Multiphoton-Polymerized 3D Protein Assay

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ABSTRACT: Multiphoton polymerization (MPP) enables 3D fabrication of micro- and nanoscale devices with complex geometries. Using MPP, we create a 3D platform for protein assays. Elevating the protein-binding sites above the substrate surface allows an optically sectioned readout, minimizing the inevitable background signal from nonspecific protein adsorption at the substrate surface. Two fluorescence-linked immunosorbent assays are demonstrated, the first one relying on streptavidin–biotin recognition and the second one on antibody recognition of apolipoprotein A1, a major constituent of high-density lipoprotein particles. Signal-to-noise ratios exceeding 1000 were achieved. The platform has high potential for 3D multiplexed recognition assays with an increased binding surface for on-chip flow cells.

KEYWORDS: two photon lithography, direct laser writing, HDL, immunoassay, functional polymers, confocal microscopy

INTRODUCTION

Functional proteome analysis holds great promise for a better understanding of diseases at the individual patient level.1 Hence, technologies allowing proteome analysis continuously gain importance. Typically, the proteome is analyzed with protein microarrays. The relative amounts of target proteins in blood sera, saliva, urine, and so forth indicate the function of an organism and disease progression.2,3 The sore spot of many research areas such as photonics, 13 chemical or physical properties.19,21,26

ABSTRACT: Multiphoton polymerization (MPP) enables 3D fabrication of micro- and nanoscale devices with complex geometries. Using MPP, we create a 3D platform for protein assays. Elevating the protein-binding sites above the substrate surface allows an optically sectioned readout, minimizing the inevitable background signal from nonspecific protein adsorption at the substrate surface. Two fluorescence-linked immunosorbent assays are demonstrated, the first one relying on streptavidin–biotin recognition and the second one on antibody recognition of apolipoprotein A1, a major constituent of high-density lipoprotein particles. Signal-to-noise ratios exceeding 1000 were achieved. The platform has high potential for 3D multiplexed recognition assays with an increased binding surface for on-chip flow cells.

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RESULTS

The 3D platforms were tested for two recognition assays. First, a streptavidin–biotin interaction is shown. Second, we show antibody recognition of apolipoprotein A1 (ApoA1) in high-density lipoprotein (HDL) particles. HDLs are transport vehicles for lipids and proteins and are responsible for cholesterol transport from tissues to the liver. Screening of
the HDL proteome has identified significant protein alterations in a variety of diseases, including cardiovascular diseases.38−42 Up to now, the characterization of the protein profile of HDL occurs with complex and time-consuming methods, for example, with liquid chromatography−mass spectrometry and size exclusion chromatography followed by reverse phase protein arrays.43 Our method represents a simplified approach for HDL protein profiling.

The photoresists used for MPP fabrication were a mixture of the monomers pentaerythritol triacrylate (PETA), 2-carboxyethylacrylate (CEA), and poly(ethylene glycol) diacrylate (PEGDA) (see the Experimental Section part for detailed information). An SEM image of a representative platform is shown in Figure 1b, with one column of binding pins shaded in blue. The platforms measure 16 μm × 16 μm × 5 μm, having a top square grid with 4 μm periodicity. Figure 1c sketches part of a scaffold (gray) with an attached binding pin (blue), which is covered with immobilized capture proteins. For a fluorescence readout, confocal sections were recorded and focused at the position of the binding pins. Fluorescence was excited by a 532 nm CW laser (Verdi-V5, Coherent) and a 660 nm CW laser (opux 660, Laser Quantum).

Representative confocal slices of a platform incubated with HDL are shown in Figure 1d. The binding pin signal in the XY slice (4 μm above the surface) is well-separated from the surface background signal, which is visible in the XZ and YZ slices. For fluorescence analysis of each platform, a mask of 25 ROIs is placed on the image (Figure 1f). Within these ROIs, the fluorescence signal from a platform was analyzed.

The first fluorescence-linked immunosorbent assay (FLISA) relies on the streptavidin−biotin interaction; see Figure 2a. First, Alexa 555-labeled streptavidin was immobilized on the binding pins, followed by passivation of remaining binding sites with fetal bovine serum. In the next step, the platform has been incubated with a biotinylated mouse IgG antibody as antigen. As a probe, a secondary ATTO 655-labeled anti-(mouse-IgG) antibody was used. Figure 2b depicts a confocal microscopy

Figure 1. Fabrication and the readout of a 3D protein assay. (a) Sketch of the setup used for MPP structuring (excitation at 780 nm) and for the confocal readout (excitation at 532 and 660 nm). (b) SEM image of a 3D platform, with hanging binding pins (5 pins shaded blue). (c) Proteins are immobilized on adhesive binding pins (blue), followed by a confocal fluorescence readout. (d) Confocal optical sections show separation of the signal at the binding pins from the surface background signal. XZ and YZ slices were taken along the horizontal and vertical arrows, respectively. (e) For data acquisition, the binding pin signal was determined in 40 regions of interest (ROIs). For each ROI (6 × 6 pixels, 200 nm pixel size), the collected average photon number was determined. (f) Twenty-five ROIs were used to determine the background signal originating from the carrier scaffold.

Figure 2. Streptavidin-based fluorescence-linked immunosorbent assay (FLISA) on a 3D platform. (a) First, Alexa 555-streptavidin is immobilized on the binding pins. A biotinylated mouse IgG antibody represents the antigen. Detection is performed with an ATTO 655-labeled secondary anti-(mouse-IgG) antibody. (b) Confocal XY-scan of Alexa 555-streptavidin (excitation 532 nm). (c) Confocal XY-scan of ATTO 655-anti-(mouse-IgG) (excitation 660 nm). Linear color scales are shown in the insets. (d) Logarithmic bar chart of Alexa 555 (green) and ATTO 655 (red) fluorescence signals from two experiments, showing the positive assay (left) and the control sample testing for unspecific binding of the secondary antibody (right, without the biotinylated mouse IgG antibody). The specific ATTO 655 signal is ~165 times higher than the unspecific signal. (e) Logarithmic profiles of confocal fluorescence images along the white dashed lines in (b,c), showing a signal to noise ratio of ~10^3.
image of a platform incubated with Alexa 555-labeled streptavidin; Figure 2c shows the corresponding ATTO 655-labeled anti-(mouse-IgG) antibody signal. To estimate the number of molecules per binding pin, single molecule signals were recorded. The fluorescence of individual molecules, sparsely distributed on glass, was collected. For individual Alexa 555-streptavidin molecules, 84 ± 52 fluorescence photons were collected. For single ATTO 655-anti-(mouse-IgG) antibodies, an average fluorescence photon count of 62 ± 43 was measured.

The reported single pin signals show a relatively large SD. This strong pin-to-pin fluctuation is mainly caused by the stochastic chemical labeling process of the proteins and lipoproteins. Typically, labeling as well as protein adhesion to the pins are stochastic processes. Furthermore, proteins as well as lipoproteins may form clusters during storage. Hence, a broad (Poissonian-like) distribution of the signals of the pins is expected. For the intensity analysis of a whole array, we averaged the signal of all 40 pins. The calculated standard error of this average is typically below 15%.

We calculated the ratio of binding pin fluorescence to individual molecule fluorescence to give an estimate for the number of molecules per binding pin (neglecting different photon emission probabilities at the interfaces of glass, polymer, and buffer). The calculated average numbers of molecules per binding pin are ~3140 streptavidin molecules and ~950 secondary antibodies.

Figure 2d displays the average fluorescence signal obtained from a positive assay and from a control sample, where the primary biotinylated mouse IgG antibody was left out. The specific antibody signal is 165 times higher, compared to the control sample. Figure 2e shows logarithmic profiles of the fluorescence images in Figure 2bc, with a signal to noise ratio exceeding 3 orders of magnitude. The selective recognition of the biotinylated antibody on streptavidin-coated binding pins proves the functionality of the immobilized proteins as well as the applicability of the platform for immunoassays.

A medically more relevant application of the new platform was tested with a FLISA of ApoA1, the main constituent of the HDL particle; see Figure 3a. First, HDL was immobilized on the structure. Passivation of remaining adhesive surfaces was performed with bovine serum albumin in this case because fetal bovine serum contains (bovine) HDL particles. The passivated platform was incubated with the chicken anti-ApoA1 antibody. For visualization of the binding, an Alexa 555-labeled anti-(chicken-IgY) antibody was used. Figure 3b shows a confocal image of a platform incubated with Alexa 467-labeled HDL. Figure 3c depicts the corresponding Alexa 555-anti-(chicken-IgY) fluorescence signal. To determine the number of molecules per binding pin, single molecule signals were measured. The collected average fluorescence photon counts of Alexa 467-HDL and the Alexa 555-anti-(chicken-IgY) antibody were 278 ± 211 and 85 ± 71, respectively. By calculating the ratio of binding pin fluorescence to individual molecule fluorescence, we get an estimated average number of ~500 HDL molecules and ~1420 secondary antibodies per binding pin. These numbers indicate that on average, three Alexa 555-labeled antibodies were attached to one HDL particle via chicken anti-ApoA1 antibodies. The selectivity of the binding was tested in control experiments without incubation with HDL or without chicken anti-ApoA1 antibodies. A comparison of a positive assay versus two control samples is shown in a logarithmic bar chart in Figure 3d. The detected signals of Alexa 467-HDL and the Alexa 555-labeled anti-(chicken-IgY) antibody are comparable for the positive assay (left). For the first control experiment (middle, unspec. binding 1st ab), HDL particles were omitted to determine the nonspecific chicken anti-ApoA1 antibody adsorption, which was detected with the Alexa 555-anti-(chicken-IgY) antibody. The Alexa 555 signal was lower by 3 orders of magnitude compared to the positive assay. For the second control (right, unspec. binding 2nd ab), the primary chicken antibody was not added to the HDL carrying binding pins. Adding the Alexa 555-anti-(chicken-IgY) antibody for detection, a more than 300 times lower signal compared to the positive assay was observed. Figure 3e depicts logarithmic profiles of the Alexa 467-HDL (red) and Alexa 555-labeled anti-(chicken-IgY) antibody (green) fluorescence signals along the dashed lines in Figure 3b,c. The selective recognition of ApoA1 proves the applicability of our platforms for HDL profiling.

DISCUSSION

An obvious advantage of a confocally readout assay, elevated 4 μm above the substrate, is the suppression of background fluorescence from unspecific binding to the substrate. Optical sectioning provided by confocal or multiphoton microscopy enables to observe a single focal plane with a shallow depth of field. The out-of-focus signal is suppressed, which enables higher contrast and sensitivity. Numerous standard biochip readers use confocal detection for 2D assays, without taking advantage of optical sectioning. Nevertheless, an existing
standard reader would allow straightforward integration of 3D assays into existing workflows.

Moreover, there is another big advantage of the proposed 3D assay: the increase in the reactive surface area. In the current case, the proteins were attached to hanging binding pins as shown in Figure 1b,c. The binding pin dimensions were ~450 nm in diameter and ~1 μm in height. The calculated surface was therefore ~1.41 μm² (assuming a cylinder with a half sphere on top), which is ~9 times larger compared to a two-dimensional spot with the same diameter (0.16 μm²). Therefore, an almost tenfold higher number of capture molecules can be presented on the same footprint by the additional extent along the axial direction. This number could easily be further improved, for example, by stacking such structures or writing of longer pins. Especially, in the case of low numerical aperture (NA) objective lenses (NA = 0.5, α = 650 nm), which are typically used in microarray scanners, the low numerical aperture (NA) objective lenses (NA = 0.5, α = 650 nm), which are typically used in microarray scanners, the binding surface in the readout volume could be increased more than 100 times by using subdiffraction structuring, as described in more detail in the Supporting Information. Consequently, on the same footprint, the steric accessibility, the amount of binding sites, and the functional density could also be increased. A higher functional density allows fabrication of even smaller devices, enabling higher throughput and lower sample consumption. Particularly, microfluidics would benefit from the increased binding surface because channels of ~100 μm width limit the available footprint. MPP can be readily combined with microfluidics to overcome diffusive limitations: higher velocities of the Poiseuille flow can be reached for elevated reactive binding pins. To improve the biocompatibility, the platforms could be fabricated out of unsensitized photoresists, avoiding the addition of potentially fluorescent or toxic photoinitiators.

So far, only micro-bead assays a 2.5D polymer living graft polymerization assay, and stacked 2D FLISA micro-arrays have been realized and introduced as 3D assays. On the one hand, because the stacked 2D FLISA consists of planar assays, the background signal is present in each section. On the other hand, micro-bead assays have limitations relating to microfluidics because the micro-bead colloids often clot and disturb the flow and mixing.

In our experiments, the lipoproteins have been purified and 5 ng of HDL has been used for incubation. If an average weight of ~210 kD is assumed, 24 fmol of HDL molecules per platform has been used for prototyping (lower concentrations have not been tested). So far, our unique methodology presented here shows the detection of HDL over 3 orders of magnitude of dynamic range via antibody binding to Apo-A1 using a three-dimensional structure. Thereby, this limit arises from the biochemical interaction/binding selectivity and not from the physical signal detection as shown previously using another system, likewise with single-molecule sensitivity.

CONCLUSIONS AND OUTLOOK

In summary, we have shown the applicability of 3D MPP platforms for proteomics. A two-component structure consisting of a protein-repellent scaffold and submicrometer-sized protein-adhesive binding pins was used as a 3D protein recognition platform. We tested the 3D platforms for two FLISA immunoassays. The first FLISA was a streptavidin–biotin dumb system; the second FLISA was designed for protein profiling of HDL particles. Using a confocal readout, we obtained a signal to noise ratio of over 3 orders of magnitude for the optical signal in both cases. In principle, the design of the 3D platform is flexible, enabling versatile applicability for molecular recognition assays, tissue engineering, and fluid dynamic analysis. The three-dimensional nature of our system increases the surface area with the detection volume and thereby the sensitivity and the magnitude of the dynamic range. Typically, the dynamic range is limited, on the one hand, by the analyte concentration and on the other hand by the number of immobilized probe molecules (specific signal) and the local background. In the case of small dot surfaces, the reaction area limits the number of probe molecules on the surface and hence the maximally obtained signal. Multiplexing has not been tested within one microarray. Multichannel microfluidics might circumvent this limitation. A multiplexed analysis may also be achieved by fabricating binding pins with different chemical functionalities. Using orthogonal chemistry would then allow placement and covalent coupling of different capture molecules. Binding pins with functional polymers could be used to immobilize gold nanoparticles, using them as anchors for label-free plasmonic sensing. Last but not least, the enhancement of the reactive surface due to the 3D geometry and the possibility of optical sectioning provides completely new access to the protein chip design.

EXPERIMENTAL SECTION

The photoresists used for MPP fabrication were a mixture of the monomers PETA (Sigma-Aldrich), CEA (Sigma-Aldrich), and PEGDA (average Mₐ 575, Sigma-Aldrich). The photoresist used for the protein-repellent scaffold consists of a monomer mixture of 4:1 PEGDA–PETA with 1 wt % IRGACURE 819 (BASF Schweiz AG) as the photoinitiator. The binding pins were structured with a photoresist composed of a 9:1 PETA–CEA monomer mixture with 1 wt % IRGACURE 819 as the photoinitiator. Figure 1a shows a sketch of the setup used for MPP lithography and confocal fluorescence microscopy. Acrylate polymerization was initiated via multiphoton absorption of 780 nm ultrashort laser pulses (50 MHz repetition rate, 100 fs pulse duration, FemtRay 780, Menlo Systems GmbH), focused with an oil immersion objective lens (Zeiss α-plan Apochromat, 100x, NA = 1.46). First, the carrier scaffold was fabricated using the protein-repellent PEGDA–PETA photoresist. Next, the binding pins were written using the protein-adhesive PETA–CEA photoresist. Detailed descriptions of the lithography setup and the fabrication process are given in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.7b13183.

HDL preparation and the labeling procedure, a detailed setup description, fabrication protocol, protein assay protocols, data acquisition protocols, and an estimation of surface enhancement by 3D MPP structuring (PDF)

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Notes
The authors declare no competing financial interest.

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