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Optimization of printing buffer for protein microarrays based on aldehyde-modified glass slides

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1. ABSTRACT

It is of great importance to efficiently immobilize probes onto a substrate with good spot quality for fabrication of protein microarrays. Printing buffers play an essential role in the fabrication process for the microarrays. In this work, antigen (Ag)/antibody (Ab) microarrays were fabricated on 3-aminopropyltriethoxysilane (APTES) modified glass slides through glutaraldehyde (GA), a bis-aldehyde homobifunctional cross-linker. Different types of buffers such as triton X-100 and glycerol and their effects on the protein immobilization were investigated for improving the quality of microspots and the immobilization efficiency on the aldehyde-activated APTES silanized slides. In addition, the performance of the optimized printing buffer was characterized with fabricated Ag/Ab microarrays. The results indicated that the optimized printing buffer, 0.01 M PBS with additional 0.003% triton X-100 and 10% glycerol could effectively eliminate non-homogeneous morphology of the microspots and significantly improve the signal intensities. The results provide an improved approach to construct high performance Ag/Ab microarrays.

2. INTRODUCTION

The microarray technology was started by Ekins and his colleagues with the ambient analyte model, and then has been developed as a tool for high-throughput DNA sequencing and gene expression analysis (1-3). However, the investigation of genetic information could not provide sufficient insight to understand complex cellular networks and protein post-translational modifications that affect cell functions directly (4). Thus, the protein microarray has been rapidly developed after the complement of 35,000-40,000 human genes now predicted. The Ab-Ag interaction based microarray is one of the most important protein chips, due to its high specificity, good sensitivity, and broad applications in proteome analysis, disease diagnostics, identification of therapeutic markers and targets, and profiling of response to toxins and pharmaceuticals(5, 6).

It is essential to efficiently immobilize probes onto the substrates with good spot quality in fabrication of Ag/Ab chips. At present, there are a number of strategies such as physical adsorption, specific affinity interaction and

covalent bindings for construction of Ag/Ab microarrays on different types of substrate (7). In these approaches, covalent binding is the most promising method since it provides the strongest attachment of probe Ags or Abs to substrate surfaces (8). Probe covalently coupling to aldehyde-activated APTES silanized glass slides was one of the most popular approaches for fabrication of Ag/Ab microarrays because of its low-cost and superior properties for optical detection (9, 10).

Some researchers have pointed out that non-homogeneous microspot morphology such as ring-like profile and smearing effects are inherent drawbacks of one-dimensional surfaces (10-12). Although the surface properties of substrates affect the spot quality of protein microarrays, an appropriate printing buffer can significantly improve the surface binding capacity, the stability of proteins and the quality of the spots produced. Some literatures investigated the effect of pH of printing buffers on the protein immobilization (13) such as application of glycerol (9), trehalose (13), saccharose (14) to prevent dehydration and denaturation of immobilized protein. Even though these supplements can prevent dehydration and improving the signal intensity, the non-uniform spot structure still occurred. The non-uniform profile of spots could result in poor reproducibility and difficulty in quantitative application of protein microarrays (12). Deng and Zhu studied the mechanism of ring-like formation on epoxy-terminated slides, and demonstrated that the non-uniform structure could be eliminated by competitive surfactant (triton X-100 as example) (12). However, such a buffer effect on aldehyde-modified slides has not been studied. The surface properties of the aldehyde-modified slides should be very different from the epoxy-terminated slides, which is more hydrophobic than the former one. Theoretically, different surface properties should have different interactions between the printing buffer and the substrate surface. Thus, the printing buffer, an essential factor for good quality and high performance of protein microarrays, is needed for further extensive investigation for construction of highly sensitive Ag/Ab microarrays based on aldehyde-modified slides.

In this work, we selected rabbit IgG and anti-rabbit IgG as model probes to investigate the buffer effect. Two supplements, glycerol and triton, were applied to printing buffers synchronously. Their effects on morphology and signal intensity of microarrays were investigated based on aldehyde-functionalized slides to optimize the printing buffer and thus to improve the spot quality and the performance of Ag/Ab microarrays. The optimized printing buffer could be very significant for efficiently and inexpensively fabricating Ag/Ab microarrays with high performance.

3. METERIALS AND METHODS

3.1. Materials and apparatus

Plain microscope slides (Menzel), Rabbit IgG, polyclonal anti-rabbit IgG, biotin-conjugated polyclonal anti-rabbit IgG, 0.01 M phosphate buffered saline (PBS, pH7.4) and 0.05M carbonate-bicarbonate buffer saline (CBS, pH9.6) were purchased from Sigma. Cy3-conjugated streptavidin was received from GE healthcare. Glycerol and Triton® X-100 were obtained from Sigma-Aldrich. 3-aminopropyltriethoxysilane (APTES) and ethanol were purchased from Fluka. Glutaraldehyde (GA, 50% solution) and Blocker™ Casein in TBS were obtained from Pierce. The deionized water used in all experiments was produced by a water purification system, Q-Grad®1, from Millipore Corporation.

VersArray chip writer™ contact system (BIO-RAD, USA) was used to array probes onto the substrate for fabrication of microarrays. ProXPRESS 2-D proteomic imaging system (PerkinElmer, USA) was employed to detect the fluorescent response of the microarrays for assessment of the biochip performance,

3.2. Preparation of glass slides

3.2.1. Cleaning and silanization of glass slides

Microscope glass slides were first cleaned in a 70/30 (v/v) mixture of concentrated H_2SO_4 and 30% H_2O_2 for 2 h in oven at 80 ℃ followed by three-time washing with deionized water and ethanol sequentially, and then were dried under nitrogen flow. The precleaned slides were immersed in 5% (v/v) APTES in 96% ethanol for 1 h at room temperature to introduce amino groups onto the slide surface, then rinsed thoroughly with 96% ethanol and deionized water to remove any non-bound silane compounds. The prepared slides were dried under nitrogen flow and baked in a vacuum oven at 120 ℃ for 2h.

3.2.2. Activation of silanized slides

The amino groups on the APTES silanized slide surface were activated via GA to covalently immobilize Ag or Ab. Specifically, silanized slides were immersed in 2.5% (v/v) GA in 0.01 M PBS (pH7.4) and kept quiescent for 2 h at room temperature. Then, these slides were rinsed thoroughly with ethanol and deionized water, followed by drying under nitrogen flow. After the aldehyde-activation, probes could be immobilized to the substrate surfaces through Schiff's base formed between aldehyde groups and primary amines of Ag/Ab.

3.3. Printing and testing of immunoassay microarrays

Probes were dissolved in the printing buffer to prepare probe solutions with different concentrations and then transferred into a 96-well plate before printing. The VersArray chip writer™ contact system was used to print probes onto the functionalized glass slide surface. Nanoliter volumes of sample per spot were delivered to the substrate for fabrication of microarrays. After printing, all slides were kept in a humid chamber at 37 ℃ for 1 h for full immobilization before further treatment. Then, the slides were washed three times for 2 min each with PBST (containing 0.01M PBS, 0.05% Tween 20) to remove any unbound probes. All array-printed slides were immersed into Blocker™ casein in TBS for 1 h for not only quenching the unreacted aldehyde groups on the slide surface, but also forming a molecular layer of casein that could reduce nonspecific binding of other proteins in subsequent steps.

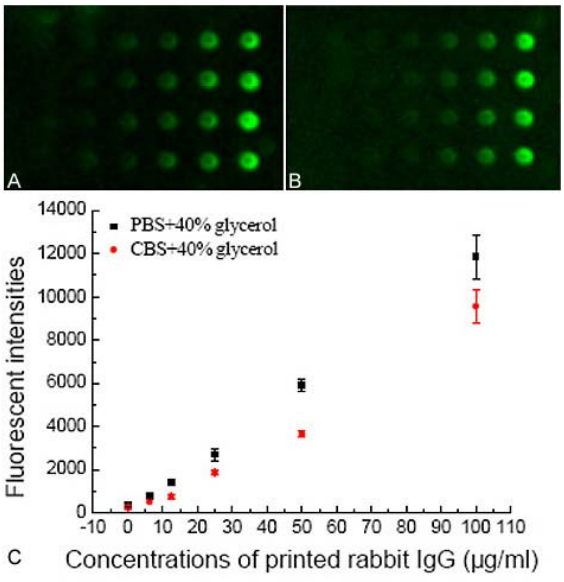

Figure 1. Immobilization of rabbit IgG in different printing buffers on aldehyde-activated APTES silanized slides. A, 0.01M PBS+40%glycerol; B, 0.05M CBS+40%glycerol; C, Fluorescent intensities versus the concentrations of immobilized rabbit IgG. Rabbit IgG was immobilized onto the glass slides and incubated with biotin-labeled anti-rabbit IgG and Cy3-labeled streptavidin. The fluorescent images were captured by an imaging system. The fluorescent intensity was employed to evaluate the performance. In figure 1 A and B, the concentrations of rabbit IgG were 0, 6.25, 12.5, 25, 50, 100 μg/ml for each column from left to right.

For antigen microarrays, 15μl of 2μg/ml biotin-labeled anti-rabbit IgG was applied to each array and incubated in a humid chamber for 1 h at 37 ℃, followed by washing three times for 2 min each with PBST. Then the Cy3-labeled streptavidin was used to produce fluorescent signals. Antibody microarrays were carried out by applying 15 μl of 2 μg/ml rabbit IgG to slide surface firstly and incubating for 1 h at 37 ℃. After washing treatment, biotin-labeled anti-rabbit IgG and Cy3-labeled streptavidin were successively added to each arrayed spot followed by incubation and washing as described above.

3.4. Optimization of printing buffer

To optimize the printing buffers, different buffers including 0.05M CBS (pH 9.6) plus 40% glycerol, 0.01M PBS (pH7.4) with different concentrations of glycerol and triton X-100 were investigated to optimize printing buffers for fabrication of Ag/Ab microarrays on the aldehyde-activated APTES silanized slides.

3.5. Imaging and analysis

After application of Cy3-conjugated streptavidin, the slides were incubated for certain duration according to the procedure described above, followed by washing with PBST and deionized water three times respectively. Then, all slides were dried under nitrogen flow. The dried slides were immediately scanned with ProXPRESS 2-D proteomic imaging system. The captured images were analyzed with ProScanArray® Microarray Analysis software. The fluorescent intensities were local background-subtracted median, which was used for downstream statistical analysis (15). The acquired data were inputted to Origin 6.0 for further analysis and plots.

4. RESULTS AND DISCUSSION

4.1. Performance of different buffers

Currently, a number of buffers including carbonate, PBS, acetate buffer and citrate were commonly used for fabrication of protein microarrays (7, 13). In this work, two buffers (0.01 M PBS, pH 7.4; 0.05 M CBS, pH 9.6) were firstly selected to explore their effect on immobilization efficiency for aldehyde-activated APTES silanized glass slides. 40% glycerol was added to each buffer to prevent protein dehydration and denaturation (9). Different concentrations of rabbit IgG (0-100 μg/ml) were prepared with the two printing buffers to spot onto the functionalized glass surface to fabricate microarrays. Biotin-labeled anti-rabbit IgG and Cy3-labeled streptavidin were applied to acquire the microarray images. The results are shown in Figure 1. It can be seen that the fluorescent signals in Figure 1 A (PBS buffer) are brighter than that in Figure 1 B (CBS buffer). Figure 1 A and B were analyzed with analysis software and the data were processed by Origin 7.0 to plot Figure 1 C, which showed the quantitative relationship of fluorescent intensities versus the concentrations of printed rabbit IgG. According to Figure 1 C, the fluorescent intensities increased with the increase of the concentrations of printed rabbit IgG, and the fluorescent intensities of PBS group were stronger than that of CBS group. Therefore, 0.01 M PBS was used as the printing buffer for further optimization.

4.2. Effect of triton X-100 on protein immobilization

During fabrication of protein microarrays in our labs, the quality of the chips are often suffered from a ring-like non-homogeneous spot morphology (Figure 1: A and B) on aldehyde-activated APTES silanized glass slides, which is identical to the reported results in (10-12). This is because of that protein molecules in microspots preferentially accumulate at air/water interfaces, especially when the droplet size decreases from macroscopic to the nanoliter and subnanoliter scale (12). According to this mechanism, the ring-like structure could be eliminated by addition of competitive surfactants to displace protein molecules at the air/water interface.

Different concentrations of triton X-100, a competitive surfactant to the protein molecules, were added in the printing buffer to investigate and optimize the effects on microspots quality and performance of protein microarrays. 50 μg/ml rabbit IgG in different printing buffers: 0.01M PBS with 40% glycerol and 0.001%, 0.003%, 0.006%, 0.009%, 0.012% triton X-100 were spotted onto aldehyde-activated APTES silanized slides. Figure 2 A and B showed the fluorescent images and the relationship between the signal intensities and different printing buffers. From Figure 2 A, it was found that the ring-like profile was almost eliminated after adding

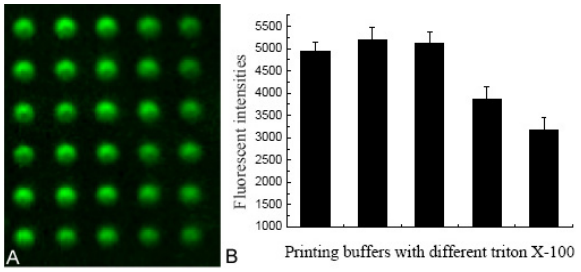

Figure 2. The effect of triton X-100 on spot quality and protein immobilization. A: Fluorescent image of microarrays. 50 μg/ml rabbit IgG were immobilized on the aldehyde-terminated slide surface by using 0.01 M PBS with 40% glycerol and different concentrations of triton X-100 as the printing buffer. From left to right columns, the concentrations of triton X-100 are 0.001%, 0.003%, 0.006%, 0.009% and 0.012%. B: The relationship of the fluorescent intensities vs. concentrations of triton X-100. The concentrations of triton X-100 correspond to figure 2 A for each column.

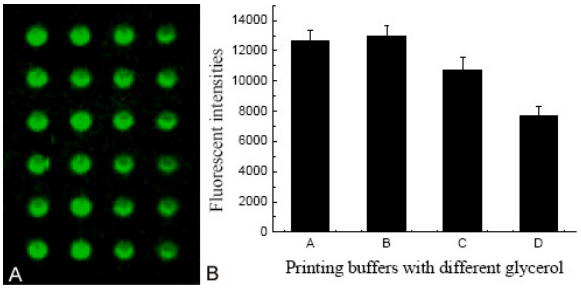

Figure 3. The effect of glycerol on protein immobilization. A: Fluorescent image of microarrays fabricated with different printing buffers. 50 μg/ml rabbit IgG were immobilized on aldehyde-terminated slide surfaces by using 0.01 M PBS containing 0.003% triton X-100 and different levels of glycerol as the printing buffer. From left to right columns, the concentrations of glycerol are 5%, 10%, 20% and 40%. B: The relationship between the fluorescent intensities and the printing buffer with different glycerol. The concentrations of glycerol correspond to figure 2 A for each column.

different concentrations of triton X-100 in the printing buffer. However, the signal intensities were different with using different concentrations of the surfactant. The signal intensities increased firstly and then dropped with the enhancement of the concentrations of triton X-100 applied. The buffer containing 0.003% triton X-100 produced the strongest fluorescent intensities (Figure 2 B).

Triton X-100, a non-ionic detergent, was widely used to reduce the nonspecific adsorption in immunoassay (16, 17). Thus, high concentrations of triton X-100 prevent the protein molecules from the substrate and result in low immobilization efficiency. In addition, high concentrations of triton X-100 could damage the activity of proteins (18). That is why that the signal intensities decreased with the enhancement of triton X-100 after 0.003% concentration (see Figure 2 B). Deng and Zhu reported that the ring-like structures could be eliminated by 0.006% triton X-100 in the printing buffer for the epoxy-terminated slides based microarrays (12). The variation could attribute to the different surface properties of these two substrates. The aldehyde-terminated slide surface is more hydrophilic than the epoxy-terminated surface that needs higher level of triton x-100 surfactant to reduce the accumulation of protein on the air/water interface. Apparently, the results in this work show that the immobilization efficiency and quality on the aldehyde-terminated slide surface are much more improved after 0.003% triton X-100 was applied. It is very useful for further improving the performance of protein microarrays and for the quantitative applications with the optimized buffer composition.

4.3. Effect of glycerol on protein immobilization

Unlike DNA, proteins (Ag and Ab) have 3-dimentional structures and easily lose their bioactivities due to dehydration and denaturation during array printing and probe immobilization. The ability to protect Ag/Ab against denaturation is one of the most challenging tasks in fabrications of Ag/Ab microarrays. Even at room temperature and in humid environment, the nanodroplets containing proteins spotted on the glass slides could quickly evaporate to cause protein dehydration and denaturation. As an approach, glycerol is typically added to the printing solution for preventing Ag/Ab dehydration and subsequent denaturation (9, 15). Thus, we added glycerol into the printing buffer to prevent evaporation of probes nanodroplets and to retain the Ag/Ab bioactivity.

Although glycerol did have positive effect on protein activity during array printing, it could also cause negative effect, particularly on glass slides (10). For further optimization of printing buffers, 50 μg/ml rabbit IgG, diluted in 0.01 M PBS with 0.003% triton X-100 additionally plus 5%, 10%, 20% and 40% glycerol respectively, were arrayed onto the glass surface to produce antigen microarrays. Figure 3 A and B showed the fluorescent image and the effect of the printing buffer with different level of glycerol on the signal intensities of printed rabbit IgG arrays. The results indicated that the buffer containing 10% glycerol had the highest signal intensities, while the buffers containing glycerol lower than 5% and higher than 20% could reduce the signals. This is possibly caused by the high viscosity of glycerol. The printing buffer could become very viscous with addition of high concentrations of glycerol. A highly viscous buffer would retard the moving of proteins and further makes the probe molecules very difficult to approach to the substrate surface. The experimental results in our work demonstrate that 10% glycerol could efficiently prevent rabbit IgG dehydration and enhance the signal intensities.

4.4. Performance verification of the optimized printing buffer

According to above results, the optimized printing buffer is 0.01 M PBS with 0.003% triton X-100 and 10% glycerol. In order to verify this conclusion, the optimized printing buffer was applied to construct antigen and antibody microarrays to verify the performance. Rabbit

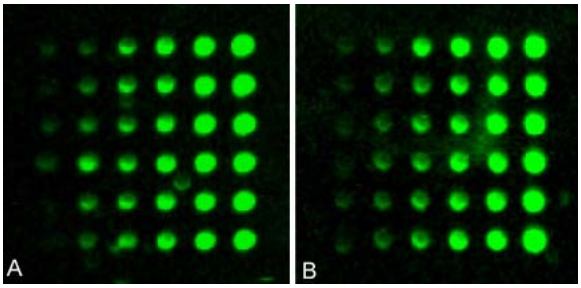

Figure 4. The performance of optimized printing buffer. A: Fluorescent image of antigen microarrays constructed with the optimized printing buffer. Different concentrations of rabbit IgG were immobilized onto functionalized slide surfaces, and then biotin-labeled anti-rabbit IgG and Cy3-conjugated streptavidin were applied respectively to produce fluorescent signals. From left to right columns, the concentrations of rabbit IgG are 0, 6.25, 12.5, 25, 50, and 100μg/ml. B: Fluorescent image of antibody microarrays constructed with the optimized printing buffer. After different concentrations of anti-rabbit IgG were immobilized onto substrate, 2 μg/ml rabbit IgG was applied firstly, and then biotin-labeled anti-rabbit IgG and Cy3-conjugated streptavidin were used. The concentrations of anti-rabbit IgG correspond to figure 4 A for each column.

IgG and anti-rabbit IgG were selected as the model proteins. Different concentrations of rabbit IgG and anti-rabbit IgG dissolved in the optimized printing buffer were printed onto the aldehyde-terminated slides surface. The results demonstrate that the ring-like non-homogenous structure is effectively eliminated with the applying of the optimized printing buffer (Figure 4 A and B). Moreover, the fluorescent signal of microarrays produced is much brighter than that with non-optimized printing buffer.

5. CONCLUSIONS

In this work, the printing buffer containing two supplements, glycerol and triton, was investigated and optimized on aldehyde-modified slide based microarrays. The results indicated that PBS produced better performance than CBS. The experimental results also demonstrated that triton X-100 had a different effect on the aldehyde-modified slide from the epoxy-terminated slide. The optimized printing buffer is 0.01 M PBS with 10% glycerol and 0.003% triton X-100, which could effectively prevent problems from non-homogeneous microspots morphology and acquire good signal intensities.

6. ACKNOWLEDGEMENT

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Abbreviation: APTES, 3-aminopropyltriethoxysilane; GA, glutaraldehyde; Ag, antigen; Ab, antibody; IgG, immunoglobulin

Key words: Protein Immobilization**;** Antigen/Antibody Microarrays Fabrication; Non-Homogenous Spot Profile; Printing Buffer Optimization

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