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Nanosphere lithography-based platform for developing rapid and high sensitivity microarray systems

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ABSTRACT

A novel gold nanoarray (NA)-based platform was developed for microarray applications. This novel approach is based upon the principle of nanosphere lithography and can be used for one-step antibody immobilization. The developed platform was checked by functionalizing with cysteine followed by capturing biotinylated antibody and detecting it with dye-conjugated steptravidin. An immunoassay was performed with spiked samples containing human fetuin A antigen. The minimum limits of detection (LOD) of human fetuin A for NA-based and conventional microarray platforms were 50 pg/mL and 50 ng/mL, respectively. The developed approach was highly reproducible and unlike conventional microarray approaches the use of a spotting system was omitted because immobilization was controlled and directed on the predefined arrays. This approach could be an ideal alternative for developing microarrays. And, the ease of the strategy also allows the high throughput production of the microarrays.

1. Introduction

Microarray, with the advent of high density printing, has become a high-throughput and cost-effective bioanalytical technique allowing the fabrication and exploitation of DNA and protein microarrays [1–4]. Various protein microarray platforms have been developed for interactome analysis that includes screening of antibodies and antigens, novel proteins, protein-nucleic acid and protein–protein interactions [5–10]. Immuno-proteomic analyses, that assess antibody-antigen interactions, are among the most established microarray applications [11–14]. These array-based assays have the potential to impact the field of infectious disease diagnostics and greatly facilitate the design of subunit vaccines [11,13]. Additionally, the development of microarray applications for the functional characterization of proteins of unknown or unproven function continues to be a major challenge [14–17]. Whereas, the array capacity for protein screening is limited to 1000–10,000, which is relatively low compared to DNA arrays [18–20]. Requirement of precision spotting and tendency of proteins to leach out from the polyion-grafted surface are two major drawbacks associated with the current microarray techniques.

The generation and immobilization of pure protein reagents are critical to the quality of protein microarrays. However, the protein attachment to the surface, that may entail covalent immobilization or adsorption, involves surface preparation which universally is performed by generating an adsorbed polylysine matrix on the support of choice [21–23]. Protein leaching and non-specificity [24] are few of the major addressable technical difficulties associated with planar microarray platforms. Despite the development of many strategies for protein capture there is an immense need to devise an efficient generic surface and a relatively easy modification procedure that can increase the reproducibility of the array performance and reduce the requirement of cumbersome and sophisticated instrumentation. In addition, such platform should also support the development of highly sensitive antibody-bound microarray platforms with a high specificity, irrespective of the nature of solid-support matrix employed.

Microarrays were reportedly developed either by spin-coating [25] or drop-coating [26,27] for grafting polylysine/silane on to the solid supports. Additionally, many reports pertaining to the development of microarrays claim to use nitrocellulose membranes [28]. We report a method to develop a generic microarray platform by first generating a highly ordered network of gold islands with nanosphere lithography (NSL) [29] followed by functionalizing these gold nanoarrays (NA/s) using either cysteine or cysteine-conjugated streptavidin, which binds to the gold surface via thiol bonds such that either, amino and carboxyl groups remain free for further chemistries or streptavidin is available for biotin-

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mediated protein capture. NSL is a potential technique to fabricate NAs with excellent size, shape, and spacing control. It also allows to use different nanomaterials with desired functionality and morphology to design customized NAs to improve performance of devices due multiple charged cites, increases electro-active working surface area and multifold signal amplification. Most recent application of NSL-based NAs is multiplex analysis and Lab-on-a-Chip for desired diagnostics application for example cancer diagnosis.

The introduction of highly ordered nanoarrays in a given spot size (~ 0.5 μm; Supplementary Fig. 1) acts as a cluster of nano-spots; each nano-spot (size ~ 25 ± 5 nm), depending upon its dimensions, is capable of holding certain number of biomolecules (Fig. 1). Therefore, this strategy obviates the use of sophisticated microarray spotters and in absence of a microarray reader it can be analyzed even with a normal fluorescence microscope with appropriate filters. In addition, this strategy will enable researchers to develop in-house microarrays on various commercially relevant solid supports. Therefore, the developed procedure could be of great utility to diagnostics and bioanalytical sciences including proteomics research.

2. Materials and methods

2.1. Materials

All reagents were of analytical grade, unless otherwise specified. Phosphate-buffered saline (PBS, 0.1 M, pH 7.2), and bovine serum albumin (BSA) were purchased from Fischer Scientific, Dublin, Ireland. Human fetuin A/AHSG kit comprising of mouse anti-human fetuin A (capture antibody), human fetuin A (antigen), biotinylated goat anti-human fetuin A (detection antibody) was obtained from RnD Systems, Minneapolis, USA. Streptavidin-conjugated fluorescene isothiocyanate (FITC) and DyLight649 was procured from Sigma Aldrich, Ireland along with 1,4-dithiothreitol (DTT), H_2SO_4, H_2O_2, n-heptane and absolute ethanol. All buffers and solutions were prepared using Milli-Q deionised water (DIW). Dilutions of human fetuin A assay components were prepared in 0.1 M PBS (pH 7.2). Microarray spotter (SciflexArrayer) from Scienion AG, Berlin, Germany, was used for spotting 0.1 uL of each sample. Fluorescence intensity of all the platforms was measured using a fluorescence microscope with appropriate filters.

2.2. Methods

2.2.1. Development of nanoarray by nanosphere lithography (NSL)

Nanoarray (NA) was developed as described by Dixit et al. (2012) [29]. Briefly, polystyrene beads (100 nm in diameter) were drop-coated in spot sizes of 0.5 mm × 0.5 mm. This coating serves as a NSL mask. Further, a gold layer of a thickness of 25 nm was sputtered on the NSL mask, which will then be lifted-off by washing the chip with *n*-heptane and absolute ethanol for 5 min. After the mask lift-off and cleaning the NA is developed. Prior to use, NA is activated with piranha (3:1 H_2SO_4: H_2O_2) treatment for 2 min.

2.2.2. Development of antibody-conjugated NAs

Biotinylated anti-human fetuin A (HFA) antibodies were reduced using DTT under the conditions described by Cherkoui et al. (2010) [30]. Each reduction was performed afresh prior to the antibody immobilization. The immobilization was confirmed with FITC-streptavidin conjugates prior to the immunofluorescence assay development. Anti-HFA antibodies were prepared at a concentration of 4 μg/ml in PBS and the developed NA chip was incubated with 100 μl of this solution at 37 °C for 1 h. The chip was then washed extensively with PBS. The anti-HFA bound chip was then blocked with 1% BSA (in PBS, pH 7.4) and incubated for 30 min at 37 °C to block all non-specific protein binding sites on the base substrate. It was then washed extensively with PBS. A ten-fold serial concentration of HFA was prepared and incubated

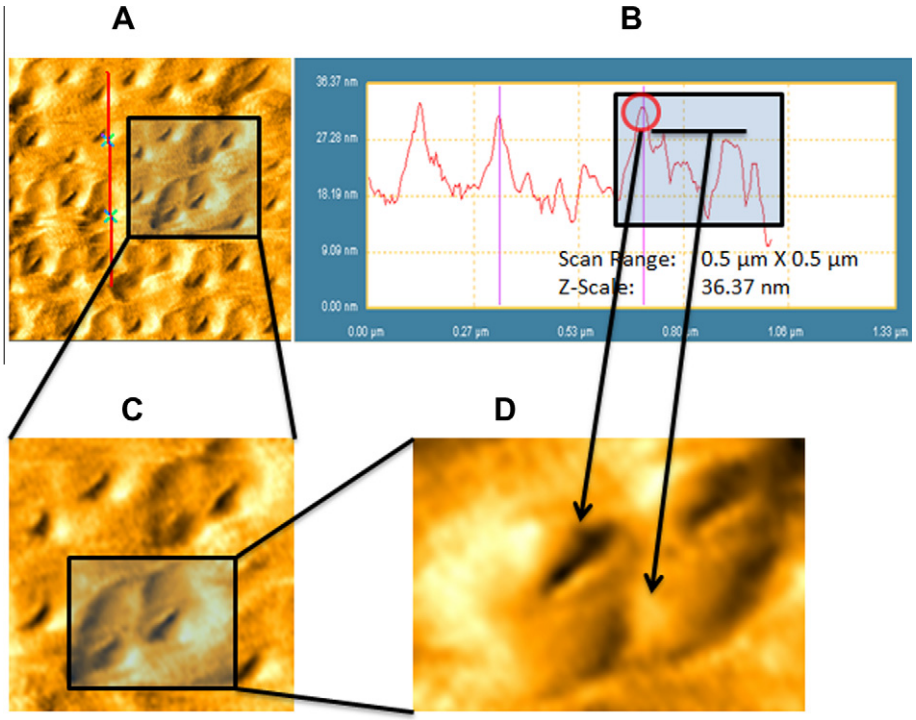

Fig. 1. Atomic Force Microscopy (AFM) image of a typical NA- based platform (A & C). AFM scans were performed for a scan range of 1.36 μm × 1.36 μm and a Z-distance of 36.37 nm. Further details pertaining to the spot size, shape and geometry is present in Supplementary material. Height profile of the streptavidin-functioanlized NAs holding holding the biotinylated antibody (B); streptavidin was immobilized on the gold NAs through thiol bond of the tagged cysteine. A close-up of the NA holding proteins (D).

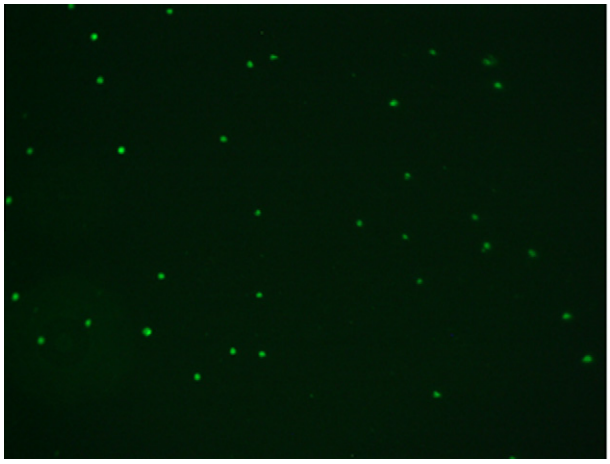

Fig. 2. Performance of the developed NA using cysteine-functionalized biotinylated anti-HFA antibody and FITC-conjugated streptavidin system. The developed NA was analyzed using a fluorescence microscope with appropriate FITC filter.

Table 1
Comparison of relative amount of antibody holding capacity of NA, PLA and GA.

Strategy	Surface area (nm^2)	Number of antibodies immobilized	Antibody/ nm^2
NA	64	23	0.35
PLA	3.14×10^{12}	6×10^9	0.19×10^{-2}
GA	3.14×10^{12}	2×10^9	0.63×10^{-3}

on the developed antibody-bound arrays for 1 h at 37 °C. Finally, it was washed extensively with PBS. The fluorescence of each chip was measured using the fluorescence microscope for FITC (Ex: 490, Em: 525) and DyLIght649 (Ex: 652, Em: 670)

2.2.3. Development of anti-HFA-bound microarray on polylysine (PL)-coated glass and activated glass

A polylysine (PL)-coated glass slide and a glass chip (both 5 mm × 5 mm) was cleaned by immersing it in 10 ml of absolute ethanol for five min followed by extensive washing with DIW. The cleaned PL glass chip was used as such for further antibody spotting; whereas, the glass surface was functionalized by immersing the cleaned chip in 10 ml of 1% KOH for 10 min and then washing extensively with DIW. Hydroxyl groups were generated on the surface based on the oxidation of the surface which was further treated with oxygen plasma in order to provide a stabilization effect to the generated functional groups.

Anti-HFA antibodies were prepared at a concentration of 4 μg/ml in PBS and 0.1 μl of this solution was spotted on to the PL-coated chip and activated glass chip using the microarray spotter and incubated at 37 °C for 1 h. The chip was then washed extensively with PBS. The anti-HFA bound chip was then blocked with 1% BSA (in PBS, pH 7.4) and incubated for 30 min at 37 °C to block all non-specific protein binding sites on the base substrate. It was then washed extensively with PBS. A ten-fold serial concentration of HFA was prepared and incubated on the developed antibody-bound arrays for 1 h at 37 °C. Finally, it was washed extensively with PBS. The fluorescence of each chip was measured using the fluorescence microscope for FITC (Ex: 490, Em: 525) and DyLIght649 (Ex: 652, Em: 670).

2.2.4. AFM based uniformity analysis of antibody-coated microarray platforms

Silicon nitride triangular cantilevers with nominal spring constant 0.12 N/m (Veeco, USA) was used for imaging using PicoPlus AFM. The cantilevers were washed in ethanol and deionized water and dried at 70 °C for 30 min. They were finally cleaned by UV treatment for 20 min. The imaging of samples was done in contact mode in air.

3. Results and discussion

Microarray is an important analytical platform, which is employed in molecular analysis and disease diagnosis. However, development of routine microarray is a complicated and laborious procedure. We have developed a simple NA-based platform (Supplementary Fig. 1), which was demonstrated for microfluidic-based applications [29]. We have demonstrated the NA platform as an alternative, cost-effective and easy method for developing microarrays with highly sensitive antigen detection.

The platform was assessed for its functionality by covalently-capturing the cysteine-functionalized and biotinylated anti-HFA antibodies on gold NAs and detecting them with streptavidin-fluorescein isothiocyanate (FITC) conjugates (Fig. 2). These NAs were developed on glass along with other analytically important polymeric solid supports such as polystyrene (PS) and cyclo-olefin (Zeonex) (data not shown). However, all the factorial comparisons associated to this study were performed for NAs on glass solid support. A comparison was performed for antibody-holding capacity of NAs with poly-L-lysine array (PLA; chemisorption) and plasma-treated glass array (GA; phsisorption). Total antibody holding capacity was in an order of PLA > GA > NA (Table 1). It was as expected because the NA coverage (presence of gold nanospots) in a given NA surface was approximately 2% of the total NA area (Supplementary information: calculations). However, ratio of the amount of protein to the available surface area for immobilization was highest in NA in comparison to the PLA and GA (Table 1).

Chip functionality was demonstrated with two immunoassay formats (direct format for HRP and sandwich format for human fetuin A using streptavidin-DyLight649 conjugate as fluorescent probe (Table 2). Fluorescence intensity measured for each subset was averaged out and normalized along with the appropriate controls. Significantly lower detection limits were obtained with both the immunoassay formats performed on NA-based microarray

Table 2
Comparison of analytical parameters of assays performed for protein antigen (human fetuin A and IgG) on PS matrix.

	Lowest detectable antigen concentration (ng/mL)	%CV
HRP detection (5 μg/mL) with different concentrations of DyLight649-conjugated anti-HRP antibody		
Assay (covalently arrayed Ab) on cysteine-functionalized nanoarray	0.7 (EC_{50}: 31)	4.15
Assay (adsorbed Ab) on commercial polylysine-grafted surface	102 (EC_{50}: 197)	11.39
Assay (adsorbed HRP) on O_2 plasma-treated glass surface	210 (EC_{50}: 325)	10.83
Human fetuin A detection (sandwich format)		
Assay (covalently arrayed HRP) on cysteine-functionalized nanoarray (on glass)	0.05 (EC_{50}: 19)	2.17
Assay (adsorbed HRP) on commercial polylysine-grafted surface (on glass)	50 (EC_{50}: 73)	9.81
Assay (adsorbed HRP) on O_2 plasma-treated glass surface	325 (EC_{50}: 428)	12.83

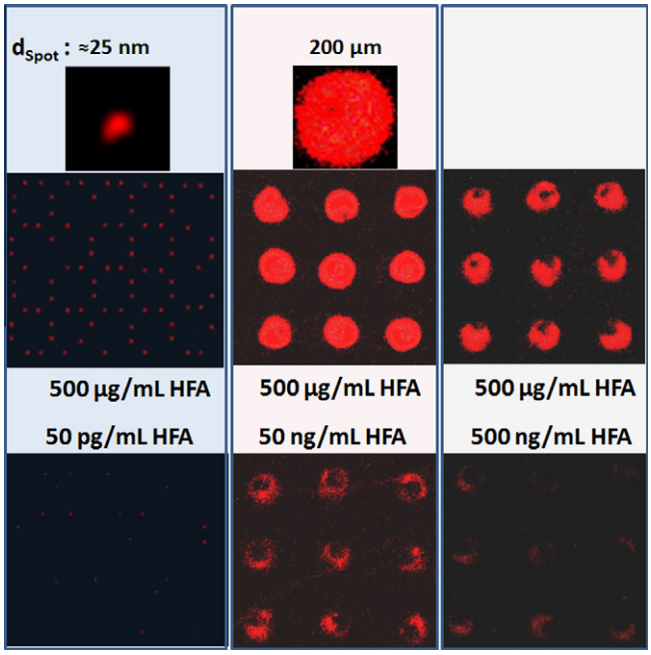

Fig. 3. Comparison of human fetuin A detection in a sandwich assay format with the developed nanoarray-based platform (a), commercially obtained poly-L-lysine glass slide (b) that was customized for microarray development, and plasma-activated glass surface. Anti-human fetuin A antibody (4 µg/mL) was captured on (a) via streptavidin–biotin interaction, (b) by chemisorption, and on (c) with passive adsorption. Nanoarray (a) was developed in a spot area of 0.5 mm × 0.5 mm such that each spot area has many individual NAs of size 25 µm; whereas, PLA and GA were developed in an area of 5 mm × 5 mm with nine spots of 200 µm each. For a spotting area of one-tenth of the size of (b) and (c), the resultant number of NA in (a) was significantly higher than the resultant spots in PLA and GA (nine each). Therefore, nanoarray-based platform was significantly effective than the array on unmodified surfaces with a LOD of 0.05 ng/mL.

platforms. This could be justified by higher protein to surface area ratio of NAs. In addition, few recent reports suggest that such gold patterns can enhance the fluorescence properties of the bound protein [31,32] or the fluorophore in their proximity [33,34]. It is evident from the improvement of detection sensitivity of the immunoassays i.e. 100-folds in direct and 1000-folds in sandwich formats, with the NA-based microarray platforms in comparison to the commercial PLA-based approaches (Fig. 3). It has also been reported that such patterned gold NAs significantly increases the surface-enhanced Raman signals [35–37]. Therefore, the same NA-based microarray platform can be employed for developing SERS-based diagnostics in conjugation with microarrays; however, that was not the focus of our current studies and could further be examined in separate studies.

The uniform distribution of the immobilized antibody-coated surface was assessed by atomic force microscopy. High uniformity in the anti-HFA antibody coating was obtained on all the three solid supports employed in this study (data not shown). It is also evident from the results that the NA-based microarray strategy is generic and can be used on chemically distinct solid supports with similar functional efficiency.

In summary, the results of this study suggest that the devised strategy enables the Pico-level detection of protein antigen in sample with improved sensitivity. NSL-based microarrays could be employed to reduce the variability of protein-antigen detection and enables the microarray development on desired solid supports without affecting cost. Besides having applications in diagnostics, biomarker screening and immunodiagnostics, this strategy may gain tremendous applications in microarray-based biochemical analysis of proteins, their interactions and molecular behaviour. In addition, it may also be used for SERS-based protein characterization due to the enhancement of Raman signal by the gold nano structures.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.144.

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