PROTEIN BINDING KINETICS IN FILTRATION-BASED PROTEIN MICROARRAY STUDIES

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INTRODUCTION

Protein microarrays, or protein chips, is one of the most promising new technologies for a wide range of biomedical applications [1,2] including protein expression profiling for disease diagnosis [3], studies of protein-protein interactions [4] and enzymatic activities [5]; and the identification of protein-binding molecules such as peptides [6], phospolipids and small molecules [7]. However, most of the current protein microarrays are formed on an impermeable solid surface such as glass coverslide, and the hybridization is performed by incubation with shaking. As such, the reaction kinetics can be limited by the slow diffusion of analyte molecules toward the surface where capture molecules are immobilized, resulting in slow hybridization kinetics and compromised assay performance.

We have developed a new protein microarray technique in which a filtration assay is performed with protein microarrays printed on protein-permeable membranes. Compared with protein microarrays on impermeable solid surfaces with an incubation-shaking method, this new technique overcomes the diffusion limit, leading to significantly improved protein binding kinetics.

MATERIALS AND METHODS

Printing of Microarray

Protein microarrays were printed on porous nitrocellulose filter membranes. Compared with modified glass/plastic surfaces and silanized silicon wafer, nitrocellulose membranes have higher protein-binding capacity and are more biocompatible for immobilized proteins [8]. Instead of using incubation and shaking with the microarray in a binding assay, the sample was filtrated through the microarray-containing filter with multiple cycles to facilitate the binding between analytes and their corresponding capture molecules. Prior to printing, antibodies as capture molecules were dialyzed into spotting buffer that containing 0.05M of sodium phosphate monobasic and 0.05 M of sodium phosphate dibasic, pH 7.4. Printing of microarrays on both nitrocellulose filter membrane and glass coverslide was performed using a non-contact Biochip^{TM} ink-jet arrayer (Perking-Elmer Bioscience). Normally 1-10 nanoliter of protein samples were dispended at a spot-spot distance of 500 μm, and the resulting spot diameter was in the range of 300-400 μm.

Labeling of Analyte Proteins

To quantify the binding kinetics, all the analyte proteins, including HSA, CEA, MigG and neutravidin were labeled with Alexa647 dyes following the protocol recommended by Molecular Probes. The conjugated protein sample was diluted in a buffer containing 0.2% BSA, 10 mM Tris, 0.9% NaCl, with pH adjusted to 7.4.

Hybridization Assay

The microarray-containing nitrocellulose filter membrane was first blocked for 15 minutes by 2% BSA in TBS buffer (Tris Buffered Saline, containing 140mM NaCl, 10 mM Tris-HCl, pH 7.4). 100-200 μl of blocking reagent was filtrated through the filter back and forth with a flow rate of 0.4 cm/s using a customized filtration device. 100-200 μl of protein sample was then filtrated through the filter in the same manner, and the filtration assay typically lasted about 1 hour. For shaking assay, 50-200 μl of sample was assayed for binding to the microarray in a 96-well microplate at 200 rpm. Two identical chips were used for each assay. Following the binding assay, protein chips were washed with the buffer for 5-10 minutes to remove non-specifically bound proteins.

Imaging and Data Analysis

Two instruments were used for imaging the assayed microarrays, and the fluorescence intensities were quantified using the software provided by the instrument manufactures. The first instrument is a confocal-based array scanner (BioChip^{TM}, Perkin Elmer) that has a focal plane of about 30 μm thick. It has a high resolution (down to 5 μm) and high sensitivity (pmol per cm^2) and can provide high quality image of microarrays. In addition, we used a FLA-3000 fluorescence imager (Fuji) which has a wider dynamic range. After quantification of

fluorescence, the signal levels were determined by subtracting the backgrounds (defined by 4 to 9 blank spots on the same array) from the original intensities.

RESULTS

Enhanced Protein Binding Kinetics

To quantify the protein binding kinetics of filtration-based protein microarray, we generated a microarray that contained five different capture molecules, including one monoclonal antibody, mouse monoclonal anti-human serum albumin (AHSA); two polyclonal antibodies, goat anti mouse IgG (GAM) and rabbit anti-CEA (ACEA); one non-antibody protein (Protein G'); and one small molecule conjugated to a protein carrier, i.e. biotin-DNP conjugated to casein (CA-Biotin), with 1.5 biotin-DNP per casein. Their corresponding analytes were human serum albumin (HSA), CEA, mouse IgG (MIgG) for both GAM and Protein G', and neutravidin. The microarrays were assayed with a sample containing all five analyte proteins labeled with Alexa647, with concentrations of 30 pM for HSA, CEA, MIgG and 100 pM for neutravidin. Two different assays were performed, both with identical microarrays on nitrocellulose filters. The first was the *filtration assay* described above, the second was an assay using incubation and shaking (*shaking assay*), in which the microarray-containing nitrocellulose filters were assayed in a microplate well under shaking at 200 rpm with a sample volume of 200 µl.

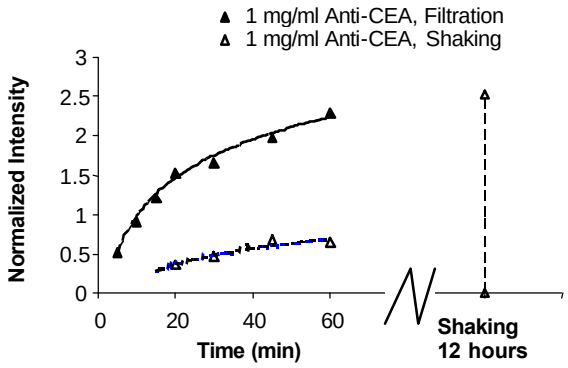

Figure 1. A comparison of the binding kinetics of filtration and shaking assays

A comparison of normalized fluorescence intensities resulted from filtration and shaking assays for CEA binding to 1mg/ml Anti-CEA spots on a microarray is shown Figure 1. It is seen that after approximately an hour, the filtration assay was approaching equilibrium, but shaking assay reached only about 20% of the signal intensity of the corresponding filtration assay. After shaking overnight at room temperature, the shaking assay yielded a signal intensity slightly higher than that obtained in the filtration assay within an hour. The degree of increase in binding kinetics was found to be different for different pairs of molecules due to their diverse intrinsic reaction rates and varied surface molar concentrations.

Analysis of Binding Kinetics

The diffusion limit in solid-phase binding assays was analyzed using a first-order kinetics model for a pair of molecules A and B that react with each other near a surface to form a complex C. When the concentration of B is low, the surface concentration of C, C_S, can be obtained approximately:

$$C_S = \frac{k_{on}}{k_{off}} A_S B_0 (1 - e^{-k_r t}) \qquad (1)$$

where A_S is the surface concentration of A, B_0 is the concentration of B in the bulk solution, k_{on} and k_{off} are the intrinsic on- and off-rate constants of the reaction, and

$$k_r = \frac{k_{off}}{1 + A_S k_{on} k_m^{-1}} \qquad (2)$$

with k_m being a parameter charactering the mass transport. Equation 2 indicates that, although increasing the surface concentration A_S of capture molecules can lead to higher values of C_S, i.e., higher signal intensity for protein chips, it also leads to a reduced value of k_r, resulting in a slower binding kinetics.

CONCLUSIONS

In summary, we have developed a novel filtration-based protein microarray technique, and studied protein binding kinetics using both experimental and theoretical approaches. The protein microarray was constructed on nitrocellulose filter membranes, and the sample was filtrated through the filter to facilitate binding between capture molecules and analytes. Compared to the current protein microarray technique, the filtration-based protein technique was found to have significantly accelerated kinetics, extended dynamic ranges, reduced backgrounds, and improved specificity.

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