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A protein multiplex microarray substrate with high sensitivity and specificity

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Abstract

The problems that have been associated with protein multiplex microarray immunoassay substrates and existing technology platforms include: binding, sensitivity, a low signal to noise ratio, target immobilization and the optimal simultaneous detection of diverse protein targets. Current commercial substrates for planar multiplex microarrays rely on protein attachment chemistries that range from covalent attachment to affinity ligand capture, to simple adsorption. In this pilot study, experimental performance parameters for direct monoclonal mouse IgG detection were compared for available two and three dimensional slide surface coatings with a new colloidal nitrocellulose substrate. New technology multiplex microarrays were also developed and evaluated for the detection of pathogen specific antibodies in human serum and the direct detection of enteric viral antigens. Data supports the nitrocellulose colloid as an effective reagent with the capacity to immobilize sufficient diverse protein target quantities for increased specificity signal without compromising authentic protein structure. The nitrocellulose colloid reagent is compatible with the array spotter and scanners routinely used for microarray preparation and processing. More importantly, as an alternate to fluorescence, colorimetric chemistries may be used for specific and sensitive protein target detection. The advantages of the nitrocellulose colloid platform indicate that this technology may be a valuable tool for the further development and expansion of multiplex microarray immunoassays in both the clinical and research laboratory environment.

Keywords

immunoassay; protein microarray; multiplex

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1. Introduction

Proteomic multiplex microarray technology offers the potential for rapid, efficient, parallel multi-target analysis on small volume samples (i.e. <50µl in a single assay) for a considerable fraction of the cost compared to traditional methodology (Ekins, 1989, Kingsmore, 2006). Multiplex microarray analysis of clinically significant proteins is versatile and amenable to meet the needs of both the basic point of care clinical service laboratory and the high throughput facility performing more sophisticated diagnostic testing. Nucleic acid microarrays have been used with a great deal of success and have been brought into the practical realm of clinical medicine (Templin et al, 2004, Hadd et al, 2005). In contrast, the problems that have been associated with protein multiplex microarrays, i.e., binding, signal to noise ratio, sensitivity, and immobilization, has delayed research and development by comparison (Anderson, 2005).

Current planar glass microarray immunoassay platforms require spotting target protein onto a solid support or substrate which immobilizes the protein target. A majority of commercial substrates rely on protein attachment chemistries that range from covalent attachment, to affinity ligand capture, to simple adsorption. While covalent and affinity interaction can provide stable immobilization of proteins, these methods require chemical modification of the surface and/or protein to affect linkage and have limitations in binding capacity. Covalent bonding between protein and substrate can denature some proteins. Thus, current commercial protein multiplex microarray substrates involve trade-offs between firmly immobilizing capture proteins, binding sufficient protein content in individual assay spots, and maintaining protein structure and function in the immobilized multiplex microarray format (Qin et al, 2010, Kingsmore, 2006, Anderson, 2005, Kusnezow et al, 2003, Olle et al, 2005, Camarero, 2007).

The physical and chemical characteristics of nitrocellulose are well defined and this reagent has been used routinely for protein binding and analysis in both macro and micro immunoassay formats, including Western Blots (Kurien, Scofield, 2009) We have developed a three dimensional protein microarray substrate that can overcome most of the challenges associated with protein multiplex microarray applications and designed a pilot study to compare and evaluate the performance characteristics of this new colloid form nitrocellulose technology and representative planar microarray substrates. Also included in this study, two nitrocellulose colloid multiplex microarrays to monitor human infectious disease exposure were prepared and evaluated. The infectious disease application was specifically selected to demonstrate technology advantage and compatibility with the clinical laboratory environment.

2. Materials and Methods

2.1 Nitrocellulose Colloid Stock Solution

The nitrocellulose colloid suspension is prepared by dissolving 5 g of 70% cellulose nitrate and 30% 2-propanol (Aldrich, St. Louis MO, USA) in 500 mls of a water miscible organic solvent such as, acetone. The clear nitrocellulose solution is then added drop-wise over a period of two hours to 5 liters of distilled water with continuous mixing, in such a way that the water insoluble nitrocellulose forms a milky colloidal suspension. The colloidal particle size range is from 0.5–1.0 µm. The particles are first washed with 0.2 M nitric acid and precipitated. Next, the colloid solution is washed with distilled water, pH neutralized, re-suspended to a final 10–20% packed colloid concentration in aqueous solution. The nitrocellulose colloid stock solution, stored at 4°C, has maintained protein binding capacity and has been stable for up to 1 year when tested. (Awdeh, et al, 2005).

2.2 Nitrocellulose Colloid Multiplex Microarray Preparation

Each target protein to be included in a multiplex microarray is individually exposed in solution, to a separate suspension of stock nitrocellulose colloid and incubated for 1 to 2 hours at room temperature for target binding. The maximum amount of protein target that may be bound to the nitrocellulose colloid was found to be approximately 25µg of bovine serum albumin per µl of packed nitrocellulose colloid. **Prior** to constructing a spotted microarray, the target protein-nitrocellulose colloid combination is blocked by treatment with “Dig-Block,” (Roche Diagnostics, Indianapolis, IN), a milk based blocking solution, for 2 hours at room temperature. Each blocked, target protein-nitrocellulose sample is then washed twice with distilled water to remove excess blocking solution. A binder solution consisting of 0.5% low melting agarose and 1.0% PEG 8000 (Fisher Scientific, Suwanee, GA) twice the volume of the packed colloid volume is then added to the blocked nitrocellulose plus target precipitate and thoroughly mixed. The suspension is transferred to a 96 well plate (Corning # 6511) and transferred to an Affymetrix 417 Arrayer for spotting in a microarray format onto clean glass 1”X 3”, microscope slides. Arraying is performed in a constant humidity environment and after spotting the arrays are dried overnight at room temperature. The microarray spots consist of a three-dimensional matrix of nitrocellulose particles displaying immobilized target proteins. The spotted arrays are then stored at room temperature, in a covered microscope slide holder box and have been processed successfully at post 6 months production. See Figure 1

2.3 Substrate Comparison Study

We conducted a comparison study of performance parameters of microarrays constructed with the colloidal nitrocellulose system and three conventional commercial substrates used for making protein microarrays. The performance metrics evaluated were: 1) protein binding capacity, 2) analyte concentration range, measurable by Cy3 fluorescence detection (working or dynamic range), and 3) lowest analytical limit of detection. For these studies, the colloidal nitrocellulose microarrays were prepared using suspensions of nitrocellulose colloid mixed with two-fold serial dilutions, out to 15X, (approximate final titer ~1:16,384) of murine monoclonal IgG, 1mg/ml, (Bioscience/OEM, Saco, ME, USA) blocked *in solution* and then spotted on to clean glass microscope slides using an Affymetrix 417 Arrayer similar to the procedure presented in Figure 1.

Additional two-fold serial dilutions of mouse monoclonal IgG antibody, 1mg/ml, (Bioscience/OEM, Saco, ME, USA) were prepared accordingly and then spotted using the same Affymetrix 417 Arrayer onto the three different commercial substrates according to manufacturers’ instructions: 1. Prolinx™, 2. FAST and 3. Streptavidin- coated slides. The commercial substrates selected represent three different surface immobilization chemistries and included: Prolinx™ polymerized slides from Prolinx, Inc. (Bothell, WA, USA), and FAST nitrocellulose coated slides from S & S/Whatman (Kent, UK) and Streptavidin-coated slides from Telechem, ArrayIt (Sunnyvale, CA, USA). In the Prolinx™ system with Versalinx chemistry, proteins covalently bound to phenyl diboronic acid P (D) BA are spotted onto slides coated with salicyl hydroxomic acid (SHA) and the proteins are immobilized on the surface by the specific complex formation of P (D) BA with SHA. FAST slides use a relatively thick (~10µm) three dimensional nitrocellulose membrane to which proteins non-specifically adsorb. Streptavidin slides are made by coating aldehyde slides with streptavidin and arrays constructed using biotin conjugated proteins (Guillaume et al, 2005). The protein microarrays, for each substrate type, were prepared by direct spotting of 0.003 µl of the serial dilution preparations (i.e. 30ng to 3 pg protein per spot). In all cases, 5 duplicate rows were spotted, with the highest concentration, 30 ng, of mouse IgG in the first column, followed by the 14 additional two-fold serial dilutions. BSA was spotted as a negative control in the last column. After blocking, each slide was incubated for one

hour with 25 μ l of 0.02 mg/ml Cy3-labeled goat anti-mouse IgG antibody (Accurate Chemical and Scientific, Westbury, NY), washed 5X in PBS containing 0.5% Tween 20 (Pierce, Rockford, IL, USA) and scanned with an Affymetrix 418 laser scanner. Specific Fluorescence Intensity (SFI) for each spot was calculated as follows: $SFI = S_f - B_f$, where S_f = target spot fluorescence intensity and B_f = BSA negative control, fluorescence intensity.

2.4 Multiplex Microarray for Detection of Pathogen-specific Antibodies in Human Serum

Routinely, clinical laboratories must test for pathogen specific antibody status and a single-plex ELISA is the most common immunoassay used. The set of pathogenic antigens selected for development of a colloidal nitrocellulose multiplex microarray to detect human pathogen specific antibodies included: Hepatitis B Core antigen, Hepatitis C Core antigen, Treponema palladium, HIV-1, HIV-2 and Human-T cell Lymphotropic virus Type I and Type II. (Recombinant proteins were purchased from Biodesign/OEM, ME, USA and viral lysates from ZeptoMetrix, Buffalo, NY, USA.) Three dilutions of human IgG (Biodesign, OEM, ME, USA) i.e. high, med, and low, containing 25, 12, and 6 μ g protein respectively, per μ l packed nitrocellulose colloid were included as positive controls. The negative control spotted in duplicate had 25 μ g human serum albumin (Talecris Biotherapeutics, Inc. NC, USA) per μ l packed nitrocellulose colloid.

The optimal experimental conditions for the human infectious disease multiplex microarray were determined to be as follows: a 1:4 test serum dilution was added and incubated for 2 hrs at room temperature and then washed 5X with PBS + 0.5% Tween 20 (Pierce, Rockford, IL). Next, 12.5 μ g/ μ l of the secondary antibody, Cy³ – labeled goat-anti-human IgG (Pierce, Rockford, IL) was added and incubated at 37°C for 1hr and washed 7X with PBS + 0.5% Tween 20.

The nitrocellulose colloid multiplex microarrays were evaluated for qualitative specificity with 30 clinical laboratory College of American Pathologists and American Association of Blood Banks (CAP/AABB) Survey human reference sera required for clinical laboratory certification and provided by CBR Laboratories, Inc. Boston, MA, USA. The processed multiplex microarrays were scanned with an Affymetrix 418 laser Scanner. Specific Fluorescence Intensity (SFI) for each spot was calculated as described above.

2.5 Colorimetric Microarray for Direct Detection of Enteric Viral Antigens

Colloidal nitrocellulose microarrays were prepared as described in Figure 1. Monoclonal antibodies of the following specificities were adsorbed to colloidal nitrocellulose: adenoviruses 40 and 41 (monoclonal antibodies IC11 and 4F2, respectively; Herrmann et al., 1987), group specific adenovirus (anti-hexon monoclonal antibody 2Hx-2, Cepko et al., 1983), astrovirus (8E7, Herrmann et al., 1988), Norwalk virus (Herrmann et al., 1995), and rotavirus (monoclonal antibody 3F7, Cukor et al., 1984). After blocking, slides were spotted as described above. 40 μ l of test sample diluted 1:10 in PBS were added to each colloidal multiplex microarray. The slides were incubated at room temperature for 1 hour and washed 6X with PBS+0.05% Tween 20. A mixture of biotinylated monoclonal antibodies (each diluted to 1:1500, a final concentration of ~0.7 μ g/ml for each antibody) was added and incubated at room temperature for 1 hour. The slides were washed 3X with PBS+Tween 20 and streptavidin-horseradish peroxidase (HPO) was added and incubated for 30 minutes at room temperature. Slides were then rinsed in PBS+Tween 20 and quickly with water, and TMB (3,3', 5,5"-tetramethylbenzidine) membrane HPO substrate was added. After about 5 minutes at room temperature the color reached peak, and water was added to stop the reaction.

3. Results

3.1 Results of Substrate Comparison

Presented in Figure 2-I, are developed microarray images that were prepared by conventional direct spotting with the nitrocellulose colloid technology (A1, A2) and onto three commercial substrates: Prolinx (B), S&S FAST (C) and Telechem strepavidin (D). In all cases, five duplicate rows were spotted, with the highest concentration of mouse IgG in the first column, followed by two-fold serial dilutions. BSA was spotted as a negative control in the last column. After blocking, each slide was incubated for 1 hour with 25 μ l of 0.02 mg/ml Cy3-labeled goat anti-mouse IgG antibody, washed in PBS containing 0.5% Tween, and scanned using an Affymetrix 418 scanner. Slide C was scanned with 10% gain because of background. It should be noted that the FAST slide has the same nitrocellulose binding characteristics as the colloid but the higher background of the FAST slide decreases the signal detection level. Slides B and D were scanned at 50 gain. A1 and A2 are the same slide scanned at 10 and 50 gain to obtain the full detection and quantitation range. Comparative fluorescence of the four different microarrays substrates is shown in the graph, Figure 2-II.

Colloidal microarrays were found to exhibit superior analytical sensitivity with a much lower limit of detection (<10 pg/spot) – more than two logs lower than the conventional spotted microarrays prepared on commercial substrates. Colloidal arrays also yielded higher signals with lower background and were capable of measuring IgG concentrations over a wide dynamic range, >3 pg to <30 ng IgG. A dose-response comparison of fluorescent signal output as a function of IgG concentration, as shown in Figure 2-II, best illustrates the colloid technology's performance characteristics that result from its high protein binding capacity – high analytical sensitivity and wide dynamic range.

3.2 Results of Multiplex Microarray for Detection of Pathogen-specific Antibodies in Human Serum

For this feasibility study only, to evaluate qualitative multiplex microarray specificity, a SFI ≥ 2 was considered positive. The results obtained with the 30 CAP/AABB Survey Samples tested and this multiplex microarray were completely concordant with the CAP/AABB Survey results and confirmed the capability of the colloidal nitrocellulose multiplex microarray to assay simultaneously and accurately detect a number of unrelated and diverse target antigens in the same sample. No significant cross-reactivity was detected.

In Figure 3, we present just four examples of the multiplex microarray developed images for the selected human pathogens. The serum sample antibody status based on the calculated SFI was determined to be as follows: **I.** Negative **II.** Anti-Hepatitis B Core and anti-HIV-1 positive **III.** Anti-Hepatitis B Core positive and **IV.** Anti-Hepatitis C Core and anti-T. Palladium positive.

3.3 Results of Colorimetric Colloidal Multiplex Microarray for Enteric Viral Antigen Detection

In Figure 4, we present examples of colorimetric developed colloidal nitrocellulose multiplex microarray experiments. This array was designed to detect and distinguish various enteric viral pathogens of humans. The two enteric adenoviruses (types 40 and 41) were each detected by a type-specific monoclonal antibody (rows 2 and 3 for adenoviruses 41 and 40, respectively) and by a group-specific monoclonal antibody that recognizes all adenoviruses (row 4) (Fig. 4-I and 4-II). SA11 rotavirus was detected by a rotavirus-specific monoclonal antibody (row 6) (Fig. 4-IV). Patterns of binding were as expected, and no crossreactivity was observed against monoclonal antibodies specific for norovirus (row 1) or

astrovirus (row 5). Adenovirus 4, a respiratory adenovirus, bound to the anti-hexon (group specific) monoclonal antibody, but did not bind to antibodies specific for the two enteric adenoviruses (Fig. 4-III).

4. Discussion

Until recently, microarray format immunoassays were developed exclusively with fluorescence. The level of training and expense of required sophisticated laser scanners limited their application in routine clinical diagnostics. The successful introduction of colorimetric and chemiluminescent protein microarray reagents that are compatible with superior protein microarray substrates, like the colloidal nitrocellulose, has expanded the number of clinical diagnostic applications and dramatically increased the number of potential service providers eligible to afford and offer testing services utilizing this technology. The advantages of a multiplex micro-immunoassay system are well documented (Walton, et al 2009). A multiplex system assay effectively delivers multiple single assay results from a single experiment. We have developed a colloidal nitrocellulose multiplex microarray system for human infectious disease testing and the data supporting feasibility is presented here.

The colloidal nitrocellulose technology also solves many major technical problems inherent in available protein microarray substrates and has many significant advantages over some protein immobilization techniques. Colloidal nitrocellulose protein multiplex microarrays do not require chemical activation of the surface to effect protein immobilization and the extremely high protein binding capacity and stability of the nitrocellulose is not altered. (Kurien, Scofield, 2009) Moreover, the protein is bound to the colloidal nitrocellulose substrate and all of the excess protein binding capacity blocked before spotting so that production, quality control, and processing are more efficient.

The fundamental principle that accounts for the enhanced sensitivity and dynamic range is that each protein spot, deposited in colloidal nitrocellulose, has the potential to trap in three dimensions an equivalent amount of target protein orders of magnitude larger than other conventional protein microarray technologies in which proteins are directly spotted/printed onto array support/substrates. The results of the protein-binding capacity experiments support nitrocellulose colloidal technology as a promising platform for highly sensitive protein-based multiplex microarray assay systems for various applications.

In the presented studies, we found that colloidal nitrocellulose protein multiplex planar microarrays, prepared using a variety of proteins, are uniform and have highly reproducible protein spots. The Affymetrix 417 Arrayer that was used in these studies uses a ring pin technology and we have calculated a spot to spot coefficient of variation of $\leq 10\%$ for this system (Data not shown). The colloidal nitrocellulose technology for multiplex microarrays is also versatile and can serve as a matrix for many diverse proteins, raising the possibility of many different applications in diagnostic laboratory medicine. Perhaps most importantly, the colloidal nitrocellulose technology, in comparison to alternative substrates, is very cost effective. Production of colloidal nitrocellulose microarrays is efficient because the substrate nitrocellulose is stable and the stock colloid solution may be stored for extended periods of time. The amount of reagent protein required to produce specific signal is equivalent to alternative technology requirements. Also, the colloid technology is compatible with most current commercial instruments for making and reading fluorescent and colorimetric microarrays. Both the instrumentation used for high-density microarray nucleotide applications, and newer, far less expensive readers can be used to image developed colloidal multiplex microarrays. Also, colorimetric developed microarrays can be read with the naked eye and a simple hand-held magnifier, a major advantage for point of care testing.

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Figure 1. Preparation of colloidal nitrocellulose multiplex microarrays

Each nitrocellulose colloid spot is a three dimensional structure on a plain glass microscope slide.

Figure 2. Comparative antibody detection capacity of four different microarray substrates
2- I. Microarrays spotted with two-fold serial dilutions of mouse monoclonal IgG were probed with Cy³-labelled goat anti-mouse IgG antibody and scanned using an Affymetrix 418 scanner. Slide C was scanned with 10% gain because of background. It should be noted that the FAST slide has the same nitrocellulose binding characteristics as the colloidal nitrocellulose but the higher background of the FAST slide, due to the thick layer, i.e. 10 μ m, of nitrocellulose, decreases the signal detection level. Slides B and D were scanned at 50 gain. A1 and A2 are the same slide scanned at 10 and 50 gain respectively, to obtain the full the detection and quantitation range.
2- II. Comparative fluorescence of the four different microarrays substrates is shown in the graph.

Figure 3. Multiplex Microarray for Detection of pathogen specific antibodies in human serum
 The Multiplex Microarray format is as follows: A1= Hepatitis B Core, A2 = Hepatitis C Core, A3 = HTLV-I, A4 = HTLV-II, B1 = T. Palladium, B2 = HIV-1 IIIB, B3 = HIV-2, B4 = Human Serum Albumin, 25 $\mu\text{g}/\mu\text{l}$ packed colloid C1 = Human IgG, 25 $\mu\text{g}/\mu\text{l}$ packed colloid, C2 = Human IgG, 12 $\mu\text{g}/\mu\text{l}$ packed colloid, C3 = Human IgG, 6 $\mu\text{g}/\mu\text{l}$ packed colloid and C4 = Human Serum Albumin, 25 $\mu\text{g}/\mu\text{l}$ packed colloid

Figure 4. Colorimetric microarray for detection of enteric viruses

Light microscope (40X) images of developed colloidal microarrays. The multiplex microarray format is as follows: Each spot in triplicate, Row 1 = anti-norovirus, Row 2 = anti-adenovirus 41 (type specific), Row 3 = anti-adenovirus 40 (type specific), Row 4 = anti-hexon of adenovirus (group specific), Row 5 anti-astrovirus and Row 6 anti-rotavirus.