Creation of Protein Nanoarrays using Dip-Pen Nanolithography

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ABSTRACT

Protein microarrays are one of the leading tools for analysing the proteome due to a range of factors including; their relatively simplistic creation and use in an assay, low cost and long shelf life. One of the primary limiting factors in conventional microarray assay systems is that they create a spot of protein ~100-300 μm in diameter. The miniaturisation of these spots will allow for the creation of much denser arrays and hence will increase the number of simultaneous protein detections possible whilst decreasing the overall physical size of the array. One of the methods for creating protein nanoarrays currently showing promise is Dip-Pen Nanolithography (DPN), a scanning probe microscopy technique. This paper outlines the creation of two types of ultra small functional protein microarrays using DPN on a nitrocellulose surface and their use to detect protein:protein interaction.

Keywords: protein nanoarrays, dip-pen nanolithography.

1 INTRODUCTION

With the entire human genome now successfully mapped efforts have now become even more focused on unlocking the mysteries of the proteome. As the term genome defined the entirety of an organism's genetic code, its DNA, an organism's proteome is a comprehensive map of its proteins. This can either be the complete proteome of all proteins possible or a cellular proteome, the proteins being expressed at a certain time or in a certain situation.

Interactions between proteins are widespread, numerous and vital for most, if not all, biological functions. Studies of these interactions (Interactomics) has lead to a greater understanding of various biological systems, such as signal transduction. Most signal transduction pathways involve the binding of an extracellular signal molecule to a cell surface receptor which triggers a series of biological events within a cell. These events frequently take the form of a cascade, where a small stimulus elicits a large response, such as apoptosis or replication.

Protein arrays are now well established as incredibly useful tools for analyzing the proteome. Their versatility, low cost and ease of use means that they can be used to probe almost any protein interaction including protein:protein, protein:drug and protein:small molecule.[1]

The wide variation in the properties of proteins (polarity, hydrophobicity etc) has given rise to a large number of different surfaces being used as the array's solid support. Different surfaces give rise to different binding methods such as adsorption (nitrocellulose,[2] poly-L-lysine[3]), absorption (hydrogel[4]), covalent bonding (various silanes[5,6]) and affinity attachment (histidine tags[7]) (Figure 1).

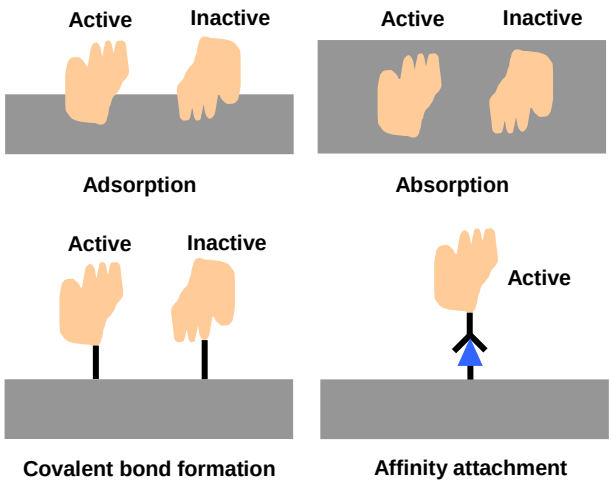

Figure 1. Schematic representation of four types of protein attachment to a surface. The diagram also illustrates the possible active/inactive conformations of the protein.

One of the primary drawbacks when attempting to fully utilise protein microarrays is the physical size of the protein spots generated. Microarray spots are typically 100-300 μm in diameter, limiting the number of spots possible per slide as well as dictating the minimum volume and concentration of the probe protein solution necessary. One of the ultimate goals in the field of proteomics is true single cell analysis and current microarrays are unsuitable for this purpose.

Protein nanoarrays are the most obvious refinement of protein microarrays. Miniaturising the protein spot to the nanoscale (<100 nm) would hugely increase the number of spots possible per unit area whilst simultaneously lowering the sample volume necessary to accomplish a positive

result. Three methods are currently significant avenues of research for fabricating protein nanoarrays; Micro (or nano) contact Printing, Electron Beam (e-beam) Lithography and Dip-Pen Nanolithography.[8] Micro (or nano) contact printing uses a specifically engineered stamp coated with an ink (e.g. protein) to create arrays on a surface.[9] E-beam lithography uses a beam of electrons to pattern a surface covered with a film called a resist followed by the removal of the resist from either the exposed or non-exposed areas.[10] Dip-Pen Nanolithography (DPN) is a recently developed offshoot of Atomic Force Microscopy (AFM). AFM is a high resolution scanning probe microscopy technique where a cantilever is brought into contact or close contact with a surface. The deflection of the cantilever as it moves across the surface is detected by monitoring by shining a laser onto the back of the cantilever, changes in the surface causing the laser spot to move. DPN uses the same technology as AFM, but an 'ink' is coated onto the cantilever which can then be 'written' onto the surface.[11-16]

2 RESULTS

The aim of this research is to integrate the existing robust nitrocellulose protein microarray assay system and DPN. By creating protein nanoarrays on nitrocellulose it should allow for much more sensitive detection of protein:protein interaction.

2.1 Towards Protein Nanoarrays

Two methods have been used to write proteins onto nitrocellulose. The first method uses a NanoInk Inkwell©. The inkwell method uses microchannels to deliver ink, in this case protein, to the cantilever tip. The tip is then brought into contact with the nitrocellulose for a set period of time, leaving a protein spot on the surface.

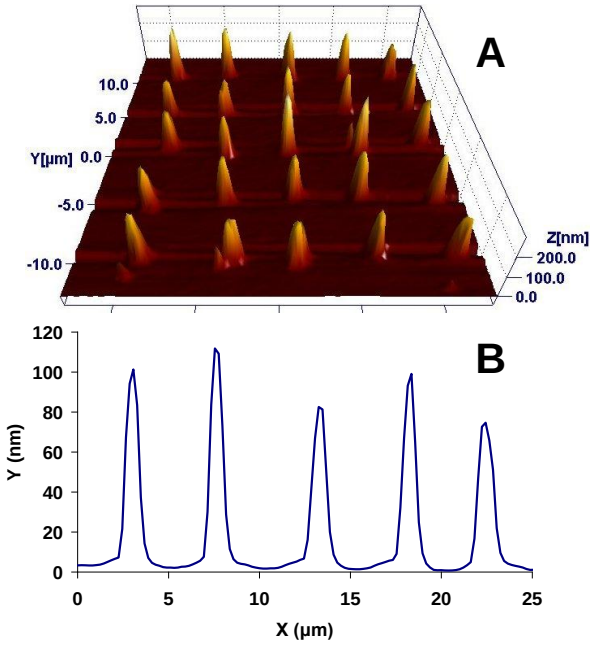

Figure 2. A – Three dimensional AFM image of ultra small protein microarray spots created by DPN. B – Line graph of the averaged data of the top row of spots seen in 2A.

Figure 2A shows the AFM image of a protein array created by DPN using the inkwell method. Figure 2B illustrates the dimensions of the spots created. The spot height is approximately 80-110 nm and spot diameter is approximately 3 µm. The protein written was extracellular signal-regulated kinase (ERK), a key protein in the MAPK (mitrogen-activated protein kinase) cascade[17,18] (see section 2.2).

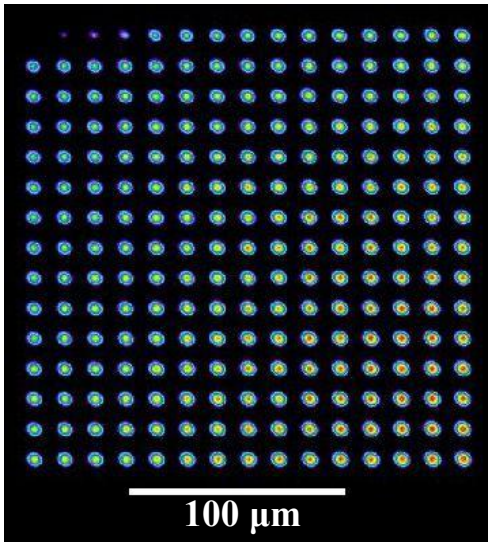

Figure 3. Fluorescence Microscopy image of a protein array written using DPN of an antibody modified with the fluorescent label Alexafluor 546.

Figure 3 shows a fluorescence microscopy image of an array of a modified antibody. These results show a significant advancement over the current microarray spot size. By comparison a single microarray spot would take up the same area as figure 3. However these arrays are still too large to be considered true nanoarrays. The second technique used created spots closer to nanoarray dimensions (figure 4). The tip immersion method involves complete immersion of the AFM tip in a protein solution for an hour prior to nanowriting.

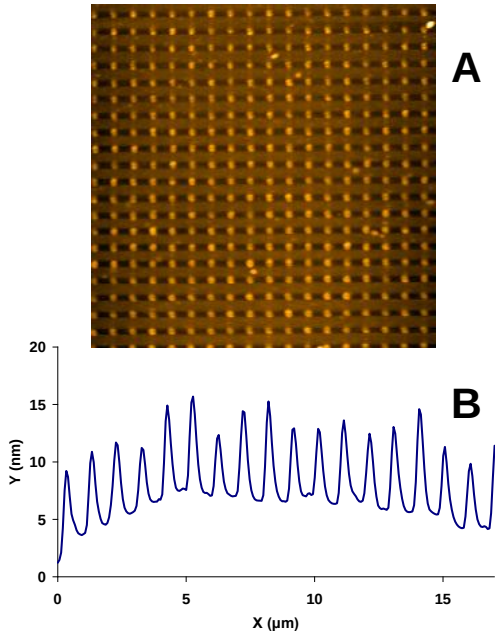

Figure 4. A – Two dimensional image of nanoscale protein spots created by DPN. B – Line graph of averaged data of the top row of spots see in 4A. The spots are approximately 6-10 nm in height and 200 nm in diameter.

These results show that protein has been successfully written onto a nitrocellulose surface using DPN. The protein arrays created are stable and not removed by washing.

2.2 Protein:Protein Interaction Probing

The protein signal pathway of interest in this research is the MAPK cascade which is at the heart of signaling networks that govern proliferation, differentiation and cell survival. Disruption of this pathway can lead to cancer.

A schematic of a protein:protein interaction microarray assay is shown in figure 5. In a microarray assay a capture protein (A) is added to the nitrocellulose surface followed by blocking of the rest of the surface to eliminate non-specific binding. A possible interactor (the probe protein) is then added (B). This protein has a tag incorporated to the protein to facilitate detection. This tag can be a short peptide sequence or a complete protein. To determine whether binding has occurred two antibodies are added. The first (primary) antibody binds to the tag on the probe protein (C). The second (secondary) antibody binds to the primary (D). The secondary antibody of modified with a fluorescent label for detection.

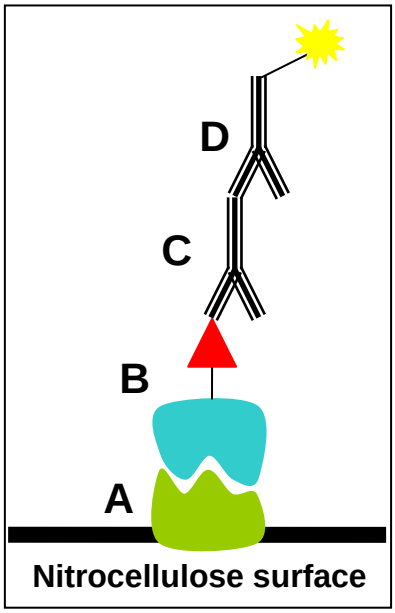

Figure 5. Schematic representation of all the proteins involved in a protein:protein interaction microarray.

This method is time consuming and uses a significant volume and number of expensive proteins, antibodies and reagents. It was hoped that by using nanoarrays and AFM/DPN is would be possible to detect the capture:probe binding event solely by changes in the topography of the surface. If this was not possible then efforts would begin on developing a detection method suited for protein nanoarrays.

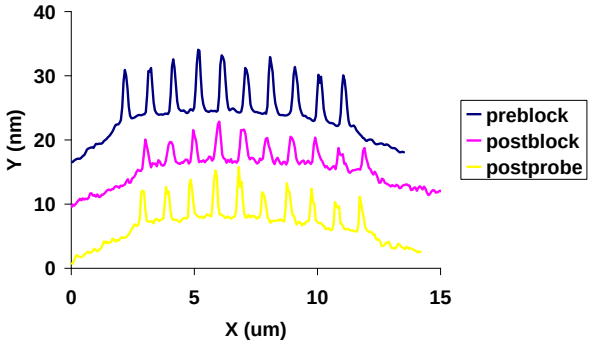

Figure 6. Stacked line graph of the peak heights of protein spots measured during the three stages of the abridged AFM assay.

Figure 6 shows the results of the assay using AFM as the sole detection method. Studying the figure shows that there is no difference in height between the blocked array and the array that has been probed with an interactor. If the

interaction had been detectable the spot height would have increased. To ensure that the interaction was occurring larger spots were created using the inkwell method and the full assay set up shown in figure 5 (figure 7).

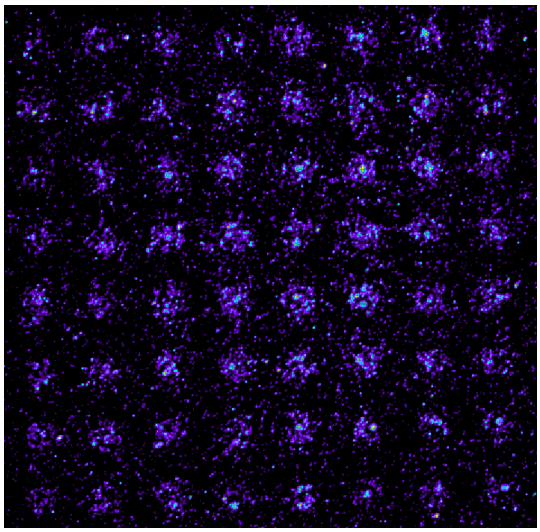

Figure 7. Fluorescence microscopy image of a probed protein array. The spot size is approximately 5 μm.

By using the inkwell method and the full assay it is clear that the capture:probe interaction is occurring but that it cannot be detected by AFM. It is possible that the protein is completely absorbed into the nitrocellulose surface and topographical detection is impossible.

3 CONCLUSION

Whilst true protein nanoarrays have not currently been created on nitrocellulose, significant progress has been made towards that goal using DPN. Using an ultra small microarray (spot size < 5 μm) has shown a positive assay result and that result demonstrates that DPN can be used to create viable protein arrays.

4 EXPERIMENTAL

All arrays were created using a Nanolithography platform (NanoInk) and AFM performed using a DPN 5000 system in contact mode. Fluorescence images were taken using a Nikon Eclipse Inverted microscope.

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