RESEARCH ARTICLE

Protein microarrays on hybrid polymeric thin films prepared by self-assembly of polyelectrolytes for multiple-protein immunoassays

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We report here the development and characterization of protein microarrays fabricated on nanoengineered 3-D polyelectrolyte thin films (PET) deposited on glass slide by consecutive adsorption of polyelectrolytes *via* self-assembly technique. Antibodies or antigens were immobilized in the PET-coated glass slides by electrostatic adsorption and entrapment of porous structure of the 3-D polymer film and thus establishing a platform for parallel analysis. Both antigen and antibody microarrays were fabricated on the PET-coated slides, and direct and indirect immunoassays on protein microarrays for multiple-analyte detection were demonstrated. Microarrays produced on these PET-coated slides have consistent spot morphology and provide performance features needed for proteomic analysis. The protein microarrays on the PET films provide LOD as low as 6 pg/mL and dynamic ranges up to three orders of magnitude, which are wider than the protein microarrays fabricated on aldehyde and poly-L-lysine functionalized slides. The PET films constructed by self-assembly technique in aqueous solution is green chemistry based, cost-effective method to generate 3-D thin film coatings on glass surface, and the coated slide is well suited for immobilizing many types of biological molecules so that a wide variety of microarray formats can be developed on this type of slide.

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1 Introduction

Protein microarrays which have burgeoned with the rapid advances in high-throughput screening, nanotechnology, and bioinformatics are becoming an important tool in proteomics, drug discovery, and disease diagnosis. Applications of protein microarrays include the studies of enzymatic activities [1, 2], protein expression profiling [3–5], and interactions of protein–protein, protein–DNA, and protein–ligand [6–10]. Currently, most of the protein microarray to date are constructed by adapting the fabrication method analogous to the existing DNA microarray technology. However, there are several additional challenges with protein-based microarrays because, in general, proteins are more sensitive to their surrounding environment than nucleic acids.

Any strategy to construct protein microarrays requires two steps: (i) deposition of proteins in parallel format on a substrate surface and (ii) immobilization of the arrayed capture probes on the substrate surface. Covalent coupling, physical adsorption, and specific affinity interaction are the

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Abbreviations: APTS, 3-aminopropyltriethoxysilane; **PAAH**, poly(allylamine hydrochloride); **PET**, polyelectrolyte thin films; **PLL**, poly-l-lysine; **PVS**, poly(vinylsulfonic acid, sodium salt); **SEM**, scanning electron microscopy

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proposed methods to immobilize proteins in array format. Although covalent linkage to an activated surface is generally the most stable method of immobilizing protein on microarray [1, 11–18], covalent coupling typically involves multiple-step surface chemistry treatments to obtain the activated substrates for subsequent protein immobilization, and extensive processing protocols have to be followed by after protein microarray fabrication. One of the more popular approaches is the slide surface functionalized with aldehyde groups. These aldehyde groups readily form aldimine (Schiff-base) bonds with primary amines in protein probes, which can be further stabilized by reduction. However, covalent coupling often results in some of the immobilized proteins to lose activity due to the direct chemical modification of the binding site and steric hindrance or strain from multiple attachment sites.

Protein microarray can be also fabricated onto a slide surface through a specific affinity interaction, where protein probes are fused with a high-affinity tag at their amino or carboxyl terminus for the attachment to the chip surface *via* this tag [19–24]. Using this method, immobilized protein probes are more likely to remain in their native conformation, while the analytes have easier access to the active sites of proteins. Besides biotin–avidin affinity interaction [19], fusion proteins containing a His tag were arrayed onto a nickel-coated glass slide [20]. Other affinity methods such as glutathione/GST and phosphonate/serine esterase cutinase ligand/protein tags were also reported [23]. However, the modification of protein probes with affinity tag and the modification of slide substrate with protein A or streptavidin require a peculiar time-consuming process, and in general, an increase in the quantity of reagents.

The most straightforward way to immobilize proteins on a surface is adsorption through noncovalent interactions (*i.e.*, hydrophobic interactions, electrostatic interactions, and entrapment in porous structures). Glass surface coated with a thin NC membrane or poly-L-lysine (PLL) such that proteins can be passively adsorbed to the modified surface through nonspecific interactions have been reported [25–28]. Recently, Lee *et al.* [28] reported fabrication of protein microarrays on a calixcrown-5-modified slide where the protein probes were bound to the crown moiety *via* host–guest interactions and hydrophobic interactions between the hydrophobic residues of a protein and methoxy groups of the calicrown-5 derivatives. The attached proteins lay on the surface in random orientation. However, it has been reported that the passive adsorption of proteins onto the PLL slides often results in protein denaturization because of the hydrophobic nature of many glass surfaces (the unfully coverage of PLL-monolayers on glass surface), nonuniform orientation of molecules, and unstable bonding (leakage). For the thick film-coated slide, such as NC, the noise level is usually higher because of the nonspecific adsorption/absorption and the auto-fluorescence of the materials. However, its simplicity of fabricating protein microarray on slide by noncovalent interactions sometimes compensates for these disadvantages.

In addition to the chemistry used to immobilize proteins, the binding capacity of protein probes on slides surface is also critical for the performance of a protein microarray since the protein samples are often very limited in supply and (unlike nucleic acid) cannot be amplified. In the use of aldehyde- and PLL-functionalized slides, the amount of immobilized protein/peptide is limited to a 2-D surface area, causing a low sensitivity and a low S/N level. Polymer-based 3-D films, such as activated agarose film [29], hydrogel polymer [30], sol-NC film [31], plasma-polymerized film [32], and protein-gel chip were reported very recently to improve binding capacity and thus the sensitivity [33, 34]. In addition to the sophisticated processes of creating such 3-D matrixes which often include photolithography or photopolymerization process, the major disadvantage of these reported 3-D protein microarrays is that the 3-D coatings often have lower reproducibility and a higher background signal caused by auto-fluorescence of the polymer materials.

Thus, there is great demand for new slide surface which provides reliable attachment of protein probes for various functional analyses. Ideally, proteins should be immobilized on a slide such that their native format and their folded conformations are preserved. Recently, there is a growing interest in developing pretreated surfaces by creation of nanostructures on protein-compatible surfaces *via* the polymer-coated substrate such as NC membranes or sol–gel methods to minimize the denaturation of immobilized proteins in protein microarrays [22, 34–36]. We now report a simple procedure to coat glass slides with polymeric thin films by self-assembly of polyelectrolyte multilayered thin films as a platform for fabrication of protein microarrays. Due to the amphiphilicity nature of polyelectrolyte, protein probes are immobilized in semiwet environment by the combination of strong electrostatic adsorption, hydrophobic adsorption, and entrapment of the porous structure, which keep protein probes in an active form. Furthermore, the multilayered polyelectrolyte thin films (PET) provide 3-D structures where high binding capacity can be achieved and the direct contact of protein with hydrophobic glass surface was avoided. In addition to this, PET film is chemically stable, and its adhesion to substrates is strong, which is critical in obtaining a reproducible immunoassay performance. We also demonstrated the fabrication of antigen and antibody microarrays on the PET-coated glass slides as well as the direct and indirect immunoassays on the protein microarrays for multiple-analyte detection.

2 Materials and methods

2.1 Reagents

Amine-reactive Alexa Fluor 488 succinimidyl esters and Alexa Fluor 488-labeled proteins of fibrinogen from human plasma (FIB, p*I* 5.5), avidin (p*I* 10.5) were purchased from

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Molecular Probes (Eugene, USA). Unlabeled proteins of HSA (HSA, p*I* 4.6), myoglobin (MGB, p*I* 7.0), lysozyme (LSZ, p*I* 11.1), α-fetoprotein (AFP), human IgG, and goat IgG, as well as Cy3-labeled streptavidin, Cy3-labeled polyclonal antibodies against AFP, goat IgG, and Cy3-labeled monoclonal antibodies against HSA, human IgG, fibronectin, and avidin were purchased from Sigma-Aldrich. Recombinant human cytokine TNF-α and IL-2, IL-6, monoclonal antibodies against cytokine TNF-α, IL-2, and IL-6, and biotinylated detection antibodies (biotinylated anti-TNF-α, anti-IL-2, and anti-IL-6) were obtained from R&D Systems (Minneapolis, MN). These reagents were chosen to represent a broad class of capture molecules, including monoclonal antibodies, polyclonal antibodies, and nonantibody proteins. Microscope glass slides (76 × 26 × 1 mm), glass cover slips, biotin-anti-goat IgG, and 5 × Denhardt's solution (containing 1 mg/mL each of Ficoll, PVP, and BSA); 3-aminopropyltriethoxysilane (APTS), as well as anionic poly(vinylsulfonic acid, sodium salt) solution (PVS) (25%), and cationic poly(allylamine hydrochloride) (PAAH, M_n 50 000–65 000) were also purchased from Sigma-Aldrich. All the chemicals of commercial origin were used without further purification. Aldehyde-modified slides (SuperAldehyde) were purchased from TeleChem International (Sunnyvale, CA) and PLL-coated slides were purchased from Cell Associates (Houston, TX).

2.2 Protein labeling

Proteins of HSA and MGB were labeled with Alexa Fluor® 488 dye by using the following protocol. Protein solutions at 0.5 mg/mL 0.1 M pH 8.3 sodium carbonate buffer and amine-reactive Alexa Fluor 488 succinimidyl esters at 10 mg/mL in 50% DMSO were prepared before labeling. Equal volume of the protein and dye solutions were mixed thoroughly by repeated pipetting. The reactions were allowed to sit in the dark for 1 h at room temperature mixing approximately every 10 min, and then quenched by the addition of one-tenth volume of 1 M pH 8 Tris-base. The reaction solutions were brought to 0.5 mL with PBS and purified with Sephadex G-50 gel filtration column (Amersham Bioscieence) where the first band passing through the column contained the labeled protein.

2.3 Slide preparation

Figure 1 illustrates the procedures for preparation of PET-coated slide. Glass slides were cleaned with 2.5 M NaOH/ethanol solution and then thoroughly rinsed with distilled water and ethanol. Cleaned slides were immersed into 1 mM of APTS/ethanol solution for 30 min to form an APTS monolayer coating on the glass surface with amino functional groups toward the outside [38]. APTS coated slides were immersed into solution of 5 mg/mL PVS, 1.0 M NaCl at pH 8.0 for 15 min, followed by washing with distilled water, and air-drying. The PVS-coated slide was then exposed to the solution of 5 mg/mL PAAH, 1.0 M NaCl, pH 6.0 for 15 min, followed by washing with distilled water, and air-drying. This procedure was repeated until the desired number of polyelectrolyte pair layers $(PVS/PAAH)_n$ were deposited on the slide with the positively charged PAAH on the outermost layer.

2.4 Scanning electron microscopy (SEM)

SEM was performed with a Hitachi-4700 scanning electron microscope.

2.5 Protein microarray fabrication and immunoassays

Protein printing solutions were prepared in 1 × PBS (pH 7.4) with different concentrations of glycerol. The protein probes were printed on PET-, PLL-, and aldehyde-coated slides at 60% relative humidity. One nanoliter of the printing solutions from a 384-well plate was printed onto PET-, PLL-, and aldehyde-coated slides with a distance of 250 μm between the centers of adjacent spots by using contact printing (PixSys 5500 robotic printer, Cartesian Technologies, Irvine, CA) in 60% relative humidity. Following printing, the slides were incubated for 2 h at room temperature. Slides were then washed twice for 2 min in a solution of PBST (containing 1 × PBS, 0.5% Tween 20) to remove any unbound probes. The slides were blocked in a 1% BSA w/v/PBST buffer for 30 min. The slides were stored in blocking buffer at 4°C unless they were used immediately. A prototype of antigen microarray containing AFP, goat IgG, and HSA and a prototype of antibody microarray containing the antibodies

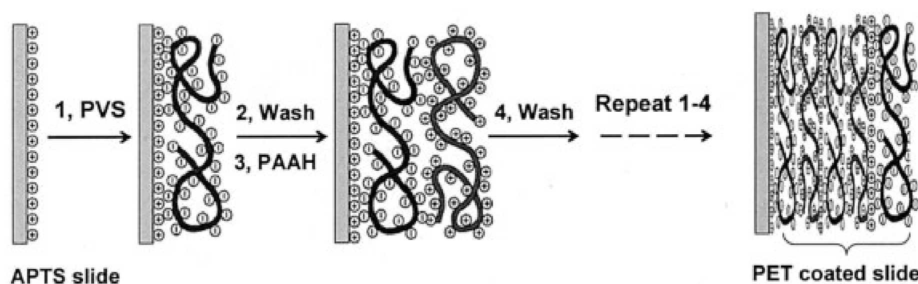

Figure 1. Stepwise assembly of polyelectrolyte multilayered thin film (PET) on glass slide.

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against human IgG, fibronectin, HAS, avidin, and IL-2 were fabricated for direct immunoassay, where anti-IL-2 antibody was used as negative control. Another antibody microarray containing antibodies against cytokine TNF-α, IL-2, and IL-6, as well as anti-goat IgG (a negative control) and biotinylated anti-goat IgG (a positive control) was fabricated for sandwich immunoassay.

Direct immunoassays were carried out by applying 15 μL of a Cy3-labeled cognate protein solution in a PBST buffer to the slide surface. The slides were then sealed in a humidified chamber for 2 h at room temperature. The microarrays were dipped briefly in PBST to remove the protein solution and the cover slip, followed by washing with PBST buffer for 10 min, and twice with 0.1 × PBS for 2 min.

For sandwich immunoassays, slides arrayed with cytokine antibodies TNF-α, IL-2, and IL-6, as well as anti-goat antibody (a negative control) and biotinylated anti-goat IgG (a positive control) were incubated with individual or a mix of cytokine TNF-α, IL-2, and IL-6 in PBST buffer for 2 h. Nine concentrations of each cytokine (100 ng/mL to 0.25 pg/mL at five-fold serial dilution) were tested to generate a dose–response curve. After the slides were washed three times in PBST for 5 min each, a 5 μg/mL mixture of detection antibodies (biotinylated anti-TNF-α, anti-IL-2, and anti-IL-6) was applied to the slides and the slides were incubated for 1 h at room temperature. Slides were washed twice in PBST buffer for 8 min each, and then incubated with 100 ng/mL of Cy3-labeled streptavidin for 1 h. Slides were again washed three times in PBST buffer for 5 min each, followed by washing twice in 0.1 × PBS for 2 min to remove the detergent.

2.6 Imaging and data analysis

Microarrays were scanned at 488 nm (Alexa 488 channel) and 530 nm (Cy3 channel) using a scanning laser confocal fluorescence microscope (ScanArray 5000 System, Packed Biochip Technologies) at 10-μm resolution. For all microarray experiments, the laser power was 80% and the PMT gain was 65%. The images were analyzed by quantifying the pixel density (intensity) of each spot using ImaGene 3.0 (Biodiscovery, Los Angeles, CA). The mean signal intensity of each spot was used for data analysis. All the fluorescent intensities in the scanned images were processed as background-corrected mean fluorescence intensities of the pixels within the spot. Statistical analysis was performed with SigmaPlot 5.0 (Jandel Scientific, San Rafael, CA) or with Microsoft Excel®.

3 Results

3.1 Effect of structural properties of PET film on immobilization of arrayed protein probes

To optimize the film thickness for the construction of protein microarrays, we spotted the Cy3-labeled streptavidin probe onto glass slides that were coated with PET films at different thicknesses. The fluorescent intensity of immobilized streptavidin was analyzed after extensive rinsing with a washing buffer. Figure 2 represents the adsorption isotherms of streptavidin interacting with the polyelectrolyte multilayers $(PAAH–PVS)_n$–PAAH, the outer layer of which being constructed by the cationic PAAH polyelectrolyte. The amount of streptavidin that immobilized the $(PAAH–PVS)_n$–PAAH film increased initially with an increase in the number of bilayers and reached a plateau when the bilayer number (n) was about 9. This correlates with an increase in the number of binding sites (the negative vinylsulfonic groups and the porous network) on the PET film. The spot size changed slightly when the number of bilayers was lower than nine but increased rapidly when the film thickness was greater than ten bilayers (Fig. 2B). This increase suggests protein surface diffusion. Thus, the optimized PET film thickness was obtained with nine bilayers of PVS/PAAH. The SEM micrograph in Fig. 3 shows that the thickness of the coated film is about 70 nm. Glass slides coated with nine bilayers of PVS/PAAH were therefore used for further study. Spots size with 1 nL of protein solution on the $(PVS/PAAH)_9$ is about 165 ± 0.8 μm of 1600 spots from ten slides.

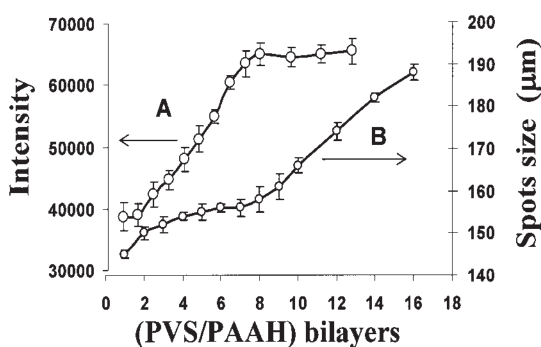

Figure 2. Effect of the thickness of a multilayered polyelectrolyte thin (PET) film on binding capacity (A) and spot size (B). A 25 μg/mL Cy3-labeled streptavidin sample was spotted onto glass slides coated with different thicknesses of PET film (presented here as the numbers of bilayers). After washing with a solution of PBST buffer, the microarray was scanned and analyzed. Data are for 36 replicates. (A) Mean fluorescent intensity ± SD and (B) diameter of spot size ± SD.

Figure 3. SEM micrograph of PET coating on glass slides.

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3.2 Effect of p*I* of protein probes

The advantage of a protein microarray is that multiple proteins can be affixed on one slide surface for simultaneous parallel analysis. The protein probes are immobilized in the porous entrapments of the PET film mainly by the electrostatic interaction between the film and the protein molecules. Therefore, it is important to confirm that this approach is generally applicable to immobilizing proteins with a wide range of p*I*s. We selected five Alexa Fluor 488-labeled proteins that have a wide range of p*I*s (from 4.3 to 11.1) and spotted them onto a PET film whose outer film was the positively charged PAAH polyelectrolyte. Under the spotting buffer and the washing buffer (pH 7.4 for both), HSA (p*I* 4.6), FIB from human plasma (p*I* 5.5), and MGB (p*I* 7.0) were negatively charged whereas avidin (AV, p*I* 10.5) and lysozyme (LSZ, p*I* 11.1) were positively charged. Figure 4A shows the scan image of the microarray after washing with the PBST buffer. It clearly shows that these proteins were strongly affixed on polyelectrolyte multilayers whatever the sign of the charge of the protein. This result becomes even clearer in Fig. 4B, where the S/Ns of $(F_P - F_G)/F_G$ are plotted against the protein concentration and p*I*s. F_P and F_G represent the fluorescent intensity of proteins labeled with Alexa Fluor 488 spotted on PET film and the background noise of the glass surface after washing, respectively. At protein concentrations higher than 25 μg/mL, a positively charged protein can also give saturated adsorption (full coverage) on PET film, as the negatively charged proteins did. Only small differences were observed for the binding of protein probes at spotting concentrations lower than 12.5 μg/mL, where the binding of the negatively charged proteins is slightly larger than that of the positively charged proteins on the positively charged PAAH surface of the PET film.

3.3 Effect of ionic strength on protein immobilization

Since the protein probes are affixed on the PET coating mainly by electrostatic interaction, the ionic strength in the buffers used for washing and immunoassay may affect the affinity of proteins on the PET film. To investigate the stability of the spotted protein adsorption, we carried out experiments by washing the spotted protein microarray for 10-min intervals with a PBS buffer (pH 7.4) having different ionic strengths (total Na^+ concentration). The fluorescent intensities before and after rinsing were recorded (Fig. 5). Less than 10% of the initially adsorbed proteins are desorbable after continuously washing with a buffer solution of similar ionic strength as the one employed for the protein layer buildup, whatever be the sign of the charge of the protein molecules. This result indicated that washing with a buffer solution of similar ionic strength as the one employed for the protein printing does not significantly modify the structure of the adsorbed protein layers. The adsorbed proteins thus appear to interact very strongly with the terminating polyelectrolyte layer. Although about 25% of the proteins immobilized on the PET film were released, when the protein microarrays were washed with a buffer solution of higher ionic strength (*e.g.*, 2 M NaCl), the immobilized proteins were strongly attached on the PET film when washed with buffer solution containing less than 0.5 M NaCl. This concentration is within the range of physiological conditions for immunoassay.

3.4 Spotting buffers

The characteristics of the spotting buffer can affect the stability of protein probes, protein-binding capacity of a slide surface, and quality of the spots produced. To optimize the spotting buffers used for the protein microarrays fabricated

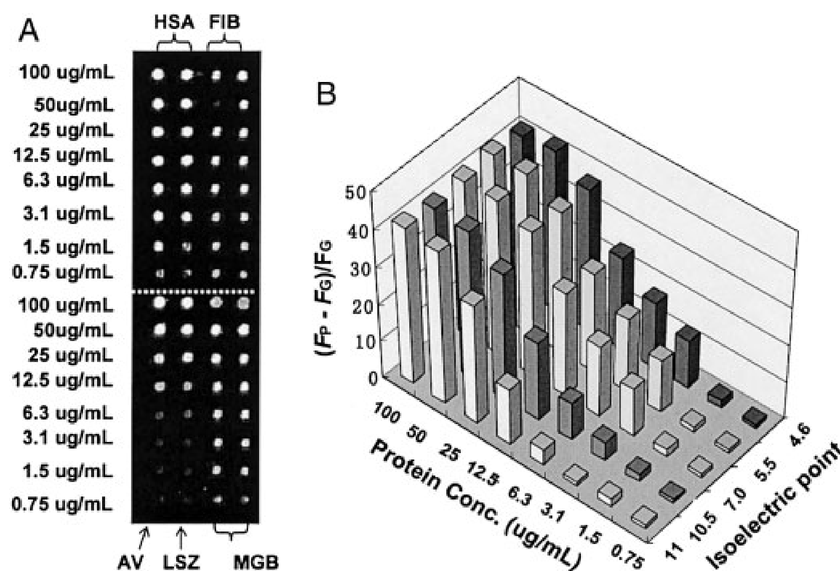

Figure 4. Relationships between the binding of protein probes onto PET surface and the p*I* of the protein probes and concentration of spotting solution. (A) Rainbow displays of scanning image of Alexa Fluor 488-labeled proteins spotted on PET slide after washing with PBST buffer; (B) relation of immobilized proteins to their p*I* and concentration of spotting solution.

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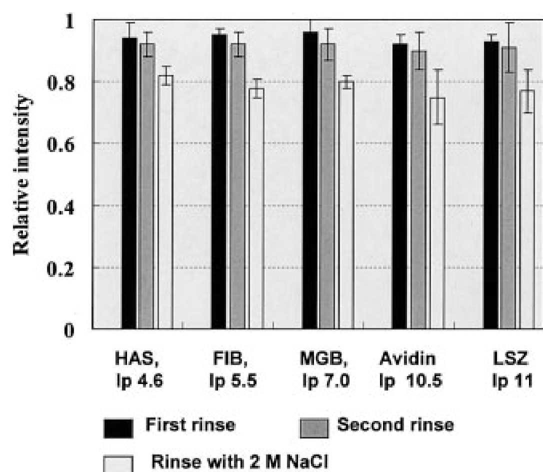

Figure 5. Effect of ionic strength in buffer solution on binding activity of protein probes. Slides spotted with 100 μg/mL of protein probes in 1× PBS buffer, 10% glycerol were rinsed with a 1× PBST buffer for 2 min, and the intensity of each spot was defined as initial spot intensity and set as 1. Slides were then rinsed with 1× PBS buffer containing different concentrations of NaCl: The first rinse was in the 1× PBS buffer (total 0.15 M Na^+) for 10 min; the second rinse was in the 1× PBS buffer (total 0.15 M Na^+) for 10 min; the third rinse in PBS buffer with total 2 M Na^+. Intensities of the protein spots after each rinse were detected and were compared with the initial spot intensities.

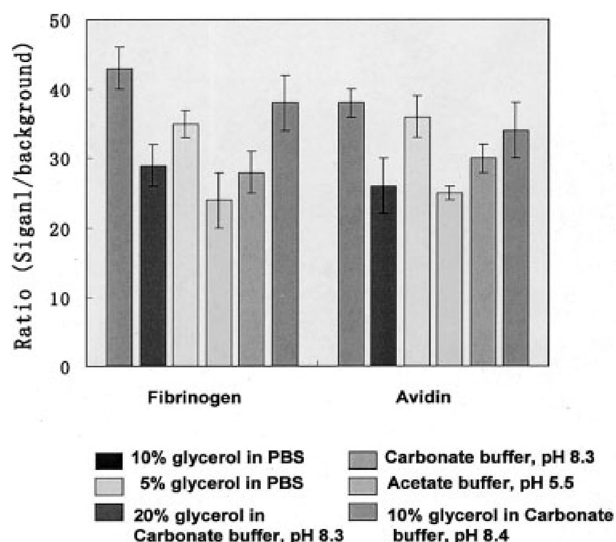

Figure 6. Effect of spotting buffers on signal-background-ratios of detection. Immunoassay fluorescent signals were obtained by incubation of the microarray containing fibrinogen and avidin spots (16 spots for each protein) with 10 μg/mL of Cy3-labeled anti-fibrinogen and anti-avidin antibodies in a PBST solution. Background signal was determined as the fluorescent intensity around the spots.

on the PET film, we tested a microarray spotted with a negatively charged fibrinogen and positively charged avidin protein in six spotting buffers having different pH values and different concentrations of glycerol as supplements (5% glycerol in 1× PBS, pH 7.4; 10% glycerol in 1× PBS, pH 7.4; 20% glycerol in 1× PBS, pH 7.4; 10 mM acetate, pH 5.5; 10 mM carbonate, pH 8.3; 10% glycerol in 10 mM carbonate, pH 8.3). Glycerol has been used as a component of the spotting buffer to prevent dehydration of the spotting solution and to improve the stability of the protein probes. As shown in Fig. 6, the best signal intensities and signal-to-background ratios were obtained with the PBS buffer (pH 7.4) containing 10% glycerol. A concentration of 20% glycerol in the PBS buffer produced spots of bad quality (smeared spots), which may be due to the presence of the glycerol on the binding sites of the slides (data not shown). The pH values of the spotting buffers do not have a major effect on the immunoassay signal intensities. Previously, Kusnezow *et al.* [37] also reported that the pH values of the spotting buffer have little effect on the binding of protein probes on the PLL and APTS slides on which the protein probes are immobilized on an amino-functionalized surface by electrostatic interaction.

3.5 Blocking reagents

An unspecific background signal caused by the binding of cognate proteins on the slide surface is one of the problems encountered in protein microarray technology. Also, if blocking reagents are not well selected to block the unused active groups on the microarray surface, protein probes on a protein microarray may be denatured or lose activity, which results in reducing the signal intensity in an immunoassay. We therefore tested several blocking buffers (5× Dehart's solution, 3% gelatin, 1% BSA, 3% nonfat milk, and 3% nonfat milk/1% BSA). Nonfat milk and BSA are classical blocking reagents. Typical results are shown in Fig. 7. The strongest effect of reducing nonspecific adsorption was found with blocking with 1% BSA in PBS for 20 min.

3.6 Antigen microarrays on PET film-coated slides

After the conditions for fabrication of protein microarrays on PET slides were optimized, we evaluated the performance of protein microarrays on PET-coated slides by generating prototypes of both antigen and antibody microarrays. The direct immunoassay was conducted on an antigen microarray using model proteins of AFP, goat IgG, and HSA. Figure 8 shows the image of an antigen microarray spotted with different concentrations of AFP, goat IgG, and HSA on a PET slide after incubation with a solution containing monoclonal mouse antibodies against AFP. While no nonspecific immunoassay was observed on the spots of goat IgG or HSA, very specific interaction between the AFP antigen and the fluorescently labeled anti-AFP were obtained. When lower concentrations of AFP were used in the spotting solution (6.25, 12.5, and 25 μg/mL), an increase in the fluorescence signal was observed (Fig. 8, rows 1–3). Saturation of the signal was reached at 25 μg/mL (Fig. 8, rows 4 and 5).

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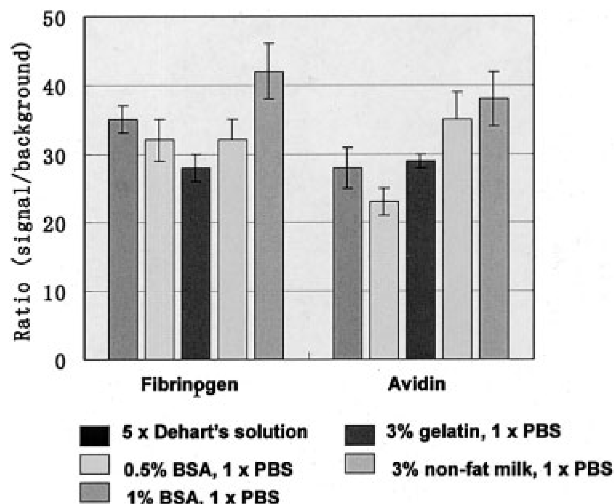

Figure 7. Effect of blocking reagents on the microarray performance. Protein microarrays containing fibrinogen and avidin spots (16 spots for each protein) were blocked with one of the blocking reagents before immunoassay. Immunoassay fluorescent signals were obtained by incubation of the microarray with 10 μg/mL of Cy3-labeled anti-fibrinogen and anti-avidin antibodies in a PBST solution. Background signal was determined to be the fluorescent intensity around the spots.

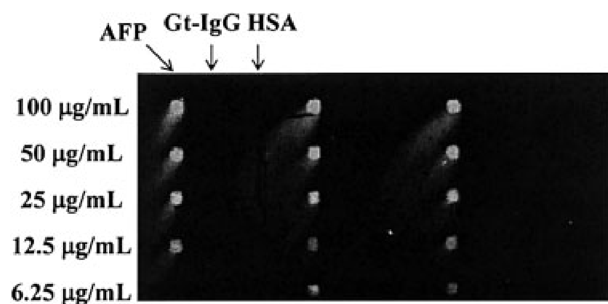

Figure 8. Antigen microarray on PET films. Protein antigens of α-fetoprotein (AFP), goat IgG, and HSA at five concentrations were spotted in a 5 × 3 array format with three replicates and was subsequently incubated with the Cy3-labeled anti-AFP antibody. Signal intensity of the bound antibody correlates with the concentration of AFP protein immobilized on the PET slide.

3.7 Antibody microarrays on PET film-coated slides

The multiple-analyte capacity of the PET slides was tested and demonstrated for parallel determination of four independent antigen analytes. A prototype of antibody microarray was tested by arraying antibodies against human IgG, fibronectin, HSA, avidin, and IL-2 on the PET slides. The anti-IL-2 antibody was used as negative control. To investigate the performance of the antibody microarray format, it was of interest to first determine the array response for the detection of individual or mixed analytes. Figure 9 shows a representative response obtained when a direct immunoassay was performed on the antibody microarray for the detection of a single analyte. The image shows the resulting fluorescence pattern that was generated when a solution containing different combinations of corresponding Cy3-labeled antigens were incubated on the antibody microarray. All four kinds of capture antibody spots simultaneously bound their specific target antigen, and no nonspecific adsorption on anti-IL-2 spot was observed. This finding suggests that the detection arrays do not have nonspecific cross-reactivity and that multianalyte immunoassays can be achieved on the PET slide with good selectivity.

3.8 Sandwich immunoassay on antibody microarrays fabricated on PET slides

Sandwich immunoassays in protein microarrays take advantage of the proven utility of ELISA. In the sandwich assay, proteins captured on an antibody microarray are detected by a cocktail of labeled detection antibodies. Each antibody is matched to one of the spotted antibodies. Thus, sandwich immunoassays are widely used for the detection of proteins found in very low concentrations, such as cytokines, growth factors, or hormones from biological specimens.

To investigate the performance of sandwich immunoassays by protein microarrays on PET slides, we generated an antibody microarray containing antibodies against cytokine TNF-α, IL-2, and IL-6, as well as anti-goat IgG (a negative control) and biotinylated anti-goat IgG (a positive control). Detection of the spotted primary antibodies was performed by incubation of the microarray with different antigen targets followed by incubation with biotinylated secondary antibodies and Cy3-labeled streptavidin. Scanning the biochip at a wavelength of 534 nm revealed specific binding of the antigen targets on immobilized antibody probes (Fig. 10A). The fluorescence resulting from the negative control was minimal (<7% of the fluorescence intensity obtained on primary protein spots), indicating a small degree of nonspecific binding of the Cy3-streptavidin on the immobilized capture antibody. The binding may be due to the multiple steps of incubation applied. The average fluorescence resulting from negative-control spots was defined as a background signal in the sandwich immunoassay and was subtracted from all fluorescent values present in the array for further data analysis.

To investigate the dynamic range of the sandwich assay performed on PET supports and to determine the detection limit, we performed several quantitative studies. For comparison, the same protein microarrays were also fabricated on the PLL and aldehyde slides, and an immunoassay was conducted under identical conditions with the microarrays on a PET slide. The microarrays were incubated with different concentrations of analytes (100 ng/mL–0.256 pg/mL at five-fold serial dilution) according to the protocol described in Section 2. The mean fluorescence intensity, plus or minus the SD at each analyte concentration, was subtracted from the fluorescence from negative-control spots to yield the

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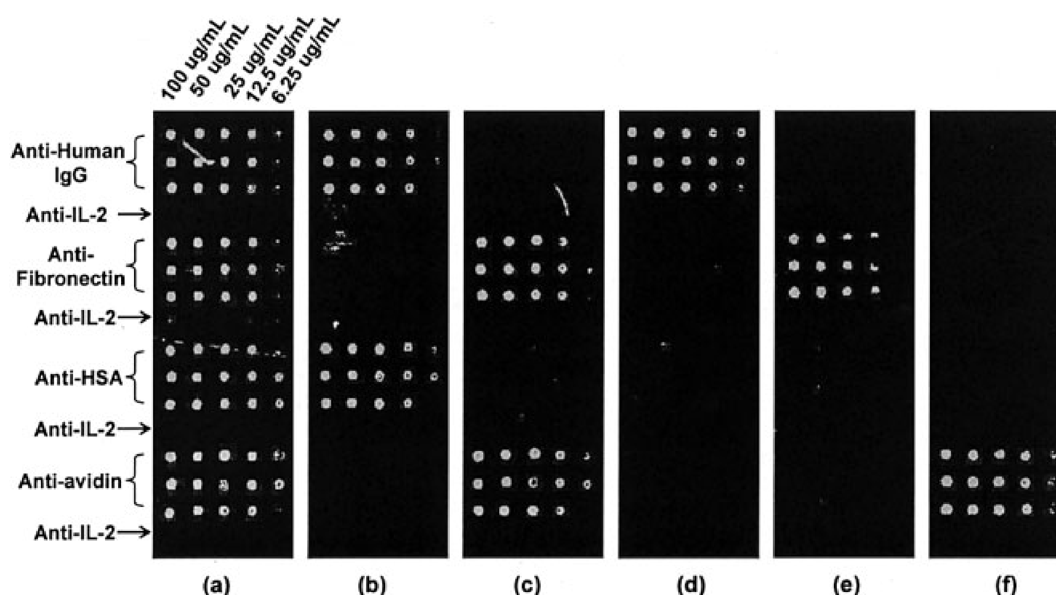

Figure 9. Multianalyte direct-immunoassay on an antibody microarray. A PET slide was immobilized with antibodies against fibronectin, human IgG, avidin, HSA, and IL-2, whereas anti-IL-2 was used as control. After blocking, the protein microarrays were incubated individually with solutions of different combinations of corresponding Cy3-labeled antigen cognates at 100 ng/mL: (a) mixed human IgG, fibronectin, HSA, and avidin; (b) mixed human IgG and HSA; (c) mixed fibronectin and avidin; (d) human-IgG; (e) fibronectin; and (f) avidin. From panel (a) to (e), all binding occurred only at the specific capture antibody sites.

background-corrected fluorescence values, which were subsequently plotted to generate dose–response curves. Figure 10Ba–c shows the typical dose–response curves of TNF-α on PET, aldehyde, and PLL slides, respectively. The data resulted in sigmoid curves having a linear range (the concentration range that gave the best fit to the linear equation $y = mx + b$) from 6.4 pg/mL to 20 ng/mL on the PET slide ($R^2 = 0.97$) and from approximately 32.5 pg/mL to 4 ng/mL on aldehyde ($R^2 = 0.98$) and PLL slides ($R^2 = 0.96$), respectively. At concentrations above 20 ng/mL, the dose–response curve began to plateau on the PET slide. The calculated LOD (the concentration corresponding to three SDs) was determined to be ~3 pg/mL on the PET slide. The intraslide variation in fluorescence, determined as the CV among the 18 spots at each concentration ranged from 3% at 100 ng/mL to 7.8% at 6.4 pg/mL. Table 1 summarizes the properties of the immunoassays for the three types of slides. Clearly, the PET slide provided a higher sensitivity and a wider dynamic range than that of the PLL or aldehyde-functionalized slides.

Table 1. Performance of sandwich immunoassay with protein microarray fabricated on PET, aldehyde, and PLL slides[a)]

Slide	PET	Aldehyde	PLL
Linear range, pg/mL			
TNF-α	6.4–20 000	32–4000	32–4000
IL-2	6.4–20 000	32–4000	32–4000
IL-6	6.4–4 000	32–4000	32–4000
LOD, pg/mL			
TNF-α	3	25	15
IL-2	6	30	28
IL-6	4	25	23
R^2			
TNF-α	0.97	0.98	0.96
IL-2	0.96	0.95	0.93
IL-6	0.97	0.97	0.93

a) R^2 is the regression square value of data that gave the best fit to the linear equation $y = mx + b$.

4 Discussion

The buildup of polyelectrolyte films by the alternating adsorption of cationic and anionic polyelectrolyte layers constitutes a novel and promising technique to modify surfaces in a controlled way [40]. Multilayered polyelectrolytes can be easily engineered on a solid substrate to form uniformly thin films with multiple internal pores that have charged surfaces. The structure has been confirmed by atomic force microscopy studies [38–45]. Previous studies [39, 43] showed that proteins embedded in or adsorbed on a polyelectrolyte multilayer retain their reactivity with respect to their antigens. Ladam [44, 45] investigated the adsorption of HSA on polyelectrolyte multilayers and found that proteins strongly affixed on both negatively and positively charged polyelec-

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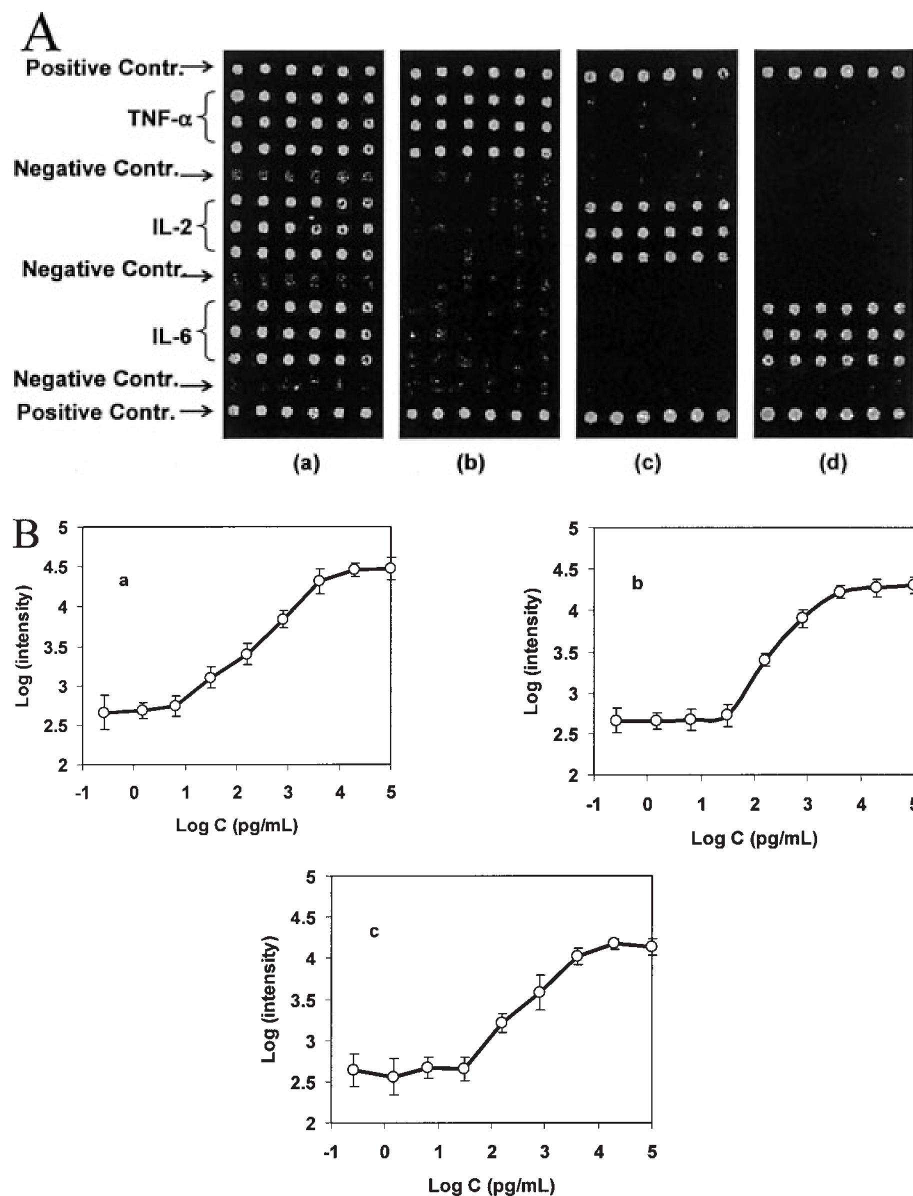

Figure 10. Sandwich immunoassay of antibody microarrays fabricated on PET slides. (A) Scanning images of antibody microarrays exposed to 390 pg/mL of (a) mixture of TNF-α, IL-2, and IL-6, (b) TNF-α, (c) IL-2, and (d) IL-6; (B) dose–response curve of cytokine TNF-α detected on (a) PET slide, (b) aldehyde slide, and (c) PLL slide. Data shown represent means ± SD of 54 replicates from three slides.

trolyte multilayers. However, to the authors' knowledge, no study of the application of polyelectrolyte multilayers as a general platform for protein microarrays has been reported up to now. This study represents a first step in this direction. Indeed, the fact that the PET thin film can be easily engineered on a glass substrate to form uniformly thin films of 10–100 nm thickness makes PET a good candidate as a general platform for biomolecular microarray fabrication. SEM micrographs showed that the coated PET film is uniform with a film thickness of about 70 nm (Fig. 3). The nature of the PET film allows the use of many types of spotting technologies to deposit the desired biological molecules. The low inherent fluorescence of the PET film can support a variety of detection strategies compatible with multiplexed analysis and high-throughput screening. More importantly, the hydrophilic matrix of the PET film can be better than planar surfaces at retaining native protein structures.

In protein microarray technology, high-quality substrates with reproducible surface properties and optimized surface chemistries are required to immobilize the capture proteins homogeneously and in a functional conformation. By analyzing the fluorescent intensities retained on the PET slides after extensive washing, we demonstrated that protein probes with a wide range of p*I*s were all stably immobilized on the PET-coated slide without chemical conjugation (Fig. 4). The fact that positively charged proteins still adsorb strongly onto a positively charged PAAH-terminated film can be explained by the following three facts: (1) Proteins

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attach to their surface domains in the presence of both positive and negative surface excess charges. (2) On a PAAH-terminating multilayered PET film, some negatively charged PVS chains can emerge at the outer surface and are thus also able to interact with the proteins because both the polyelectrolyte chains of PVS and PAAH are flexible. Proteins adsorbed by interactions with underlying PVS chains eventually are also possibly caused by the interdigitation of neighboring layers [44, 45]. (3) Protein adsorption saturation occurred at a high spotting concentration. This implies that the large coverage observed, even when proteins adsorb on polyelectrolyte films of same surface charge, must be due to protein diffusion along the film. The existence of such surface diffusion is indeed confirmed by recent diffusion studies of albumin on the polyelectrolyte multilayers [45]. More than 90% of the initially adsorbed proteins were retained stably on PET after continuously washing with a buffer solution of similar ionic strength as the one employed for the protein layer buildup (Fig. 5). This finding indicates that the washing with a buffer solution of similar ionic strength as the one employed for protein printing does not significantly modify the structure of the adsorbed protein layers. This result also implies that protein microarrays fabricated on PET slides can undergo incubation and washing procedures as required in immunoassay.

Figure 6 indicates that the pH value of the spotting buffer does not have a major effect on the immunoassay signal intensities. Previously, Kusnezow *et al.* [37] had also reported that the pH of the spotting buffer had little effect on the binding of protein probes on the PLL and APTS slides, where the protein probes are immobilized on an amino-functionalized surface by electrostatic interaction.

Unlike the biosensors on PET film, where only one type of biomolecular probe was immobilized, the microarray contains multiple probes. To achieve specific signals from the multiple probes in protein microarrays, the unspecific signals from the nonspecific adsorption of slide surface (antigen binding in the absence of antibody) and the nonspecific reactivity of the protein probes with target samples have to be blocked. In this paper, we found that the use of classic blocking reagents of 1% BSA in PBS for 20 min can strongly block nonspecific adsorption of the slide surface (Fig. 7). To avoid nonspecific reactivity of the protein probes with target samples is one of the most severe challenges in protein microarray technology. It has been reported that high concentrations of capture antibody lead to nonspecific cross-reactivity [49]. Although we have not observed the cross-reactivity of protein probes with the protein concentrations in the spotting buffer in this investigation, optimization of capture protein concentration is suggested for other protein probes.

The capture proteins can be immobilized onto the PET slides at random orientations by the combination of strong electrostatic adsorption, hydrophobic adsorption, and entrapment of the porous structure, which do not result in conformational changes and thus without loss of binding activity of the protein probes. Random orientation of protein immobilization avoids steric hindrance of oriented proteins and the occlusion of binding to specific epitopes. Although some data suggest advantages in oriented protein immobilizations [46], others showed no significant differences between the oriented and nonoriented immobilization of protein function [47]. Due to the amphiphilic nature of polyelectrolyte, protein probes are immobilized on PET film in semiwet environment which keep protein probes in an active form.

The sandwich assay format is an array analog of the widely applied ELISA technique. The unlabeled target protein of interest is bound first by the immobilized capture antibody, and then a biotinylated detection antibody binds to the captured target protein, forming a capture/target/detection protein "sandwich." The target is then detected indirectly by measuring the intensity of the streptavidin-conjugated label bound to the detection antibody. The obvious disadvantages of the sandwich method are the multiple steps, including several washings/incubations, and the need for detection antibodies. In addition to the simplicity of handling the PET slides, the sandwich immunoassay on a PET slide also showed high sensitivity and a wider dynamic range than the microarray fabricated with PLL and aldehyde slide (see Fig. 10). The LOD was shown to be as low as 1–10 pg/mL of the analyte protein. Assays performed on three independent slides demonstrated an average variation of 7.0% in the fluorescence values at each concentration tested (data not shown). The LOD on a protein microarray depends on the slide surface chemistry, the affinity constant of the antibody–antigen pair, and the detection system. Direct comparison of the LOD from reported literature would be difficult. MacBeath has shown the LOD of FKBP 12 to be approximately 150 pg/mL on aldehyde glass [7], while Wagner has reported an LOD of 200 fg/mL for IL-10 on the Zymomix microarray system [48]. Recently, Li [49] has reported detection of cytokine at levels as low as 10 pg/mL using a protein microarray fabricated on an NC-coated slide. Lin [3] reported a sensitivity of LOD down to pg/mL for cytokine detection with protein microarrays on hydrogel film-coated slides. The sensitivity and dynamic ranges of immunoassays on protein microarrays fabricated on PET surface are comparable to these reported ELISA methods and could be found to have potential application in clinical detection [50, 51]. With the same antibody–antigen pair, the PET slide provided higher sensitivity and a wider dynamic range than monolayer-functionalized aldehyde or PLL slides. The higher sensitivity and wider dynamic range of the PET slide are most likely due to the combination of the higher probe binding capacity of the PET film due to its 3-D nature and the higher level of probe accessibility of the PET film. Unlike the thick polymer-film-coated slides, such as the NC-coated slides, on which the proteins were retained mainly by hydrophobic adsorption, the PET film's affinity for spotted protein probes results from a combination of electrostatic adsorption, hydrophobic adsorption, and retention in the

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pores. The PET film also has a lower fluorescence background than the thick NC film. Moreover, the binding capacity and bioassay sensitivity of the protein microarrays on PET can be further increased by using dendrimeric polymers [52, 53], such as polyamidoamine starburst polymers, as starting materials for preparing PET slides.

In summary, we have demonstrated the use of PET films as a highly stable generic platform for the fabrication of protein microarrays *via* spontaneous one-step direct immobilization of the protein biomolecules. This provides a new method for the fabrication of protein microarrays. Microarrays on PET-coated slides have a uniform spot morphology and provide the performance features needed for developing assays for genomic and proteomics analysis. The PET immobilization chemistry presented here combines the several advantages of a 2-D monolayer coating with those of a 3-D thick polymer coating. First, the preparation of PET films is simple and easy to handle. The PET film can be deposited on a solid substrate using a layer-by-layer self-assembly technique. No chemical synthesis steps or handling of toxic coupling reagents are required in preparing the coating film. Second, the noncovalent adsorption of capture proteins on PET film minimizes the denaturation of the biological function of the proteins. The PET slides do not require any special handling or storage precautions. This feature provides the convenience for the individual laboratory researchers to conduct microarray studies for multiple projects. Third, compared with conventional aldehyde or PLL slides, the PET film has higher sensitivity and wider dynamic range.

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