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Cluster of differentiation antibody microarrays on plasma immersion ion implanted polycarbonate

E. Kosobrodova [a,*], A. Mohamed [b], Y. Su [c], A. Kondyurin [a], C.G. dos Remedios [b], D.R. McKenzie [a], M.M.M. Bilek [a]

[a] Department of Applied Plasma and Physics, School of Physics, University of Sydney, Sydney, 2006, Australia
[b] Muscle Research Unit, Discipline of Anatomy and Histology, Bosch Institute, University of Sydney, Sydney, 2006, Australia
[c] Australian Center of Microscopy and Analysis, University of Sydney, Sydney, 2006, Australia

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ABSTRACT

Plasma immersion ion implantation (PIII) modifies the surface properties of polymers, enabling them to covalently immobilize proteins without using linker chemistry. We describe the use of PIII treated polycarbonate (PC) slides as a novel platform for producing microarrays of cluster of differentiation (CD) antibodies. We compare their performance to identical antibody microarrays printed on nitrocellulose-coated glass slides that are currently the industry standard. Populations of leukocytes are applied to the CD microarrays and unbound cells are removed revealing patterns of differentially immobilized cells that are detected in a simple label-free approach by scanning the slides with visible light. Intra-slide and inter-slide reproducibility, densities of bound cells, and limits of detection were determined. Compared to the nitrocellulose-coated glass slides, PIII treated PC slides have a lower background noise, better sensitivity, and comparable or better reproducibility. They require three-fold lower antibody concentrations to yield equivalent signal strength, resulting in significant reductions in production cost. The improved transparency of PIII treated PC in the near-UV and visible wavelengths combined with superior immobilization of biomolecules makes them an attractive platform for a wide range of microarray applications.

1. Introduction

Monoclonal antibodies are the fastest growing group of biotherapeutics [1]. They can target specific molecules in patients and provide an efficient and safe treatment of cancer [2], autoimmune disease [3], anemia [4] and heart disease [5]. Each year, hundreds of monoclonal antibodies and fusion proteins undergo clinical evaluation. An ideal antibody therapeutic has to be absolutely specific to the target and must not cause an immune response in the human body. Antibody cross-reactivity resulting in non-target binding is commonly tested by Western blot [6], immunocytochemistry [7], immunohistochemistry [8], competitive ELISA [9], and flow cytometry combined with immunofluoresence [10]. These methods are labor- and time-consuming and require expensive equipment. In recent years, a number of studies [1,11,12] have shown that protein microarrays can be successfully applied for rapid and sensitive analysis of antibody cross-reactivity and epitope mapping.

Although these microarrays provide valuable information about antibody–antigen interactions, they cannot fully imitate a complex immune system with its cell–cell interactive relationships. Due to the foreign nature of biotherapeutics, even successful medicines can be hemotoxic and cause immune responses and other adverse events in patients [13]. Anti-cluster of differentiation (CD) antibody microarrays not only allow testing of antibodies for cross-reactivity, but also provide the ability to directly observe the reactions of living human white blood cells to foreign antibodies without complicated sample preparation and cell staining. Anti-CD antibody microarrays printed on nitrocellulose (NC) coated glass slides have already demonstrated good results in leukocyte immunophenotyping for leukemias [14–16], lymphomas [16], stable/unstable angina [17], colorectal cancer [18], and HIV infection [19]. The high degree of correlation of these results with flow cytometry demonstrates the reliability of antibody microarrays [20].

A simple, rapid and label-free method of detection can be used to detect large immobilized objects such as white blood cells using anti-CD antibody microarrays. When the slide is scanned using transmitted light, the presence of the attached cells is detected as a reduction in the transmitted intensity. This method was previously implemented using NC-coated glass slides [16]. However, the sensitivity of the microarrays was limited by a background signal due to the low light transmittance of NC, even when wet. The use of a transparent platform for antibody immobilization can reduce the background signal and so significantly improve the sensitivity when transmitted light is used for detection.

In this study, we tested transparent plasma immersion ion implantation (PIII) treated polycarbonate (PC) as a platform for anti-CD antibody microarrays. PIII treated polymers have a high protein-binding capacity

* Corresponding author. Tel.: +61 2 9114 1338.
E-mail address: elenak@physics.usyd.edu.au (E. Kosobrodova).

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[21,22]. Proteins form a continuous layer covering the entire surface of a PIII treated polymer [23] and become covalently immobilized to the surface [24,25]. This immobilization without the need for chemical linker molecules has recently been shown to be due to the presence of radicals created in the polymers by the energetic ion bombardment of PIII [26]. The radicals retain their bonding activity for over a year [27,28]. To estimate the performance of anti-CD antibody microarrays based on PIII treated transparent PC, we investigated intra-slide and inter-slide reproducibility, the dynamic range of detection and the density of bound cells and compared these parameters to the same parameters obtained with commercially sourced Medsaic DotScan™ microarrays printed on NC-coated glass slides and with other materials previously used as protein microarray platforms.

2. Materials and methods

2.1. PIII treatment of PC substrates

Polycarbonate (PC) sheets with a nominal thickness of 1.0 mm were purchased from Goodfellow Cambridge Limited, UK. The PC sheets were cut to conform to standard glass slides with a surface area of 25 × 75 mm^2 and were treated with PIII as described in Ref. [27] for 40–1600 s corresponding to ion implantation fluences of $5\cdot10^{14}$–$2\cdot10^{16}$ ions/cm^2.

2.2. Transparency

The transmission spectra of untreated and 40–1600 s PIII treated PC as well as NC-coated glass slides (both dry and wet) were measured using a Cary 5E UV–Vis–NIR spectrophotometer in the wavelength range of 200 nm to 800 nm.

2.3. FTIR ATR spectroscopy

Untreated and PIII treated PC sheets were cut into four 1 cm × 2.5 cm strips each. Two of four samples were placed into Falcon tubes with 1 mM phosphate-buffered saline (PBS), pH 7.4, stored overnight at room temperature and washed for 10 s in milli-Q-water (Millipore). One of the two samples was dried overnight. The second sample was boiled in 1% SDS for 30 min, washed for 30 min in milli-Q water and dried overnight. The other two samples were incubated with mouse IgG (1 mg/ml) overnight at room temperature, washed in PBS for 30 min and for 10 s in milli-Q-water to remove unbound IgG. Then, one of these samples was dried overnight at room temperature and the second one was boiled in 1% SDS for 30 min, washed in milli-Q-water for 30 min and dried overnight. FTIR ATR spectra were measured from the samples using a Digilab FTS7000 FTIR spectrometer fitted with a multibounce ATR accessory with a trapezium germanium crystal at an incidence angle of 45°. To obtain sufficient signal/noise ratio and resolution of spectral bands, 500 scans were taken at a resolution of 4 cm^{-1}. The presence of mouse IgG on the polymer surface was inferred by the appearance of absorptions associated with amide vibrations characteristic of polypeptides. These characteristic amide absorptions were seen in the spectra taken from samples incubated in IgG after subtraction of spectra taken from the equivalent (boiled/not boiled in SDS) samples incubated in PBS in the absence of IgG. The spectra were analyzed using Digilab software.

To estimate the amount of mouse IgG bound to the PC samples before and after SDS boiling, we subtracted FTIR spectra of the samples stored in PBS from the spectra of the samples incubated in IgG. The subtracted spectra were normalized on the intensity of the 1505 cm^{-1} absorbance line of PC in the original (before subtraction) IgG containing spectrum to eliminate the effects of differences in contact pressure during the measurements. Then the mean absorbance of 3315 cm^{-1}, 1650 cm^{-1} and 1540 cm^{-1} lines corresponding to Amide A, Amide I and Amide II was calculated with normalization coefficients of 0.14, 1 and 0.47, respectively. This method of estimation of the protein amount was previously described in detail in Ref. [29].

2.4. CD antibodies and cells

Mouse IgG and rat anti-mouse CD antibodies (CD2, CD3, CD29, CD184, CD244.1 and CD326) were purchased from Sapphire Bioscience (Waterloo, NSW, Australia). Antibody solutions were reconstituted as recommended and stored in aliquots with 0.1% w/v BSA at 4 °C. The antibody concentrations ranged from 15.8 to 500 μg/ml.

Blood samples were drawn from healthy patients with approval of the Human Ethics Committee of the University of Sydney. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation as described in Ref. [30], washed in PBS and resuspended in PBS containing 1 mM EDTA to a density of $1.2\cdot10^7$ cells/ml. Viability of PBMCs was measured prior to hybridization using trypan blue to identify dead cells. All samples contained more than 75% living cells.

3. Production of CD antibody microarrays

The antibody spots were contact printed on the surface of the PC slides using a Bio-Rad robot, dried at room temperature for 1 h and stored at 4 °C. All printed spots had a diameter of 300–350 μm with a distance between the spots of 1 mm. The slide surface was the blocked with 5% w/v skim milk (Diploma; Bonlac Foods, Melbourne, Vic., Australia) in PBS for 90 min at room temperature. The slides were washed in PBS for 2 min. The microarrays were incubated with 200 μl of PBMCs ($0.19–12\cdot10^6$ cells/ml) for 30 min at room temperature. Unbound cells were washed off in PBS. The bound cells were fixed for 20 min in 3% w/v formaldehyde in PBS, washed in PBS and stored in PBS at 4 °C before analysis.

3.1. Data recording and analysis

The microarrays were scanned by a Medsaic DotReader™. Images were analyzed using DotScan software with an 8-bit pixel gray scale from 0 to 256. The intensity of spots and background signal was evaluated using Matlab software. Relative spot intensity (RSI) for a particular spot was calculated as the mean spot intensity (MSI) less the mean background (MB) on the same slide.

The intra-slide coefficient of variation (CV_{intra}) was calculated as the ratio of the standard deviation to the mean RSI values for the 12 spots of the same antibody on a single slide. To eliminate both non-biological variations introduced during microarray production and biological variations due to different blood sources, the mean RSI values of each antibody on each slide were normalized to the mean RSI value for the CD29 spots on the same slide. Then the inter-slide coefficient of variation (CV_{inter}) for a particular antibody was calculated as the ratio of the standard deviation across 6 slides to the mean normalized RSI values for spots of that antibody.

Bound PBMCs were imaged by a digital camera connected to an optical microscope (Axioplan 2 Imaging, Zeiss, Australia) with 20×/0.22 HD DIC and 10×/0.25 HD DIC objectives, in transmitted light mode. The density of bound cells was estimated using ImageJ software.

SEM images were also used to investigate interactions of human PBMCs with rat anti-mouse CD antibodies. Samples were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer and 0.1 M sucrose solution (1:1) at 4 °C overnight and rinsed three times in 0.1 M sodium cacodylate and sucrose buffer. Then the samples were post-fixed with 1% osmium tetroxide solution in 0.1 M sodium cacodylate and sucrose buffer at RT for 1 h. The samples were rinsed three times with sodium cacodylate and sucrose buffer and dehydrated in graded ethanol solutions (70%, 80%, 90%, 100% and 2× dry ethanol) for 10 min each. The samples were dried in 100% hexamethyldisilazane for 3 min, air-dried for 1 min and then transferred to a desiccator for 25 min. The samples