Microarrays Incorporating Gold Grid Patterns for Protein Quantification

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ABSTRACT: Protein microarrays are miniaturized two-dimensional arrays, incorporating thousands of immobilized proteins, typically printed in minute amounts on functionalized solid substrates, which can be analyzed in a high-throughput fashion. Irreproducibility of the printing techniques adopted, resulting in inconsistently and nonuniformly deposited microscopic spots, nonuniform signal intensities from the printed microspots, and significantly high background noise are some of the critical issues that affect protein analysis using traditional protein microarrays. To overcome such issues, in this study, we introduced a novel gold grid pattern-based protein microarray. The grid patterns incorporated in our microarray are equivalent to the spots used for protein analysis in conventional protein microarrays. We utilized the signal intensities from the grid patterns acting as spots for quantifying the protein concentration levels. To demonstrate the utility of our novel design concept, we quantified as low as 66.7 ng/mL of bovine serum albumin using our gold grid pattern-based protein microarray. Our grid pattern-based design concept for protein quantification overcame the signal nonuniformity issues and ensured that the dominance of any distorted signal from a single spot did not affect the overall protein quantification results as encountered in conventional protein microarrays.

INTRODUCTION

Proteomics investigates, under a certain fixed set of conditions, the complete protein complement of a biological system. Over the past decade, proteomics has rapidly grown as a key technique to examine biological pathways and mechanisms and has also assisted in identifying the critical agents involved in such mechanisms. This vital examination of the biological pathway has enabled to elucidate the key bioactive players that govern them in native functions and most notably in new diagnostic and therapeutic discoveries.

Among the numerous tools employed in proteomics, protein microarrays have garnered noteworthy attention as a promising tool capable of furnishing fundamental details about proteins, ligands, analytes, receptors, binding partners, and protein–protein interactions. Protein microarrays, also called as protein chips, typically immobilize multiple proteins on a solid substrate and elucidate protein interactions utilizing miniaturized assays. Attributes of the protein microarray, such as high sensitivity and specificity, high throughput, and low utilization of biological samples, make it an ideal candidate to discover and validate new interaction partners, relative protein assessment in complex samples, biochemical pathway mapping, and discovery of biomarkers for diagnostic and therapeutic usage. 1−5

Over the years, a large number of protein microarray designs have been developed to cater to the demands of various applications and research studies. These array designs can be categorized into three types, namely, functional, 6−11 analytical, 12 and reverse phase. 13−15 Functional protein microarrays aim at the study and description of the function and interaction of various proteins, and analytical protein microarrays are developed for the quantitative detection of analytes in various samples. Reverse-phase protein arrays immobilize an individual complex test sample in each array spot contrary to analytical protein microarrays. To develop such array designs, several fabrication techniques have been explored as these are key to their overall performance and reliability. 6 These range from conventional contact 16−18 and noncontact-based printing 19 to lithography 20−24 and microfluidics, 25−27 which are as diverse as cell-free protein expression-based printing.

Printing reproducibility has been one of the most crucial issues affecting the traditional protein chips. Such an issue results in distorted and nonuniform signals from the minute amount of proteins deposited on the chip surface (spots) and impacts the overall reliability. 28 Furthermore, the signal quality from the printed spots is also governed by the uniformity and quality of the chip surface on which the spots are printed. Improper and poor-quality chip surfaces generate a signifi-
cantly large amount of background noise, in turn influencing the final signal quality.

To address the issues of traditional protein chips, in this study, we report a new design of quantitative protein chip developed using a facile lithographic fabrication method. As opposed to the conventional protein chip design, typically consisting of arrays of circular patterns (spots), our protein chip consists of arrays of grid patterns (spots) with varying areas. Six different grid pattern areas were fabricated and were grouped to form a subwell. The subwells were next arranged in a matrix format having four columns and four rows to form a well. In total, 16 wells were incorporated on our protein chip. Finally, to evaluate the performance of our protein chip, multiple concentrations of bovine serum albumin (BSA) were quantified.

**RESULTS AND DISCUSSION**

We employed a facile lithographic fabrication approach to develop a novel protein chip consisting of grid grid pattern-based array design. A gold surface is used for immobilization of proteins on the solid supports. The functional groups such as thiol (−SH) in a protein have strong affinity to gold by forming a gold–sulfur interaction. The pictorial representation of our protein chip design concept is shown in Figure 1A. The chip consists of a two-dimensional array of sample analysis zones which we refer as wells. In total, 16 wells were accommodated on a single chip, thus permitting to analyze 16 different samples at a time. Each well comprised 16 subwells patterned in a 4 × 4 array configuration. Furthermore, each subwell contained six gold grid patterns with different surface areas. Each individual gold grid pattern acted as an independent microscopic spot, thereby facilitating each subwell with six spots to quantify the protein concentration levels.
The actual fabricated protein chip with 16 wells, optically magnified images for subwells, and different gold grid patterns are presented in Figure 1B–D. The grid patterns typically consisted of gold microlines having a width of 2 μm patterned in a mesh format. The pitch between the microlines was varied to obtain grid patterns with different surface areas. Each gold grid pattern surface area was expressed as a percentage of the subwell area. Typically, the subwell had an area of 1.44 mm². Microlines with pitch values of 8, 14, 20, 35, 50, and 70 μm resulted in grid patterns having surface areas of 5.48, 7.54, 10.52, 17.36, 23.44, and 36.00%, respectively. Care was taken while fixing the separation distance between two subwells to avoid any possibility of contamination. The mask layouts for the protein chip and different gold grid patterns are provided in the Supporting Information, Figure S1.

Conventional protein chips utilize the signal intensities of spots, arrays of circular patterns, to analyze the proteins. In contrast, we analyzed the proteins by plotting a curve based on the intensity of the signals obtained from the six functionalized gold grid pattern surface areas. The gold grid patterns incorporated in our chip design are equivalent to the spots utilized for sample analysis in traditional protein microarrays. The novelty of our work lies in designing the grid patterns as spotting zones and evaluating the protein concentration levels based on the signal intensity curves of the grid patterns.

We next tested the performance of our protein chip by evaluating the BSA concentration levels of 6.7; 66.7; 666.7; 6666.7; 66,666.7; and 666,666.7 ng/mL. In brief, the BSA antibody was first immobilized on the gold grid patterns followed by blocking, biotin labeling, hybridization, and finally measuring the signal intensities from the different gold grid patterns to quantify the protein concentration levels. The fluorescence image of different concentrations of BSA along with the negative control and biotin acting as a control is as shown in Figure 2. Figure 3 summarizes the BSA concentration levels quantified using the grid pattern-based protein chip. To determine the protein concentration levels, the averaged signal intensities from different gold grid patterns incorporated in subwells of a single well were plotted using Mapix (Innopsys, Inc),
France). As shown in Figure 3A, distinct linear-fitted curves were obtained based on the various BSA concentration levels. Reliability of traditional protein microarrays is known to be governed by the specific printing technique adopted for depositing microscopic amounts of proteins (spots). Such dependence is considered as the prime source of irreproducibility, ultimately resulting in nonuniform signals from the spots. Moreover, the undesired background signal in conventional protein chips also affects the result of protein quantification. On the contrary, the novel protein chip we developed quantified the protein using a linear-fitted curve based on the uniform signals obtained from the microlines of the gold grid patterns, as shown in Figure 3B. Because the signals to plot the linear curve were obtained from the grid patterns with varying areas, any possibility of experimental bias or influence of nonuniformity in signal intensities, as encountered in conventional chips to evaluate the protein concentration, was minimized. The protein chip we propose uses a microline which makes a slope using signals from spots that have six different gold area ratios in order to determine the quantification of the protein. It is expected that the slope will change at a constant rate depending on the concentration. Our novel grid-based design ensured that the slope of the linear curve was not dictated by the distorted signal intensity of any specific single spot resulting in stable quantitative values.

We also tested the strength by which the protein binds to the grid patterns incorporated in our protein chip. The typical wash duration we employed was for 15 min and 5 min, repeated for 3 times. To test the binding strength, we performed wash for durations of 5, 10, and 15 min and later quantified the signal intensities using NIH ImageJ. The fluorescence image and signal intensities for different wash durations are shown in Figure 4A,B. Extending the wash duration from 5 to 15 min resulted in the reduction of fluorescence, as indicated by decreased signal intensity, as shown in Figure 4B. However, no significant reduction was noted, which clearly demonstrates that the protein binds relatively strongly to the grid patterns even after employing a long wash duration of 15 min. In addition, this shows that specific binding was performed well with little nonspecific binding.

Over the years, protein microarrays in their diversified forms have emerged as an impressive screening and identification tool. However, in spite of compelling advances, the present day technical challenges have impeded the widespread acceptance of this promising tool in various fields. Some of the issues that require immediate attention include inconsistently and nonuniformly deposited microscopic spots, significantly high background noise, and nonuniform signal intensities from the printed microspots, to name a few. Such obstacles need to be overcome soon for protein microarrays to realize their true potential and satisfy the demands of various fields, or it risks ending up as a mere niche application. We believe, our novel grid pattern-based design will enable in making the existing technology more robust and practically adoptable. Even though protein microarrays may not have achieved their pretechnology hype, yet there exists a little doubt that this tool will inevitably prevail in several fields.
CONCLUSIONS

The numerous benefits of miniaturized assays owing to the printed microscopic spots, in addition to the attraction for printing thousands of such microspots as discrete assay units on a single solid surface, have facilitated protein microarrays to cater to a wide spectrum of applications, low consumption of reagents, and to achieve higher throughput than conventional immunoassays. Over the years, numerous fabrication approaches have been employed to develop protein chips with superior performance and improved reliability levels. In this study, we fabricated a protein chip having gold grid patterns for quantifying the protein concentration levels. We first demonstrated the ability to quantify the protein using the signal intensities of six different gold grid patterns and quantified as low as 66.7 ng/mL BSA concentration level and later assessed the protein binding strength to the gold grid patterns. With our novel grid-pattern-based design concept, experimental bias and the impact of signal nonuniformity on the evaluation of protein concentration was minimized. We believe, our protein chip design will lead to new directions for the development of future generation of protein microarrays having wide-scale acceptance in several fields.

EXPERIMENTAL SECTION

Protein Chip Fabrication. The schematic illustration of the process flow for fabricating the protein chip is shown in Figure 5. The process began with cleaning CZ silicon wafer P-type boron with piranha solution prepared by mixing 70% sulfuric acid (Duksan, Korea) and 30% hydrogen peroxide (Duksan, Korea) in a ratio of 3:1. Next, negative photoresist DNR-L300-40 was spin coated at 4000 rpm for 40 s followed by soft baking at 95 °C for 90 s on a hot plate. Exposure with a chrome mask was performed for 6 s on a mask aligner (MA-6). Post exposure, the wafer was baked at 110 °C for 90 s on a hot plate. The patterns were later developed using AZ 300 MIF for 60 s followed by hard baking at 95 °C for 60 s. Next, titanium 200 Å and gold 1000 Å were deposited on the patterned wafer using an E-beam evaporator (Maestech Inc., ZZS550). The final step consisted of liftoff process wherein the wafer was sonicated in acetone for 5 min followed by methanol and isopropyl alcohol wash for 3 min each and later rinsing the wafer with deionized water.

Surface Treatment. The gold grid-patterned chip was soaked for 30 min in piranha solution and then washed with distilled water. Next, a second wash was performed with 1% SDS solution (Biosesang, Korea) and 1% potassium hydroxide (Samchun Chemical, Korea) using an ultrasonic cleaner (Hwashin Tech, Korea). After washing with distilled water, the chip was dried with nitrogen gas.

Antibody Immobilization. To form a protein G layer on the gold grid patterns, 0.1 mg/mL cy3-protein G (RayBiotech, USA) was reacted with the chip for 1 h. These patterns were washed 3 times and 5 min each with 1× phosphate-buffered saline with 0.1% Tween detergent (PBST). After washing, the antibody dissolved in PBS (Invitrogen, USA) and 1 M magnesium chloride (Biosesang, Korea) was added onto the gold surface and allowed to react for 2 h. In this paper, 2 mg/mL BSA antibody (Thermo Scientific, USA) and various concentrations of BSA (Sigma-Aldrich, USA) peptides were used. Next, the chip was washed 3 times, 5 min each with PBST, and later reacted with 5% Difco skim milk (BD Bioscience, USA) in PBST for 1 h to remove the nonspecifically bound antibody. In addition, biotin was attached to the antigen using a biotin labeling kit (Full Moon BioSystems, USA) and allowed to react for 2 h on an antibody-immobilized gold grid pattern. After washing the chip with PBST for 3 times, 5 min, it was bound to cy3-streptavidin (BioLegend, USA) for 1 h and finally detected by a fluorescence scanner (Innoscan, France). It scanned at 100% PMT gain with 3 μm resolution.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c01549.

Mask layout for fabricating gold grid pattern-based protein chip and six different gold grid pattern designs functioning as six individual spots for protein quantification (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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