Short Technical Report

Method for Printing Functional Protein Microarrays

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ABSTRACT

Piezoelectric dispensing of proteins from borosilicate glass capillaries is a popular method of protein biochip fabrication that offers the advantages of sample recovery and noncontact with the printing substrate. However, little regard has been given to the quantitative aspects of dispensing minute volumes (1 nL or less) at the low protein concentrations (20 µg/mL or less) typically used in microprinting. Specifically, loss of protein sample due to nonspecific adsorption to the glass surface of the dispensing capillaries can limit the amount of protein delivered to the substrate. We demonstrate the benefits of a low ionic strength buffer containing the carrier protein BSA that effectively minimizes the ionic strength-dependent phenomenon of nonspecific protein adsorption to borosilicate glass. Over the concentration range of 20–2.5 µg/mL, the dispensing of a reference IgG in 10 mM PBS including 0.1% BSA resulted in the deposition of 3.6- to 44-fold more IgG compared to the deposition of IgG in standard 150 mM PBS in the absence of BSA. Furthermore, when the IgG was dispensed with carrier protein, the resulting spots exhibited a more uniform morphology. In a direct immunoassay for cholera toxin, capture antibody spots dispensed in 10 mM PBS containing 0.1% BSA produced fluorescent signals that were 2.8- to 4.3-fold more intense than antibody spots that were dispensed in 150 mM PBS without

BSA. Interestingly, no differences were observed in the specific activities of the capture antibodies as a result of printing in the different buffers. The implications of these results on the future development of protein biochips are discussed.

INTRODUCTION

Following the progress of DNA-chip technology, microarrays have emerged as a means to pose questions not only at the level of the gene but also at the level of the protein. One of the chief goals of protein biochips is the ability to analyze the complexity of a system by identifying and quantifying multiple ligands within a single biological sample. To that end, protein microarrays have emerged for numerous applications including the screening of recombinant antibody libraries (2), the monitoring of protein-protein interactions (4,12,14), and the detection of clinically relevant cytokines (5,7). Driving the development of protein biochip fabrication has been a number of basic deposition techniques for the delivery of the protein sample to the substrate. These techniques have included microspotting using a transfer stamp (6), ink-jetting using a desktop jet printer (10), and piezoelectric (non-contact) dispensing (7). The main disadvantages of the microspotting technique are low transfer efficiency and the need to modify transfer protocols to adapt to different surfaces. Ink-jetting exposes the sample to heat and can compromise the structure, and therefore the function, of the capture molecules to be dispensed. Piezoelectric

dispensers have become popular microprinting platforms because they do not contact the printing substrate and they allow for the recovery of that portion of the sample that is not dispensed. These printers aspirate sample into a borosilicate glass capillary that is surrounded by a piezoelectric element collar. Upon application of a voltage to the collar, the sample in the capillary is compressed, producing a droplet typically less than 1 nL in volume. Despite the benefits of the noncontact method employed by the piezoelectric dispenser, little regard has been given to the quantitative aspects of dispensing minute volumes of protein solutions (1 nL or less) at the low concentrations (20 µg/mL or less) that are often used in microarray printing. Here we describe a microarray printing buffer that minimizes the loss of protein sample due to nonspecific adsorption to the borosilicate glass capillary.

MATERIALS AND METHODS

Preparation of Proteins

Purified polyclonal mouse immunoglobulin G (IgG; Sigma, St. Louis, MO, USA) and goat anti-cholera toxin antibody (Biogenesis, Brentwood, NH, USA) were reacted with biotin-LC-NHS ester (Pierce Chemical, Rockford, IL, USA) at a molar ratio of 5:1 (biotin:antibody) for 30 min at room temperature. Unincorporated biotin was removed by size exclusion chromatography using Biogel P-10 (Bio-Rad Laboratories, Hercules, CA, USA). Fluorescent labeling of biotiny-

lated mouse IgG or cholera toxin with Cy5 (bifunctional derivative; Amersham Biosciences, Piscataway, NJ, USA) was accomplished by reaction of the protein and Cy5 at a 5:1 molar ratio for 30 min at room temperature. Excess dye was removed by size exclusion chromatography. The Cy5:protein ratio was determined by spectrophotometry to be 2.8 for the IgG and between 1 and 1.5 for the cholera toxin.

Microarray Surface Preparation

Conventional glass microscope slides (Daiggerbrand, Wheeling, IL, USA) were cleaned using the KOH/methanol method described previously (1). The cleaned slides were silanized, and NeutrAvidin™ Biotin-Binding Protein (Pierce Chemical) was covalently immobilized onto the glass surface according to Reference 8. The NeutrAvidin-coated slides were stored in blocking buffer [10 mM phosphate, 10 mM NaCl, 1% (w/v) BSA, 0.01% (w/v) NaN₃] at 4°C for up to four weeks before use.

Microarray Printing

Biotinylated antibodies were prepared in the buffer under investigation and were deposited onto the NeutrAvidin surface using a BioChip Arrayer I™ (Perkin Elmer Life Sciences, Boston, MA, USA) housed in an atmospherically isolated chamber with a relative humidity of 45% at room temperature. The instrument was programmed to dispense antibody solutions at 1 nL/spot and consistently yielded spots approximately 230 µm in diameter. To control for intercapillary variability, all dispensing manipulations were performed using the same glass capillary tip. Between each sample dispense, an aspirating rinse step was performed in which the tip was rinsed liberally with 0.1% Tween® 20 (Sigma) in distilled water, followed by a final rinse with distilled water to remove any residual protein. Arrays that were printed with biotinylated Cy5-labeled IgG to compare different dispensing buffers were washed and dried immediately after printing (see below). Arrays that were printed with biotinylated antibody for the detection of Cy5-labeled cholera

toxin were stored at 4°C just long enough to prepare reagents for the assay (less than 30 min).

Comparison of Different Dispensing Buffers

Biotinylated Cy5-labeled mouse IgG was dispensed at concentrations ranging from 2.5 to 20 µg/mL in PBS (137 mM NaCl, 3 mM KCl, 10 mM phosphate, pH 7.4). The ionic strength of the PBS buffer was adjusted to final values that ranged from 10 to 150 mM by dilution with sterile, distilled water (pH 7.4). All solutions were checked before use to ensure the maintenance of pH at 7.4. BSA, when present, was used at a final concentration of 0.1% (w/v). After all samples were dispensed, nonspecifically bound proteins were removed from the slide with two washes with 150 mM PBS containing 0.05% Tween (PBST). This was followed by two washes with distilled water. Finally, the slide was dried under a stream of nitrogen before imaging.

Detection of Cholera Toxin

Direct detection of Cy5-labeled cholera toxin was achieved using a modification of the flow-based microarray immunoassay system described previously (3). Briefly, 15 replicate spots of biotinylated goat anti-cholera toxin capture antibody were deposited in a linear orientation for later alignment within one of the six sample channels of a poly-(dimethylsiloxane) (NuSil Technology, Carpinteria, CA, USA) flow module. As an internal printing control, five replicate spots of biotinylated Cy5-labeled mouse IgG were printed within each sample lane. All antibodies were dispensed at a concentration of 5 µg/mL in either 150 mM PBS or 10 mM PBS containing 0.1% BSA. Cy5-labeled cholera toxin, at concentrations of 50, 200, and 500 ng/mL in PBSTB (PBST containing 0.1% BSA), was flowed through individual channels of the flow module at a rate of 0.3 mL/min. Nonspecifically bound cholera toxin was removed by rinsing the channels with 1 mL PBSTB at a rate of 1 mL/min. All solution transfers were achieved using a peristaltic pump. At the completion of the assay, the slide

MICROARRAY *Technologies*

was washed and dried as described above before imaging.

Microarray Imaging and Analysis

Microarray images were acquired at 10 μm pixel resolution using a ScanArray Lite™ microarray scanner and ScanArray image acquisition software (version 2.1; Perkin Elmer Life Sciences). The fluorescence intensity of the spots was determined using the QuantArray™ analysis package (version 2.1; Perkin Elmer Life Sciences). In all analyses, the fixed circle quantitation algorithm was used to locate and quantify the fluorescence intensity of each spot and its surrounding background. Each spot was enclosed by a circular spot mask (230 μm diameter) and a circular background mask (inner diameter 240 μm ; outer diameter 300 μm). The final spot intensity was calculated as the mean intensity of the pixels located within the spot mask minus the mean intensity of the pixels in the background mask. Images were formatted for publication using Slidewrite Plus (version 5.0; Advanced Graphics, Encinitas, CA, USA).

RESULTS

Effect of Buffer Composition on the Amount of IgG Dispensed During Piezoelectric Printing

Different buffer conditions were tested to assess their effect on the amount of protein delivered from the borosilicate glass capillary tips of a piezoelectric noncontact microarrayer. Specifically, we investigated the influence of buffer ionic strength and carrier protein on the amount of IgG dispensed from the capillary. Figure 1A shows the results of a representative experiment in which 1 nL spots of biotinylated Cy5-labeled mouse IgG were dispensed to the surface of the same NeutrAvidin-coated microscope slide using PBS buffer that ranged in ionic strength from 10 to 150 mM. The left panel shows an array that was printed in the absence of the carrier protein, BSA, while the right panel depicts an array that was printed in the presence of 0.1% BSA. As shown in Figure 1A

(left), it was apparent that in the absence of BSA, the amount of IgG protein dispensed from the tip was inversely proportional to the ionic strength of the buffer. At each IgG concentration tested, a consistent increase in the fluorescence intensity of the spots was observed as the ionic strength of the printing buffer decreased from 150 to 10 mM. This trend was most dramatic at the lowest IgG concentration tested and became less pronounced as the IgG concentration increased. For example, at an IgG concentration of 2.5 $\mu\text{g/mL}$, the fluorescence signal of the spots dispensed in 150 mM PBS was 6.6-fold less than that of the spots dispensed in

10 mM PBS. When the IgG concentration was increased to 20 $\mu\text{g/mL}$, however, this difference became less dramatic. At this higher IgG concentration, the fluorescence intensity of the spots dispensed in 150 mM PBS was only 1.5-fold less than the intensity of the spots dispensed in 10 mM PBS (Figure 1B).

Interestingly, when 0.1% BSA was added to the printing buffer, the amount of IgG dispensed from the capillary was independent of the ionic strength of the buffer (Figure 1A, right). The plot of fluorescence intensities in Figure 1C illustrates that, for a given IgG concentration, there was less than a 1.1-

Figure 1. Effect of printing buffer on the amount of IgG dispensed from piezoelectric glass capillaries. (A) Comparison of arrays printed using different printing buffers. For each IgG concentration (indicated at left), 10 replicate spots of biotinylated Cy5-labeled mouse IgG were dispensed in PBS buffer at four different ionic strengths (indicated at right; typical for each IgG concentration). The absence or presence of 0.1% (w/v) BSA in the printing buffer is noted above each respective array. All spots were deposited to the surface of the same NeutrAvidin-coated glass microscope slide. The color scale denotes the range of fluorescence intensities registered by the scanning laser microarray reader. (B and C) The fluorescence intensities of the arrays in panel A are shown graphically. The arrays were printed in the absence (B) or presence (C) of BSA. For each IgG concentration, the bars correspond to the following ionic strengths of PBS: 150 mM (open bar), 100 mM (single-hatched bar), 50 mM (cross-hatched bar), and 10 mM (solid bar). The results shown are representative of duplicate slides and are expressed as $\bar{x} \pm \text{SEM}$.

fold difference in the amount of IgG protein dispensed from the capillary over the range of ionic strengths tested. The scale in Figure 1C (50 000 fluorescence units) is 2.5 times larger than the scale in Figure 1B (20 000 fluorescence units), emphasizing that the addition of BSA resulted in the deposition of a greater *proportion* of IgG compared to deposition in the absence of BSA. This difference was dependent on both the ionic strength of the buffer and the concentration of the IgG dispensed. When 10 mM PBS was used, the presence of BSA resulted in the deposition of 2.5-fold more protein at an IgG concentration of 20 $\mu\text{g/mL}$ and 6.6-fold more protein when the IgG concentration was 2.5 $\mu\text{g/mL}$ compared to when BSA was absent. An even more dramatic difference was observed when the buffer was 150 mM PBS. In this instance, the addition of BSA resulted in the deposition of 4.1-fold more protein when the IgG concentration was 20 $\mu\text{g/mL}$ and 51-fold more protein when the IgG was dispensed at a concentration of 2.5 $\mu\text{g/mL}$. It was also observed that the absence of BSA resulted in fluorescent spots whose morphology resembled a “doughnut” or “halo” (Figure 1A, left).

When BSA was included in the buffer, however, the spots displayed a much more uniform shape and fluorescence signal (Figure 1A, right).

Detection of Cholera Toxin by Capture Antibodies Deposited in Different Printing Buffers

The functional implications of dispensing capture antibodies in two different printing buffers were assessed by performing a direct immunoassay for cholera toxin. Figure 2A shows the results obtained when 15 replicate spots of biotinylated goat anti-cholera toxin antibody were printed in either 150 mM PBS or in 10 mM PBS containing 0.1% BSA. As an internal printing control, five replicate spots of biotinylated Cy5-labeled mouse IgG dispensed in the same buffer were included in each sample channel. The sample channels were exposed to 50, 200, or 500 ng/mL Cy5-labeled cholera toxin. A comparison of the fluorescence intensities of the internal printing controls revealed that the capillary dispensed approximately 3.4-fold less mouse IgG when it was printed in 150 mM PBS compared to when it was printed in 10 mM PBS

containing BSA (Figure 2B, inset). Similar differences were also observed in the fluorescence intensities resulting from the goat anti-cholera toxin capture antibody spots. Upon exposure to 200 and 500 ng/mL Cy5-labeled cholera toxin, the capture antibody spots printed in 150 mM PBS were 4.3- and 4.2-fold less intense, respectively, relative to the anti-cholera toxin capture antibody spots dispensed in 10 mM PBS containing BSA. When exposed to 50 ng/mL cholera toxin, the difference between the fluorescence values decreased slightly to 2.8-fold.

DISCUSSION

Here we investigated the influence of the dispensing buffer used during the fabrication of antibody microarrays on the fabrication of antibody microarrays with a noncontact microarray printer. Specifically, we examined how the ionic strength of the printing buffer and the presence of carrier protein affect both the amount and function of antibody protein that is dispensed from the borosilicate glass capillaries of a piezoelectric microarrayer. Piezoelectric dispensers have become popular devices

Figure 2. Deposition of anti-cholera toxin capture antibody in a low salt buffer containing BSA results in capture of a larger proportion of labeled analyte. (A) A direct detection assay was performed for cholera toxin. The antibody spots printed in the three sample channels to the left were dispensed in printing buffer consisting of 150 mM PBS without 0.1% (w/v) BSA (indicated by “-BSA”). The antibody spots printed in the three sample channels to the right were dispensed in printing buffer consisting of 10 mM PBS with 0.1% (w/v) BSA (indicated by “+BSA”). All antibody spots were printed at 5 $\mu\text{g/mL}$. Rows marked “1-3” denote the position of replicate spots of biotinylated goat anti-cholera toxin antibody, while the rows marked “C” indicate the position of replicate spots of biotinylated Cy5-labeled mouse IgG used as an internal printing control. Each sample channel column was exposed to 50, 200, or 500 ng/mL Cy5-labeled cholera toxin as indicated. (B) The fluorescence intensities of the arrays in panel A are represented graphically. The bars correspond to antibodies printed in the following buffers: 10 mM PBS containing 0.1% BSA (open bar) and 150 mM PBS (single-hatched bar). The inset shows the fluorescent intensities of the control biotinylated Cy5-labeled IgG printed in the two buffers. The results are plotted as the $\bar{x} \pm \text{SEM}$ and are representative of replicate assays.

for microarray printing applications because they do not require contact with the substrate and they allow for the recovery of unused sample. However, little or no regard has been given to the quantitative aspects of dispensing protein samples in minute volumes (typically 1 nL or less) using the borosilicate glass capillaries that are commonly employed to dispense the samples.

Using a biotinylated Cy5-labeled IgG as a reference protein, we demonstrated that both the ionic strength of the printing buffer (PBS) and the presence of carrier protein (BSA) can have a profound effect on the amount of IgG that is dispensed from the glass capillary. PBS was chosen because it is a commonly used buffer system for the microprinting of proteins. Over a range of ionic strengths that spanned from 10 to 150 mM, the amount of IgG protein dispensed from the capillary varied inversely with the buffer ionic strength, and this effect was most pronounced at the lowest IgG concentrations tested. This result is perhaps not surprising when one considers that the nonspecific adsorption of proteins to borosilicate glass surfaces, particularly at the low protein concentrations investigated herein, is a well-documented phenomenon (9,11,13). Still, it was notable that the addition of BSA, which effectively increased the total protein concentration, completely eliminated this ionic strength effect. Furthermore, including BSA in the 10 mM PBS printing buffer resulted in the deposition of a significantly higher proportion of IgG (as much as 44-fold more protein when the IgG was printed at 2.5 µg/mL) compared to 150 mM PBS without the carrier protein. We also noted an overall improvement in spot morphology when BSA was included in the printing buffer, namely the disappearance of the "doughnut"-like morphology of the spots. It is likely that increasing the total protein concentration reduced the effect of drying at the edge of the microspots.

We demonstrated the functional benefit of our observations in the direct detection of Cy5-labeled cholera toxin in which the capture antibody spots deposited in 10 mM PBS containing BSA produced fluorescent signals that were 2.8- to 4.3-fold more intense than those deposited in 150 mM PBS. As these ra-

tios were comparable to those seen in the deposition of the internal control IgG, it was apparent that the low ionic strength buffer containing BSA had no effect on the specific activity of the capture antibodies. PBS (10 mM) containing BSA simply resulted in the deposition of a greater amount of protein.

It is anticipated that the benefits of the observations described herein will extend beyond the relatively simple assay system demonstrated in this report. With the growing demands of proteomics and high-throughput drug screening, increasing numbers of proteins will be deposited in arrays as capture molecules. Thus, capture reagents with extremely restricted specificities must be found to reduce cross-reactivity in the array format. Many of these molecules will be derived from recombinant technologies (e.g., phage display) and will be prone to common problems such as low expression levels and low concentrations post-purification. To develop these precious reagents for use in biochips, it is likely that dispensing will be performed using piezoelectric printers that permit sample recovery. The use of a low ionic strength buffer and carrier protein will aid not only in these instances but also in any printing application where one wishes to minimize loss of sample due to nonspecific protein adsorption.

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