The inset shows a fluorescence micrograph of the entire array. The bright spot in the center of the array results from the initial deposition of a single droplet through contact with a slotted pin. Traces within the array derive from spotting or retraction of solution during incubation. (e) Confocal microscope image (z-stack rendered in 3D) of a high-density pillar array (\( p = 10 \mu m, d = 5 \mu m, h = 33 \mu m \)). Scale bar: 20 \( \mu m \). (f) Fluorescence micrograph and intensity profile of an underfilled micropillar array (\( p = 40 \mu m, d = 18 \mu m, h = 48 \mu m \)). A gradual change in \( I_v \) can be observed along the principal directions of the propagating liquid film (indicated by the white arrows in the image). The intensity profile covers 13 pillars for which absolute values of \( I_v \) decrease by more than 95%. Scale bar: 100 \( \mu m \). Micropillar arrays shown in (d–f) were modified with biotinylated goat anti-human ALB and subsequently exposed to Cy3-labeled streptavidin.
incorporate a porous nitrocellulose membrane strip which promotes flow of sample and reagents by capillarity.19 A change in contrast is generated at the test and control lines which is strong enough to allow for rapid, visual detection of affinity-based interactions without the need for auxiliary readout equipment. In recent years, paper-based substrates have gained increasing attention as a possible alternative to nitrocellulose membranes in lateral flow devices for their ease in adapting immunoassays to a range of nonstandard formats.20

Herein, we investigate the possibility of conducting immunoassays on 3D templates in the form of polymer micropillar arrays (Figure 1). Micropillar arrays exhibit high surface area, promoting capillarity for autonomous movement of liquid—a trait that is attractive for the development of synthetic microfluidic paper substrates.21,22 We fabricate micropillar arrays in Zeonor—a cyclic olefin polymer (COP) that allows for replicating microstructures with excellent lithographic definition (Figure 1a,b)—using thermoforming processes such as hot embossing and injection molding.23,24 Thus, the polymer is suitable for translation and potential production scale-up so that disposables can be obtained in an economically viable fashion.23,24 Zeonor is durable, nontoxic, and optically transparent in the visible and near-infrared regime, while intrinsic fluorescence is also low.23,25 These characteristics render Zeonor and other COP formulations attractive for fabricating polymer-based microfluidic systems for analytical, diagnostic, and life science applications.25,26–29 We previously have shown that periodic arrays of micropillars produced in Zeonor can be used as templates for colorimetric DNA detection.25,29 The approach takes advantage of higher surface-to-volume ratios that these arrays provide compared to planar substrates, making it possible to attach more probe molecules per surface area and so increase the signal obtained by the assay. In a similar way, Suzuki et al. have shown that reactivity and sensitivity of ELISA can be enhanced by forming a film-stack reaction field within a 96-well plate format using micropillar arrays.30 Guo et al. have fabricated thiol-ene micropillar scaffold sheets and obtained stronger fluorescence signal and lower limit of detection (LOD) for immunoassays than by using standard nitrocellulose substrates.22

Another key function of micropillar arrays is their capacity to induce wicking—a phenomenon in which liquid is being drawn by microstructures, resulting in the formation of a thin fluid film.32,33 The propagation of liquid is governed by capillary forces, interfacial pinning, and contact angle hysteresis and shows a strong dependency on surface tension. Wicking has been used as a pumping mechanism for inducing and controlling flow in passive, capillary-based microfluidic systems.34–36 Here, we apply this concept to distributing and confining solubilized proteins (Ag or capture Ab) on the activated COP substrate in a spatially controlled fashion since wicking leads the liquid to self-align and propagate uniformly across the array when placed at a random location within the pillar region. Interfacial pinning thereby prevents uncontrolled spreading of liquid beyond the structural limits, allowing multiple, independent probes to be used in close proximity.

The fact that COP is hydrophobic and chemically inert makes it necessary to functionalize the surface so that proteins can be attached covalently while also promoting wicking of aqueous protein solutions during spotting. Exposure to oxygen plasma has long been used as an effective means for introducing chemical functionality to nonreactive polymers through the formation of polar, oxygen-containing groups such as hydroxyl (−OH) and carboxylic acid (−COOH), among others.25,26,37–39 These groups are compatible with well-established procedures for binding biomolecules such as oligonucleotide probes, enzymes, and proteins covalently on surfaces.40 Here, we functionalize the surface with (3-aminopropyl)triethoxysilane (APTES) to bind proteins covalently using glutaraldehyde (GA) as a cross-linker (Figure 1c). The silanization process involves the reaction of nucleophilic hydroxyl groups generated upon oxygen plasma treatment with the alkoxy moieties in APTES to form a stable silanol (−O−Si−) bond.41 Amine functionalization using APTES has been extensively used for attaching proteins on silicon or glass,24,41–43 but several examples exist where this method has been successfully applied to polymer surfaces, including planar substrates,44–46 MTPs,45,46 and microfluidic systems.45,46 Amine-reactive binding using GA is a convenient and reliable method for covalent immobilization of proteins on solid supports44,48 as aldehyde reacts rapidly with primary amines to form a Schiff’s base linkage.49 Covalent binding—as opposed to noncovalent immobilization based on bioaffinity or physisorption—is preferable when dense and stable protein coverage is envisaged.48 We demonstrate the utility of the approach by performing solid-phase immunoassays in direct, indirect, and sandwich format for a variety of clinically relevant markers, such as alkaline phosphatase (ALP), tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and SARS-CoV-2 spike (S) protein, among others, using both colorimetric and fluorescence-based detection schemes. Colorimetric detection allows for rapid and direct visualization of signal without the need for sophisticated imaging equipment. Fluorescence-based detection, on the other hand, is more sensitive and provides a larger dynamic range for conducting immunoassays in a quantitative fashion.1

**MATERIALS AND METHODS**

**Microfabrication.** Micropillar arrays were produced in Zeonor ZF14-188 (Zeon Specialty Materials, San Jose, CA) by using hot embossing. A master mold was first prepared from a 6 in. silicon wafer (Silicon Quest International, Santa Clara, CA) by using standard photolithography. AZ 3312 positive resin (Integrated Micro Materials, Argyle, TX) was applied by spin-coating followed by baking the wafer at 90 °C for 120 s. The photolithography film was exposed to UV light at 365 nm (Hg i-line) by using a high-resolution chrome-on-quartz photomask (Front Range Photomask, Las Vegas, NV), followed by a postexposure bake at 110 °C for 120 s. Development in AZ-MIF-300 (Merck KGaA, Darmstadt, Germany) was done by immersion for ~1 min. The wafer was rinsed with deionized (DI) water and dried with a stream of nitrogen gas. A hard bake was then performed at 110 °C for 120 s. Topographic structures in silicon were produced by deep reactive-ion etching (Oxford Instruments, Bristol, UK) using variable processing parameters to achieve the desired depth. The remaining photore sist was stripped by using acetone (Sigma-Aldrich, Oakville, ON), followed by drying with a stream of nitrogen gas and exposure to oxygen plasma (Plasmalab80 Plus; Oxford Instruments) for 120 s. Topographic structures in silicon were produced by deep reactive-ion etching (Oxford Instruments, Bristol, UK) using variable processing parameters to achieve the desired depth. The remaining photoresist was stripped by using acetone (Sigma-Aldrich, Oakville, ON), followed by drying with a stream of nitrogen gas and exposure to oxygen plasma (Plasmalab80 Plus; Oxford Instruments) for 7 min. The cleaned silicon master mold was coated with a thin, antiadhesive layer formed from 1H,1H,2H,2H-perfluoroctyltrichlorosilane (Sigma-Aldrich) by using deposition from the vapor phase under reduced pressure. The master mold was replicated with a prepatterned fluorinated ethylene propylene (FEP) sheet (McMaster-Carr, Elmhurst, IL) through hot embossing using an EVG S20 system (EV Group, St. Florian am Inn, Austria), operated at a temperature of 185 °C, an applied force of 15 kN, and a pressure of 10−2 mbar. Sheets of ZF14-188 were embossed with the FEP mold at a temperature of 145 °C, an applied force of 15 kN, and a pressure of 10−2 mbar.

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Table 1. Ab/Ag Combinations Used for Solid-Phase Immunoassays on COP Micropillar Arrays

| assay no. | ELISA type | Ag | primary Ab | secondary Ab | detection |
|-----------|------------|----|------------|--------------|-----------|
| 1 direct  | mouse IgG<sup>a</sup> | sheep anti-mouse IgG (Cy3)<sup>a</sup> | goat anti-rabbit IgG (HRP)<sup>b</sup> | fluorescent |
| 2 indirect | human ALB<sup>b</sup> | rabbit anti-human ALB<sup>b</sup> | goat anti-rabbit IgG (HRP)<sup>b</sup> | colorimetric using TMB membrane peroxidase substrate<sup>c</sup> |
| 3 indirect | human PTH<sup>e</sup> | rabbit anti-human PTH<sup>e</sup> | goat anti-rabbit IgG (HRP) | colorimetric using TMB membrane peroxidase substrate |
| 4 indirect | S/RBD<sup>d</sup> | rabbit anti-SARS-CoV-2 S protein | goat anti-rabbit IgG (HRP) | fluorescent using streptavidin-Cy3 |
| 5 sandwich | S/RBD | rabbit anti-SARS-CoV-2 S protein | rabbit anti-S/RBD (biotin)<sup>d</sup> | fluorescent using streptavidin-Cy3 |
| 6 sandwich | human ALP<sup>b</sup> | mouse anti-human ALP (biotin)<sup>f</sup> | mouse anti-human ALP (biotin)<sup>f</sup> | fluorescent using streptavidin-Cy3 |
| 7 sandwich | human TNF-α<sup>e</sup> | mouse anti-human TNF-α (biotin)<sup>f</sup> | mouse anti-human TNF-α (biotin)<sup>f</sup> | fluorescent using streptavidin-Cy3 |
| 8 sandwich | human IFN-γ<sup>f</sup> | goat anti-human IFN-γ Ab (biotin)<sup>f</sup> | goat anti-human IFN-γ Ab (biotin)<sup>f</sup> | fluorescent using streptavidin-Cy3 |

<sup>a</sup>Jackson ImmunoResearch Laboratories, West Grove, PA.  
<sup>b</sup>Abcam, Cambridge, UK.  
<sup>c</sup>Kirkegaard and Perry Laboratories, Gaithersburg, MD.  
<sup>d</sup>GenScript, Piscataway, NJ.  
<sup>e</sup>BioLegend, San Diego, CA.  
<sup>f</sup>R&D Systems, Minneapolis, MN.  
<sup>g</sup>Thermo Fisher Scientific, Waltham, MA.

10<sup>−2</sup> mbar. All fabrication steps were performed in a clean room (class 1000) environment.

Surface Modification and Spots. COP micropillar array substrates were exposed to oxygen plasma at a pressure of 50 mTorr, a power of 200 W, and a gas flow rate of 10 sccm for 4 min. Freshly oxidized substrates were incubated with a 2% (v/v) solution of APTES (Sigma-Aldrich) in DI water for 1 h, followed by rinsing with DI water and drying with a stream of nitrogen gas. The samples were then immersed in a 2% (v/v) solution of GA (Sigma-Aldrich) in DI water for 1 h. Upon removal from the GA solution, the samples were rinsed with DI water, dried with a stream of nitrogen gas, and immediately used for spotting. Spots were made by transferring ~200 nL of Ab (or Ag) solution onto the pillar array by using spotted pins (hydrophobic-coated, 500 nL capacity; V & P Scientific, San Diego, CA). The spotting solution typically contained 50 μg/mL of Ab or Ag (Table 1) in PBS or DI water, although higher and lower concentrations were also used occasionally. Biotinylated goat anti-human albumin (Abcam) was also spotted directly onto micropillar array substrates for testing and calibration purposes. The spotted samples were kept in a Petri dish (Sigma-Aldrich) at room temperature for 12 h (overnight). A water-saturated wipe was added to maintain a high degree of humidity inside the Petri dish. The substrates were subsequently rinsed with PBST-B (0.01 M phosphate-buffered saline (PBS), pH 7.4; Wisent Bioproducts, Saint-Jean-Sur-Richelieu, QC), 0.05% (v/v) Tween 20 (Sigma-Aldrich), and 0.5% (v/v) protein blocking reagent (Bio-Rad, Mississauga, ON)). Substrates used for sandwich ELISA were incubated with a 1% (v/v) solution of ethanolamine (Sigma-Aldrich) in PBST-B for 5 min to inactivate the remaining aldehyde functionalities on the surface. They were then immersed in 10× Casein Blocking Buffer (Sigma-Aldrich) for 5 min to limit nonspecific adsorption of assay components on the surface as much as possible. Substrates were finally rinsed with DI water and dried with a stream of nitrogen gas.

Immunomas. Components used for solid-phase immunomas are detailed in Table 1. The substrate was flooded with solution typically containing 5 μg/mL of either Ab or Ag in PBS and incubated for 1 h at room temperature. Once completed, the substrate was rinsed with PBST-B and DI water, followed by drying with a stream of nitrogen gas. Fluorescence-based immunomas were performed by using streptavidin-Cy3 (Amersham, Chicago, IL) at a concentration of 20 μg/mL in PBS. After incubation for 5–30 min, the substrate was rinsed with DI water and dried with a stream of nitrogen gas. Colorimetric immunomas were conducted using streptavidin-HRP (Sigma-Aldrich) diluted 1:50 (v/v) in PBS; incubation with the substrate typically lasted for 30 min. Samples were rinsed with PBST-B, dried with a stream of nitrogen gas, and incubated with 3,3′,5,5′-tetramethylbenzidine (TMB) membrane peroxidase substrate for 20–30 min. Human serum samples (Sigma-Aldrich) were diluted 1:1 (v/v) with PBS to produce a concentration series (from 50 pg/mL to 5 μg/mL) by using human IFN-γ as a spike-in analyte.

Instrumentation. Contact angles were measured on planar Zeonor substrates by using a contact angle goniometer (Model 200-F1) from Ramé-Hart Instrument Co. (Netcong, NJ) with DI water as the probe liquid. Images of advancing and receding drops were recorded with a CCD camera and analyzed using DROPIimage Standard software. Scanning electron microscopy (SEM) images of micropillar arrays were recorded by using a Tabletop Microscope TM3030Plus (Hitachi High-Technologies, Mississauga, ON) operated at a voltage of 15 kV. Pictures of colored spots were taken against white background and analyzed by using ImageJ software (downloaded from https://imagej.nih.gov/ij/; National Institutes of Health, Bethesda, MD). Fluorescence imaging was performed by using an Eclipse Ti-E inverted microscope (Nikon Instruments, Melville, NY) equipped with an iXon Ultra CCD camera (Andor Technology, Belfast, UK). An X-Cite XYLIS Broad Spectrum LED illumination system (Excites Technologies, Waltham, MA) or a C2+ confocal module (Nikon Instruments) were used for excitation. Imaging was done with the microscope objective oriented perpendicular to the horizontal plane of the substrate with the focal plane set to the top of the micropillars. Image acquisition and confocal z-stack reconstruction were done by using NIS-Elements Advanced Research software (Nikon Instruments). We typically acquired images of two segments per spot which were analyzed to determine mean fluorescence values and standard deviation (SD).

RESULTS AND DISCUSSION

Characterization of Functionalized COP Substrates and Patterning of Abs. In addition to providing chemical functionality, the oxygen-containing groups generated on Zeonor during plasma exposure increase surface free energy<sup>50</sup> and promote wetting of the polymer substrate by aqueous solutions. Contact angle goniometry revealed that oxygen plasma treatment renders the COP substrate hydrophilic with both θ<sub>adv</sub> and θ<sub>rec</sub> decreasing to <10° (Table 2), as is expected for a surface that is rich in polar, oxygen-containing species.<sup>25,29</sup> A noticeable increase in θ<sub>adv</sub> has been observed upon incubation of the oxidized substrate with both APTES and GA solution, resulting in θ<sub>adv</sub> = 25°–29° and 40°–44°, respectively. The chemical modification process thus reduces hydrophobicity of the polymer substrate to some extent. For this reason, we avoided the use of organic solvents and grafted APTES by using a purely aqueous solution, as has previously been shown to provide an effective and convenient way for amino modification of silica surfaces.<sup>51–53</sup> It is also worth...
Table 2. Wetting Properties of Planar (Nonstructured) Zeonor Substrates

| Surface Condition                  | $\theta_{sw}$ (°) | $\theta_{ad}$ (°) |
|-----------------------------------|-------------------|-------------------|
| pristine                          | 107.3 ± 0.4       | 90.1 ± 0.8        |
| after O$_2$ plasma treatment      | <10               | <10               |
| after O$_2$ plasma treatment and incubation with APTES | 26.9 ± 1.6       | <10               |
| after O$_2$ plasma treatment, incubation with APTES and incubation with GA$^a$ | 42.1 ± 1.7       | 12.1 ± 1.9        |

$^a$Average of at least four measurements performed on two different substrates. $^b$See Materials and Methods for details.

noting that we did not perform an annealing step which is frequently used to stabilize the APTES layer grafted on the substrate. We observed that annealing was generally associated with higher contact angles (data not shown), which is consistent with literature reports. We did not perform annealing to maintain a wettability regime that promotes fast, wicking-based propagation of aqueous buffer solution across the micropillar array. Indeed, the wetting properties obtained after GA activation allow for complete filling of the patterned area in a single spotting step. As a result, protein can be distributed across the entire array, as shown by the example in Figure 1d. Here, a biotinylated Ab was selectively immobilized on the micropillar array, and its presence was revealed by incubation of the substrate with streptavidin-Cy3. The sharp transition between high and low fluorescence regions suggests that liquid upon spotting remained well confined within the structural boundaries of the array. Control experiments in which we tested wicking on oxygen plasma-treated COP micropillar arrays without APTES and GA modification revealed that the tendency of solution to spread outside the pillar segment was very high (data not shown). The medium hydrophilicity obtained by the chemical activation process using APTES and GA therefore provides an effective means for achieving the delicate balance desired for a spotting process that aims at homogeneously distributing protein solution by applying a single deposition step while also limiting unwanted propagation of liquid into the surrounding area as much as possible.

As shown in Figure 1d, surface topography strongly influences fluorescence signal obtained from solid-phase immunoassays on micropillar array substrates. Fluorescence intensity displayed by the vertical portions (e.g., side walls) of the pillars ($I_v$) is strongly accentuated in comparison to the signal derived from the horizontal portions of the array, which is consistent with literature reports. For a pillar height ($h$) of 48 μm, $I_v$ is about 6 times the fluorescence intensity of the floor ($I_f$) and 50 times that of the substrate outside the patterned area ($I_o$), which accounts for the signal deriving from both nonspecific adsorption and intrinsic fluorescence of the polymer matrix. The fluorescence intensity displayed by the top of the pillars ($I_t$) slightly deviates from $I_v$ because both surfaces are located at different levels relative to the focal plane. Also, interference with the signal from the side walls is more pronounced for the top segments of the pillars than for the floor given that the diameter ($d$) is only 18 μm. Confocal microscopy imaging suggests that fluorescence signal is distributed over the entire 3D surface of the array, as shown by the example in Figure 1e. Here, the interpillar spacing was...
reduced by implementing a smaller value for \( d \) and the pitch \( (p) \), which translated into enhanced overall signal where contributions from \( I_f \) are largely diminished. These findings suggest that the mean fluorescence signal intensity \( I_m \) is dependent on the proportions of lateral and horizontal segments within the array. Geometrical parameters can therefore be used to tune fluorescence signal by design—an option for which a more detailed account is provided below.

Forming a liquid film that covers the structured area evenly during spotting is a prerequisite for obtaining uniform distribution of protein molecules on the surface. The capacity of the micropillar array—that is, the combined volume of the cavities—can be calculated from the dimensions (e.g., width and length) of the structured area, \( h \), and the fill factor, accounting for the space that is occupied by pillars only. The capacity of the elongated arrays shown here (1 mm in width, 4.5 mm in length, \( h = 48 \) \( \mu \)m, 20.4% fill factor) has been estimated at 164 nL. Applying \( \sim 200 \) nL in a single spotting step at ambient conditions allowed for immersing the array completely in the liquid phase (Figure 1d,e). Increasing the volume considerably above array capacity (e.g., to 500 nL) results in liquid overflowing the structural area. Excess volume thereby causes \( I_f \) to extend outside the array so that the transition between regions of high and low fluorescence becomes more gradual (not shown). However, overfilling does not constitute an impediment to performing solid-phase ELISA on these substrates.

Reducing the volume for spotting, on the other hand, leads to partial filling of the array, which, in turn, results in nonuniform distribution of fluorescence signal across the patterned area. Previous studies have shown that the presence of pillars induces stick–slip behavior to the propagation of the contact line from one row to the next.\(^{33}\) As shown by the example in Figure 1f, \( I_f \) decreases gradually in underfilled arrays. The orientation of the gradient suggests that liquid is wicking preferentially along the direction of the square lattice in both \( x \)- and \( y \)-directions. Ishino et al. have postulated that in a complete wetting regime a thin (molecular) layer propagates on the surface ahead of the liquid film impregnating the micropillar array.\(^{54}\) The gradual change in fluorescence along the flow direction implies that the spreading layer is thinning out as the contact line moves across the array. Confocal microscopy imaging revealed indeed that the level of immersion—reflected by the fluorescence on the sidewalls of the pillars—decreases from 48 \( \mu \)m initially (corresponding to the entire pillar height) to the floor level where the contact line comes to a halt. Differences in \( I_f \) between rows suggest that the fill level is cascading as the liquid spreads along the horizontal plane of the substrate, while the tendency to migrate upward on the vertical pillar walls is very low. We did not attempt an in-depth investigation of the phenomena related to wicking in underfilled arrays as it would exceed the scope of this work.

The effect of the Ab spotting concentration on the signal obtained from fluorescence and colorimetric detection is shown in Figure 2. Fluorescence signal increases over the entire range of concentration investigated here (e.g., from 1 to 100 \( \mu \)g/mL) and displays a sigmoidal response as a result of streptavidin-Cy3 binding to the biotinylated Ab immobilized on the surface (Figure 2a,b). The finding suggests that saturation of the surface with the biotinylated Ab has not been
reached. Thus, Ab concentration constitutes a sensitive factor in the outcome of the assay, which is in agreement with literature data showing that optimum performance for APTES-modified glass slides was obtained when Ab concentration in the spotting solution was around 1 mg/mL.42 For comparison, we also conducted a colorimetric assay by first incubating the substrate with streptavidin-HRP and then with TMB. The appearance of blue colored spots (Figure 2c) indicates binding of the streptavidin-HRP to the biotinylated Ab. The reaction product obtained by TMB conversion is poorly soluble and accumulates at the polymer surface. The colorimetric response of the assay shows less dependency on Ab concentration than its fluorescence-based counterpart: A steep transition of the signal is followed by convergence and plateau formation for Ab concentrations >5 μg/mL (Figure 2d). We have previously shown that color intensifies as the reaction proceeds with time. As the deposit grows and pillars become increasingly decorated with color pigments, the reaction slows and the signal reaches saturation.30 In such a scenario, Ab concentration no longer affects signal intensity in a significant way.

Structure/Signal Relationship for Fluorescence-Based Immunoassays on COP Micropillar Arrays. In our previous work, we have demonstrated that array configuration can significantly impact colorimetric signal resulting from an immunoenzymatic DNA detection process involving TMB conversion.30 To compare variations in

| array no. | p (μm) | d (μm) | UC area (μm²) | pillar density (per mm²) | h = 5 μm | h = 33 μm | h = 48 μm | h = 57 μm |
|-----------|--------|--------|--------------|-------------------------|----------|----------|----------|----------|
|           |        |        |              |                         | d/h      | R        | d/h      | R        |
| 1         | 50     | 40     | 4330         | 462                    | 1:0.12   | 1.29     | 1:0.82   | 2.92     |
| 2         | 20     | 12.5   | 693          | 2886                   | 1:0.40   | 1.57     | 1:2.64   | 4.74     |
| 3         | 10     | 5      | 173          | 11561                  | 1:1.00   | 1.91     | 1:6.60   | 6.99     |

Table 3. Characteristics of COP Micropillar Arrays Used within This Work

Figure 4. Protein detection assays performed on COP micropillar array substrates. (a, b) Direct ELISA: scheme and fluorescence micrograph (Cy3 channel) obtained from IgG detection assay #1 implemented on array #2 (p = 20 μm, d = 12.5 μm, h = 33 μm). Scale bar: 200 μm. (c, d) Indirect ELISA: scheme and photograph showing the outcome of ALB detection assay #2. Arrays with uneven numbers were modified with human ALB while those with even numbers were left empty (outlined in white for clarity). Array configuration: p = 40 μm, d = 18 μm, h = 48 μm. Scale bar: 2 mm. (e, f) Sandwich ELISA: scheme and fluorescence micrograph (Cy3 channel) derived from SARS-CoV-2 detection assay #5 implemented on array #3 (p = 10 μm, d = 5 μm, h = 33 μm). Scale bar: 100 μm. Components used in each assay are detailed in Table 1.
microscale structure, we relied on the concept of the unit cell (UC) which defines the smallest segment that is repeated periodically throughout the array. All characteristic array parameters—d, p, h, and the tilt angle (θ)—are represented in the UC. Herein, we use a similar approach to investigate the relationship between microscale structure and fluorescence signal generated for Ab detection (Figure 3 and Table 3). The test matrix was composed of three different arrays showing variation in p (50, 20, and 10 μm) and d (40, 12.5, and 5 μm) over four levels of depth (h = 5, 33, 48, and 57 μm). For all arrays, we maintained a hexagonal lattice with the smallest segment of the polymer micropillar array. The fluorescence micrographs in Figure 3a suggest that signal intensity increases when (i) reducing the UC dimensions on the lateral plane (through lower values of p and d) and (ii) extending topography along the vertical dimension (through higher values of h). Image analysis reveals that fluorescence response obtained from the assay differs by more than 1 order of magnitude (Figure 3b) for the most extreme parameter combinations tested here—that is, low-density array #1 with the most shallow structures (h = 5 μm) and high-density array #3 with the deepest structures (h = 57 μm). This finding is consistent with the assumption that higher overall surface area leads to a larger number of Abs that are recognized by the assay.

We calculated the ratio (R) between the topographic surface area (SA) and the UC area for each array to account for design changes in a 3D parameter space. As shown in Figure 3c, fluorescence intensity scales linearly with R for all array configurations investigated in this work. A linear fit of the data set accounts for high statistical significance ($R^2 = 0.9896$). The trend differs from that observed for colorimetric assays performed in a diffusion-limited reaction regime for which disparities in the temporal and spatial distribution of color pigments lead to convergence of the signal for arrays with high pillar density and aspect ratio (d/h).56 For these arrays, color pigments are primarily deposited at the top of the pillars, limiting influx of reagents into the deeper portions of the wells. In such a scenario, even considerable changes in array morphology may yield only marginal improvements in the colorimetric signal. In the case of fluorescence detection as performed by using assay #1, incremental changes in p, d, and h translate into a scalable and predictable variation in signal, provided that similar experimental conditions for the preparation and processing of these substrates are maintained. The establishment of this relationship potentially allows for tuning design parameters such that desired signal intensity is obtained over a wide dynamic range compatible with a diverse set of assays, samples, and readout instruments.

**Immunoaassays.** We validated the three most common ELISA formats—direct, indirect, and sandwich—in a qualitative fashion using both colorimetric and fluorescence-based detection schemes (Table 1 and Figure 4). We implemented these assays using micropillar arrays of intermediate or high pillar density for proof-of-concept demonstration. For direct ELISA (Figure 4a), we immobilized Ag on the polymer surface and revealed its presence by using a fluorescently tagged detection Ab. The corresponding fluorescence image (Figure 4b) reveals that mouse IgG can be detected faithfully by using sheep anti-mouse IgG conjugated with Cy3 (assay #1). For indirect ELISA (Figure 4c), Ag was spotted onto the polymer surface and revealed its presence by using a nonlabeled primary Ab followed by a two-step detection process involving a nonlabeled primary Ab specific to the target and an HRP-conjugated secondary Ab in conjunction with TMB as colorimetric substrate. The image in Figure 4d shows blue spots with high contrast and uniformity deriving from assay #2, confirming the presence of human albumin (ALB) on the modified arrays. Nonmodified arrays show faint colorimetric background deriving from nonspecific adsorption on micropillars. The same result was obtained for assays #3 and #4, where parathyroid hormone (PTH),55 which is involved in regulating serum calcium concentration, and SARS-CoV-2 S protein56 were detected by using a target-specific primary Ab along with an HRP-labeled secondary Ab. For sandwich ELISA (Figure 4e), capture Ab was immobilized on the micropillar array to bind target Ag from solution, which was revealed by

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**Figure 5.** Quantitative detection of IFN-γ in serum using sandwich ELISA on COP micropillar array templates. (a) SEM images (top and close-up cross-sectional view) of array #3 (p = 10 μm, d = 5 μm, h = 57 μm) at different magnifications. The scale bar in the inset corresponds to 20 μm. (b) Fluorescence micrographs of the array obtained by performing assay #8 (Table 1) using different spike-in target concentrations. (c) Plot of fluorescence intensity as a function of IFN-γ concentration. Each condition was validated by using four independent tests (n = 4). LOD was estimated at ~1 pg/mL by using linear regression of the data set.
using a biotinylated secondary Ab and Cy3-conjugated streptavidin. This approach is exemplified by assay #5 which allows for sensitive detection of SARS-CoV-2 S protein (Figure 4f) using a tailored Ab pair. Other proteins successfully detected in sandwich ELISA format include ALP, \(^{57}\) a glycoprotein catalyzing the hydrolysis of phosphate monoesters, as well as TNF-\(\alpha\) \(^{58}\) and IFN-\(\gamma\) \(^{59}\) which both are cytokines involved in immune and inflammatory responses (assays #6–8). All of these markers are used for assessing human physiological conditions and monitoring disease progression in patients.

We tested quantitative detection of IFN-\(\gamma\) in serum using calibrated samples with spike-in concentrations ranging from 50 pg/mL to 5 \(\mu\)g/mL (Figure 5). Experiments were conducted by using high-density array #3 (11561 pillars per mm\(^2\)) with an aspect ratio of 1:11.35 (Figure 5a) to maximize calibrated samples with spike-in concentrations ranging from 50 pg/mL (Figure 5). Experiments were conducted by using high-density array #3 (11561 pillars per mm\(^2\)) with an aspect ratio of 1:11.35 (Figure 5a) to maximize calibrated samples with spike-in concentrations ranging from 50 pg/mL to 5 \(\mu\)g/mL (Figure 5). LOD was estimated at ~1 pg/mL by using linear regression of the data set and the signal of a nonspecific (negative) serum control sample plus 3 \(\times\) SD as a cutoff value. \(^{60}\) For healthy individuals, plasma levels of IFN-\(\gamma\) are typically in the range of 10 pg/mL, while infection or disease can upregulate IFN-\(\gamma\) to 1000 pg/mL and above. \(^{61,62}\) The LOD obtained here compares well with conventional ELISA kits such as BioNote ELISA and QuantiFERON-TB Gold In-Tube test which have been shown to detect IFN-\(\gamma\) in cultural supernatants from patient samples at 9.3 and 20 pg/mL, respectively. \(^{63}\) Ultimately, LOD is determined by the antibody–antigen binding constant. Most antibody–antigen pairs exhibit binding constants between 10\(^5\) and 10\(^12\) per M, which translates into an equally wide range of detection limits (e.g., from 10\(^3\) to 10\(^{-2}\) pg/mL, respectively). \(^2\) Signal amplification using tyramide \(^{64}\) or tailored nanoparticle conjugates, \(^{65}\) for example, have been shown effective in enhancing assay sensitivity beyond the limits of conventional ELISA formats.

Although the signal obtained from the negative serum control sample was low (e.g., less than half of that obtained for 50 pg/mL), we performed two additional experiments to validate whether background is specific to IFN-\(\gamma\) or not. For example, we exposed a micropillar array substrate modified with IFN-\(\gamma\) capture Ab to nonspecific serum, followed by incubation with biotinylated mouse anti-TNF-\(\alpha\) (taken from assay #7). In addition, we spotted TNF-\(\alpha\) capture Ab on another micropillar array substrate which was incubated with a nonspecific serum control sample. Both substrates were then exposed to streptavidin-Cy3 to probe for nonspecific binding and potential cross-reactivity. Fluorescence signals collected from these arrays were comparable to that of the negative serum control in Figure 5c, suggesting that IFN-\(\gamma\) was not initially present in the serum at detectable levels. The findings also confirm that the specificity of the assay is very high while nonspecific adsorption on both spotted and blank regions of the substrate is relatively low.

**CONCLUSIONS**

In this work, we have shown that microstructured polymer substrates can serve as 3D templates for performing immunoassays using colorimetric and fluorescence-based detection schemes. The configuration of the array thereby plays a key role with regard to the signal obtained by the assay, which, in turn, allows for tuning assay response by geometrical design. Quantitative detection of IFN-\(\gamma\) in serum using a fluorescence-based sandwich immunoassay revealed an estimated LOD of ~1 pg/mL, which is equivalent, if not superior, to what is achieved by standard ELISA formats. The result confirms that low concentration of a target analyte can be detected in a complex sample matrix, provided that a template with adequate pillar density and aspect ratio is used. The hydrophilic nature of the activated substrate along with the use of buffer solutions containing blocking agents has kept nonspecific adsorption of Ab and Ag moieties at relatively low levels, as suggested by the contrast and accuracy achieved for colorimetric and fluorescence signals. The possibility of tuning fluorescence intensity predictably by using geometrical parameters of the array opens a development pathway for using micropillar substrates to screen different protein markers in clinical samples in parallel even when large differences in their physiologically relevant concentrations are anticipated. However, the strength of the approach—that is, producing these 3D substrates in polymer materials rapidly and inexpensively by using high-fidelity microfabrication—also provides practical limitations with regard to feature size, density, and aspect ratio that are achievable. The micropillar polymer templates presented here are compatible with integration into microfluidic systems, either in the form of a coupon or by replicating the micropillar structures monolithically into fluidic circuits. These polymer-based, 3D immunoassay substrates can be incorporated directly into microfluidic chips to aid automation of the analytical process or the deployment low-cost devices for point-of-need testing. Performing assays in a flow regime would improve uniformity in the distribution of molecules along the vertical height of the pillars, while also shortening the response time compared to passive incubation where adsorption processes are limited by diffusion.

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