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Molecular patterning on carbon based surfaces through photobiotin activation†

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We have demonstrated the site-specific adhesion of photobiotin as a method of producing protein micropatterns. These patterns were created by the selective UV irradiation of a thin film of deposited photobiotin. The UV activated areas of photobiotin were then developed using fluorescently labelled avidin. The size of pattern produced is an order of magnitude smaller than those previously reported by this method. The patterns were characterised using atomic force microscopy (AFM) to determine their microstructure. It was found that the AFM could discriminate between the areas of protein immobilised to the surface through the activated photobiotin, and the bare substrate surface where the inactivated photobiotin had been removed during the washing process. The potential of these patterns as sensing surfaces is demonstrated through the creation of a spatially patterned immunosensing surface. In this case, a biotinylated antibody was bound to the surface and the pattern developed using a second antibody specific to the immobilised biotinylated antibody. This technique could thus provide a simple and efficient method of producing high density immunoassay systems.

Introduction

The development of patterned arrays of biomolecules is a requirement within a wide variety of fields including biosensing, tissue engineering and bioelectronics. Micro-patterned surfaces have become increasingly important to biosensor systems, with patterned arrays of antibodies, DNA and enzymes proposed to monitor the levels of biomolecules *in situ*.[1–4] As interest in patterning has increased, so the number of different approaches to patterning has grown. Methods currently used include photoactivation and deactivation of light sensitive molecules,[1,2,5–10] immobilisation of macromolecules to self-assembled monolayers,[11] soft lithographic techniques such as microfluidic network patterning and microcontact printing,[12] inkjet technology[13] and traditional photolithographic procedures.[14] For a comprehensive review of approaches the reader is directed towards the review of protein patterning by Blawas and Reichert.[15]

Photochemical activation of light sensitive molecules is a method that offers great versatility and a number of different molecules have been utilised to create patterned surfaces including benzophenones,[16] nitroveratryloxycarbonyls,[1] caged biotin[9,17] and *N*-(4-azido-2-nitrophenyl)-*N*-(*N*-*d*-biotinyl-3-aminopropyl-*N*-methyl-1,3-propanediamine).[2,6,10] The use of biotin derivatives to create patterns of protein is particularly attractive for two reasons. It offers the exploitation of a strong non-covalent biological interaction between biotin and the glycoprotein avidin (K_a = 10^{15} M^{-1}).[18] Furthermore, the tetrameric nature of avidin allows for further binding of biotinylated ligands to the pattern architecture thus creating higher assemblies of molecules upon the sample surface.[19]

Photobiotin (Fig. 1) is a heterobifunctional molecule that was initially designed to non-specifically label proteins and oligonucleotides. It consists of three distinct regions; a biotin group, a linker arm and a photoactivatable aryl azide moiety. Upon exposure to UV light in the region 350–370 nm the aryl azide converts to an aryl nitrene. This reactive species is capable of covalently attaching to C=C and C–H bonds,[20] rendering it ideal for patterning upon carbon rich substrates. As recommended by Hengsakul and Cass,[6] the extended long-arm photobiotin is used in the study. Between the tertiary amine and the biotin group the extended linker contains [–$(CH_2)_3$NH–CO–$(CH_2)_5$–NH–] while the molecule used in previous studies[6] has [–$(CH_2)_3$NH–]. This increased linker length of 1.6 nm is thought to reduce the effects of steric hindrance between the biotin and terminal group due to the position of the biotin binding pocket of avidin which is situated 0.9 nm within the protein.

In this study, we show that a variety of carbon surfaces can be patterned using a similar method to that described by Hengsakul and Cass.[6] However, our modifications, and in particular the use of a smaller mesh size photomask, allow the creation of patterns an order of magnitude smaller than those previously observed. These patterns have been analysed by atomic force microscopy

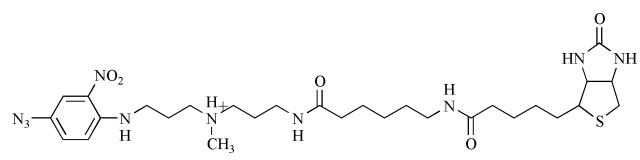

Fig. 1 The structure of long-arm photobiotin used in this study. The photoreactivity of the molecule is provided by its aryl moiety whilst the biotin group located at the other end of the molecule provides the ability to interact with avidin molecules. The two parts of the molecule are joined together by a carbon linker of approximately 1.6 nm.

† Electronic Supplementary Information available. See http://www.rsc.org/suppdata/an/b0/b008475l/

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(AFM), enabling the direct characterisation of their nature and structure at a sub-micron scale. We also demonstrate how these patterns, *via* the biotin–avidin crosslinking system, can be developed to bind additional ligands to facilitate the production of spatially confined, biomolecular arrays for biosensor development.

Experimental methods

Materials

Long-arm photobiotin was obtained from Vector Laboratories (Peterborough, Cambridgeshire, UK). Avidin–rhodamine isothiocyanate (1–2 mol of rhodamine isothiocyanate per mol of avidin), avidin–fluorescein isothiocyanate (FITC) (2–4 mol of fluorescein isothiocyanate per mol of avidin), bovine serum albumin (BSA) and polycarbonate coverslips (Hybrislips®) were all obtained from Sigma Aldrich (Poole, Dorset, UK). Anti-goat immunoglobulin G (IgG), H & L chain specific (Rabbit) fluorescein conjugates and anti-mouse IgG, H & L chain specific (Goat) biotin conjugate were obtained from Calbiochem (Nottingham, UK). The photomasks used were 50 hexagonal mesh copper and 400 square mesh nickel grids (Agar Scientific, Stansted, Essex, UK). A Nikon (Kingston, Surrey, UK) 100 W mercury lamp (USH-102-D) was used as the light source to activate the photobiotin.

Pattern formation on graphite

A 200 μL drop of photobiotin dissolved in deionised water (10 μg mL^{-1}) was left to dry on a freshly cleaved highly ordered pyrolytic graphite (HOPG) surface for 12 h in a dessiccator. A 50 hexagonal mesh grid was placed on the graphite and the sample irradiated for 30 min using slightly defocused light from the 100 W mercury lamp, located 10 cm above the surface. A quartz plate was positioned between the sample and lamp to minimise sample deformation from heat damage. After irradiation, the sample was incubated in 500 μl of avidin–FITC (0.5 mg mL^{-1}) for 30 min. The surface was then washed thoroughly with deionised water and dried under nitrogen. Pattern formation was confirmed by examination with a Nikon Diaphot 300 inverted microscope employing epifluorescence. The fluorescent molecules were visualised using a 470–490 nm bandpass excitation filter, a 505 nm dichroic mirror and a 520 nm emission filter. AFM images of the pattern were obtained using a Topometrix Lumina (Thermo Microscopes, Sunnyvale, CA, USA) using tapping mode in air.

A control sample was treated in the same manner as outlined above, but was not irradiated. The sample was simply incubated with fluorescently labelled avidin, washed and examined under the fluorescent microscope.

Patterning polycarbonate (Hybrislips®) and polystyrene

A 200 μL solution of polystyrene (10% m/v) in toluene was spun cast at 2000 rpm upon a 22 × 50 mm glass cover slip. The polycarbonate Hybrislips® were cleaned thoroughly in hexane followed by ethanol to remove surface contamination. Both substrates were treated identically to the graphite with the following exceptions; the photomask used was changed to a smaller mesh size (400 square mesh grid) to facilitate the increased pattern resolution, avidin incubation time was reduced to 10 min to further reduce non-specific binding and the composition of avidin solution was changed to include a 1 : 1 ratio of avidin–FITC with BSA blocker (3% m/v in 0.1M PBS pH 7.4).

Immunoglobulin patterned surface

An avidin-patterned surface was produced upon polycarbonate by the method outlined above. The surface was incubated with biotinylated Goat anti-mouse IgG (0.25 mg mL^{-1}) for 10 min. The surface was then washed in deionised water and incubated with fluorescein conjugated Rabbit anti-goat IgG (0.5 mg mL^{-1}) for 10 min. The surface was again washed with deionised water and dried under nitrogen prior to examination by fluorescence microscopy.

Results and discussion

Patterns on graphite

A typical photomicrograph of a photobiotin pattern surface developed with avidin–FITC is displayed in Fig. 2. The image shows that the pattern contains a number of cracks; a common feature of such patterns created on this substrate. The cracking may be attributable to contraction of the protein film during drying. The image demonstrates a series of hexagons, slightly obscured by the presence of material in-between the patterned regions. The presence of this material is believed to be partially due to photobiotin activation by scattered UV light, despite the protection offered by the bars of the grid. This material may also be present due to non-specific binding of the avidin–FITC to graphite. Collimated light could be employed in future to reduce this effect. In addition, the amount of material present within these bar regions may be decreased by the addition of a blocking step to prevent the non-specific binding of avidin to unexposed regions of the pattern. This blocking step takes the form of simultaneous incubation of bovine serum albumin with avidin–FITC to the freshly activated photobiotin surface. Non-specific interaction of the avidin–FITC with the bare substrate is seen in the control pattern where some random patches of fluorescence are observed (data not shown). No ordered patterns, however, are visible on these samples, therefore confirming that the presence of avidin–FITC in hexagonal arrays upon graphite is created by the photoactivation process.

Atomic force microscopy of protein patterns on graphite

AFM images of graphite photobiotin pattern (Fig. 3a and b) clearly illustrate that it is possible to distinguish between patterned and non-patterned regions despite the presence of non-specific binding seen in the fluorescent photomicrograph. Fig. 3a shows an AFM image of bare substrate flanked either side by avidin bound to the photobiotin pattern. Fig. 3b is a higher resolution image of the same pattern showing the interface region of the avidin layer on the graphite surface. This

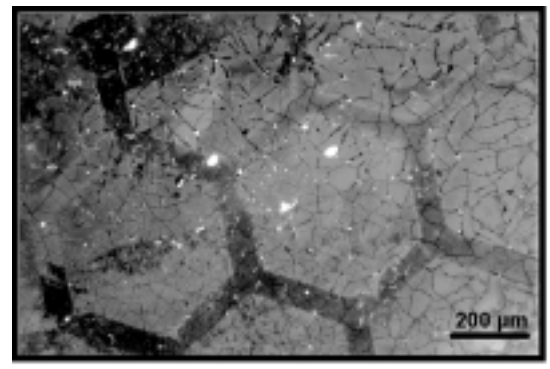

Fig. 2 Photomicrograph of a hexagonal pattern produced on graphite developed using avidin–FITC. The grid used is a 50 mesh hexagonal grid, producing hexagons approximately 450 μm in dimension.

illustrates a protein network, similar to other AFM images of an avidin film.[21] Analysis of a section of this AFM image reveals that the mean height difference between the avidin layer and bare graphite is approximately 7 nm. This result indicates the presence of avidin as a monolayer given that the dimensions of avidin are 5.6 × 5 × 4 nm.[22]

Patterns on polymer surfaces

Patterns produced on polycarbonate employing the same method as used to pattern graphite show similar results to those described above, particulary, the presence of non-specific binding of the avidin–FITC to non-patterned regions of the substrate. To counteract this it was decided to utilise the blocking step to facilitate the creation of more clearly defined patterns. Fig. 4 demonstrates a photobiotin pattern created upon polystyrene using BSA as a blocking agent co-incubated with the labelled avidin. It is evident that the introduction of this step significantly decreases the non-specific binding of avidin to non-activated regions of the substrate. The grid size used for generating these patterns was also decreased, using this method patterned squares of 40 μm could be produced. This value is approximately an order of magnitude smaller than those previously reported using a similar methodology.[6] This patterning protocol was repeated with a polycarbonate substrate which demonstrated similar results (image deposited as Electronic Supplementary Information†).

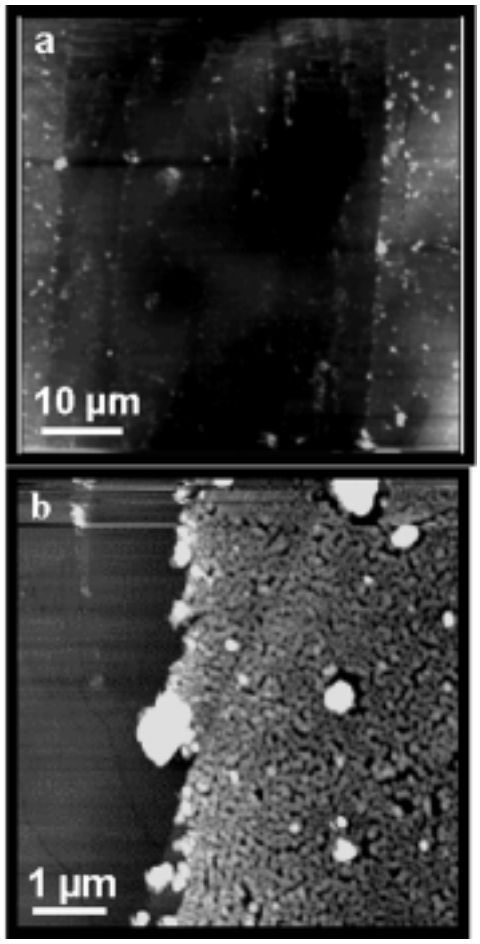

Fig. 3 AFM images from two different samples showing regions of hexagonal patterns on graphite. (a) is a low-resolution image of a bar region flanked by regions of immobilised proteins on either side. (b) is a high-resolution image clearly demonstrating the presence of avidin molecules immobilised on the graphite surface.

Atomic force microscopy of protein patterned polymer surfaces

Fig. 5 illustrates an AFM image of a photobiotin pattern upon a polycarbonate substrate. Analysis of the topography between the avidin squares and the bar regions containing the BSA blocker reveals minimal height difference. Fig. 5a indicates that the topographical height of the BSA containing bar region is greater than that of the avidin containing squares, a finding supported by literature values for the dimensions of avidin and BSA.[22,23] The observed contrast between bound avidin and the surrounding BSA blocked bar regions indicates that this level of

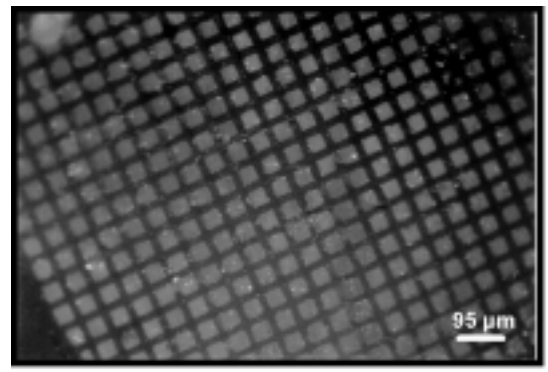

Fig. 4 Photomicrograph of a square pattern on polystyrene spun cast onto a glass slide. The pattern was produced using a 400 square mesh grid as a photomask and developed using avidin rhodamine. The squares are approximately 40 μm in size.

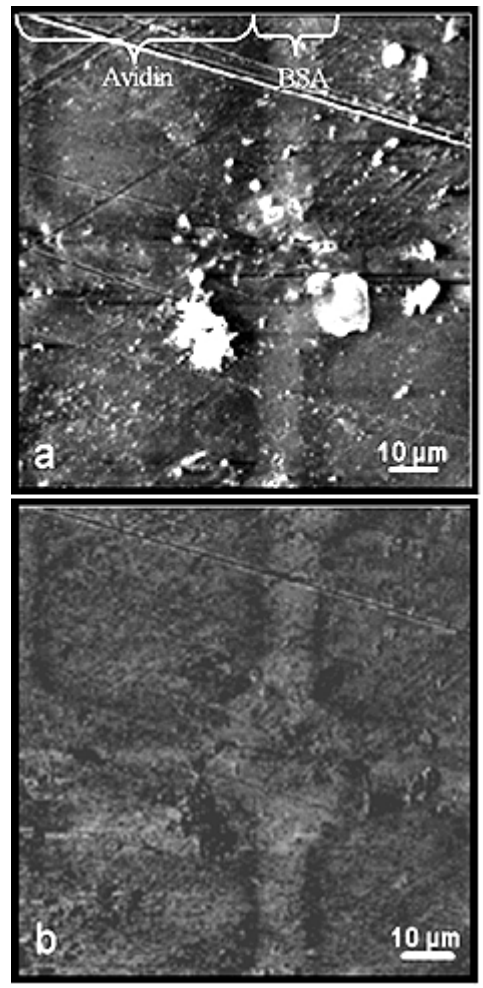

Fig. 5 (a) AFM topography and (b) phase image of a pattern produced on polycarbonate showing a collection of 4 patterned squares.

specificity of binding is due to light activation of the photobiotin molecules. This occurs in the square regions only and could not be accounted for by random non-specific adsorption of avidin to the surface. The contrast differences between patterned squares and non-patterned bar regions are more easily distinguishable in the simultaneously acquired phase image (Fig. 5b). Phase images are produced by monitoring the phase lag of the AFM tip vibration compared to the drive signal of the piezo which vibrates the tip as it scans over the surface. Whilst normal topography images contain information about the height of a feature, phase images contain information about the tip–sample interaction resulting from physical effects such as adhesion,[24] surface stiffness[25] and viscoelasticity.[26] The low phase contrast seen in Fig. 5b is to be expected as there is only a slight difference in physical properties between the BSA in the bar region and the avidin immobilised in the squares.

Immunoglobulin patterns

In order to demonstrate the potential of this patterning procedure to fabricate a sensing surface, an avidin immobilised surface was incubated with two complementary antibodies. Fig. 6 shows a schematic of the layers of protein in the pattern while Fig. 7 shows a photomicrograph of the observed pattern. The fluorescence seen is due solely to the presence of the fluorescein conjugated Rabbit anti-goat IgG, since no other fluorescent molecules are used in this procedure. The localisation of the fluorescence to individual squares confirms the molecular order of the sample and is as described in Fig. 6. This type of pattern is an example of how these materials can be utilised to create a sensing surface. The fact that the biotinylated species bound to a patterned surface retain their functionality has been shown previously[6,10] but the patterns created in this study show that it possible to build ordered structures upon the patterned surface.

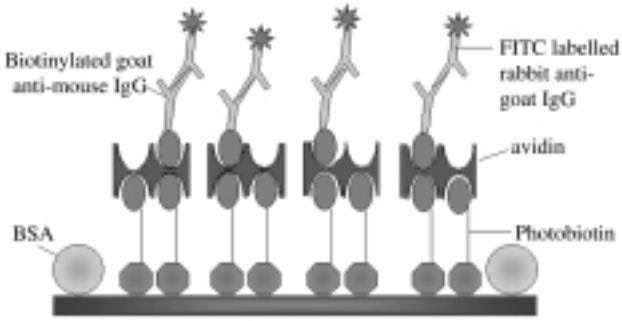

Fig. 6 Schematic of the structure of antibody assembly built upon the polycarbonate surface.

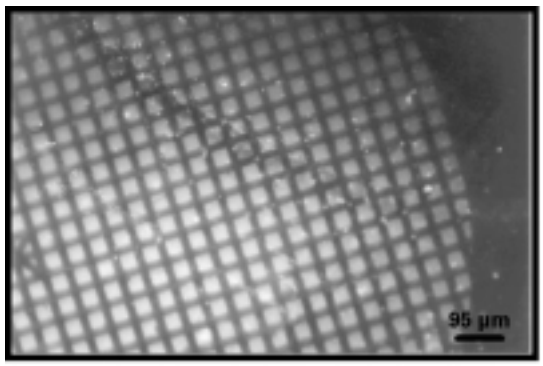

Fig. 7 Photomicrograph of antibody assembly built upon polycarbonate. The pattern was produced using a 400 mesh grid and developed using a biotinylated immunoglobulin G and a fluorescently labelled anti-immunoglobulin G.

Conclusions

Photobiotin has been shown to bind to carbon rich substrates in such a way that controlled protein arrays can be fabricated. The patterns produced in this study are an order of magnitude smaller than patterns previously reported using a similar methodology. Analysis using a surface sensitive technique has been shown to be capable of confirming the nature and structure of the patterns on a sub-micron scale. The potential to build complex structures on the avidin patterns has been shown and the possibility of using this methodology to create sensing surfaces of high molecular order has been demonstrated.

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