Micro/Nanopatterning of Proteins via Contact Printing using High Aspect Ratio PMMA Stamps and Nano- Imprint Apparatus

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Micro- and nano-scale protein patterns have been produced via a new contact printing method using a nanoimprint lithography apparatus. The main novelty of the technique is the use of Poly(methyl methacrylate) (PMMA) instead of the commonly used poly(dimethylsiloxane) (PDMS) stamps. This avoids printing problems due to roof collapse, which limits the usable aspect ratio in micro-contact printing to 10:1. The rigidity of the PMMA allows protein patterning using stamps with very high aspect ratios, up to 300 in this case. Conformal contact between the stamp and the substrate is achieved because of the homogenous pressure applied via the nanoimprint lithography instrument, and it has allowed us to print lines of protein ~150 nm wide, at a 400 nm period. This technique, therefore, provides an excellent method for the direct printing of high density sub-micrometer scale patterns, or, alternatively, micro-/nano-patterns spaced at large distances. The controlled production of these protein patterns is a key factor in biomedical applications such as cell-surface interaction experiments and tissue engineering.

Keywords: Micro-contact printing, Protein, Poly(methyl methacrylate), Nanoimprint.

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INTRODUCTION Micro-contact printing (μ CP) has been developed for rapid patterning of molecules over large areas onto a variety of surfaces (e.g. gold surfaces with thiols, glass with silanes) [1]. Increasingly, there is an interest in biomedical field for the controlled patterning of proteins on surfaces as a key factor for cell-surface interaction, cell differentiation or tissue culturing experiments [2, 3]. Nano-scale patterns are desirable for cell-surface interaction experiments, especially to examine the effect of nano-patterned protein structures on the organisation of proteins in the cell [4]. Streptavidin is an example of a protein patterned at the nanoscale using a dodecylphosphate, to which proteins can bind, and which has been selectively adsorbed onto Indium tin oxide (InSnO) structures defined by electron beam lithography and reactive ion etching (RIE) [5]. Poly(L-lysine)-g-poly(ethylene glycol) is used to passivate the surrounding silicon oxide (SiO_2) against protein adsorption. In this way, protein patterns with dimensions of 200 nm were produced. Ferretin spots of 100 nm diameter have also been produced using a colloidal lithography technique [6], whereas avidin has been immobilised at sub-micrometer dimensions on a thin poly(methyl methacrylate) PMMA film that has been electrostatically charged by an atomic force microscope (AFM) [7]. A number of other techniques have been used for nanoscale patterning [8, 9], but, as with the above, most of these techniques involve complicated apparatus and are time consuming, due to the fabrication procedures used or their serial nature.

At first μ CP seems to be an excellent candidate for the transfer of macro-molecules, such as proteins, to surfaces at small scale. This technique, that uses a PDMS stamp, is parallel in nature, involves process conditions which are non-detrimental to the (bio) molecules, requires no specialist equipment and can be undertaken by a non-skilled scientist at the bench-top. Unfortunately, conventional micro-contact printing suffers from problems that limit pattern resolution [10], such as restrictions on the structure aspect ratio (defined as the ratio of structure separation versus structure height) or diffusion of the molecules to be printed. With respect to the aspect ratio limitation, a

number of factors can adversely affect printing using stamps with high aspect ratio structures. Deformation of the stamp under the applied pressure, due to the weight of the stamp [11] or interfacial adhesion [12], can cause contamination of areas of the substrate not intended for patterning through “roof collapse” or “pairing” [13]. Attractive forces, such as electrostatic charges between the neighbouring structures or between the structure and the bulk of the elastomer [14], can exacerbate structural collapse, and these forces are increasingly important as the dimensions of the structures decrease. These problems have been limiting the available aspect ratios for printing to a maximum of 10:1 [15], thus making the printing of nanopatterns difficult.

We have recently shown that the problem of structural collapse can be alleviated if the micro-contact printing is carried out in a liquid medium that supports the stamp during printing [13]. Renault and co-workers have shown that it is also possible to circumvent this problem, thus making contact printing of proteins possible at nano-dimensions, by using domed structural features in their stamps [16]. In this way, the structural collapse is avoided, allowing the printing of antibodies (whole G immunoglobulins) and green fluorescent proteins at the nano-scale. Unfortunately, the geometry of the domed structures limits the density of the printed features and the authors produced 100 nm wide features separated by a relatively large distance of 600 nm. Similarly, sub-100 nm patterns have been obtained using V-shaped stamps of h-PDMS, a high Young's modulus PDMS silicone [17]. The large Young's modulus value provides increased polymer stiffness and minimises roof collapse, but in this case the separation of pattern features is about 2.5 μm .

Here we describe the development of a new contact printing technique for patterning proteins on surfaces at the micro-/nano-scale by using stamps with aspect ratios much higher than those used in conventional μCP . To achieve this, we use poly(methyl methacrylate) (PMMA) stamps and pressure applied by nanoimprint lithography apparatus.

MATERIALS AND METHODS Positive Poly(methylmethacrylate) stamps, where the structures protrude from the bulk PMMA surface, were obtained by replication from silicon-based moulds fabricated by UV photolithography and deep reactive ion etching (DRIE) or focused ion beam (FIB) techniques [18], and coated with an anti-adhesion fluorosilane layer [19]. FIB modification has been used to produce moulds with different aspect ratios: 100:1 ($20 \times 20 \mu\text{m}^2$, $40 \mu\text{m}$ period, 200 nm high, 200:1 ($40 \times 40 \mu\text{m}^2$, $80 \mu\text{m}$ period, 200 nm high) and 300:1 ($60 \times 60 \mu\text{m}^2$, $120 \mu\text{m}$ period, 200 nm high), which were used to explore the limits of the new printing technique. The PMMA stamps were produced via a nanoembossing technique in which PMMA sheets ($125 \mu\text{m}$ thick, Goodfellow, UK) were used as supplied and embossed in a nanoimprint lithography apparatus (Obducat, Sweden) as described previously [20]. The free-standing stamps were then simply peeled off the silicon mould. The stamps contained microstructures, with aspect ratios suitable (i.e. smaller than 10) and unsuitable (i.e. larger than 10) for conventional micro-contact printing, and nanostructures. For the printing process, the PMMA stamps were “inked” with proteins by immersion on 0.1 mg/ml protein solution (Streptavidin fluorescently labelled with Texas-Red® or Neutravidin, Molecular Probes) in PBS for 1 hour. Excess of protein solution was removed using dry nitrogen and the stamps were used immediately for printing. Washing steps for the stamps were omitted for fear of washing off the protein from the stamp. We assume the drying to be sufficient for these first experiments as the results show there is little ink contamination in-between the printed areas using the nanoimprint assisted printing (see later). Figure 1 depicts the nanoimprint assisted protein contact printing methodology.

Figure 1. Assisted printing technique using the nanoimprinter apparatus. (1) The protein-inked PMMA stamp is positioned over the PMMA substrate and pressure is applied to produce conformal contact (2). After the pressure is released, the protein is seen to transfer to the PMMA substrate (3).

The unsupported, inked stamp is carefully placed on the substrate (flat glass or non-structured PMMA) with the inked structures to be printed over the substrate surface. The nanoimprinter was then used to apply an even pressure to the back-side of the stamp. The printing was completed at room temperature (294 K) for 600 s and the pressure was adjusted (between 7.5×10^5 and 30×10^5 Pa) to find the optimum printing conditions. (Unless otherwise stated in the results, the pressure used for the imprinting in each case was 7.5×10^5 Pa.) For comparative studies neutravidin protein patterns were obtained using conventional μ CP. Poly(dimethyl siloxane) stamps (PDMS; Sylgard 184, Dow Corning) were replicated from silicon moulds by curing the prepolymer mixture (base/curing agent, 10:1) for 1h at 90°C. After inking the PDMS stamp with 0.1 mg/ml of neutravidin in PBS for 1h, excess protein solution was removed by a stream of dry nitrogen, then the stamp was immediately brought into contact with the PMMA surface for ~1 min as described previously [21].

Protein patterns were imaged using fluorescence microscopy (Nikon E1000). A staining procedure was used as proof that the patterned neutravidin was still active. The staining procedure was achieved by immersion of the PMMA samples with neutravidin patterns in a PBS solution containing 0.25 mg/ml of biotin-4-fluorescein (Molecular Probes) and 2.5% of BSA (Sigma-Aldrich) for 10 min, followed by several rinses with deionised water. Fluorescence intensity profiles were obtained using Image L software (Image L 1.33 U, National Institutes of Health, US). Direct

characterization of the stamps and the protein patterns was performed via tapping mode atomic force microscopy (Nanoman, Veeco).

RESULTS Figure 2 gives examples of PMMA stamps with 5 μm and 2.5 μm diameter structures and aspect ratios of 6.5 and 3.125 respectively, produced by nanoembossing. These stamps have then been used to print streptavidin (or neutravidin) on the surface of flat PMMA substrates via the new contact printing method using the nanoimprint apparatus. The proteins were contact printed at pressures of 3×10^6 and 7.5×10^5 Pa (Figure 2a and b).

Figure 2. Protein transfer to a PMMA substrate. AFM images of PMMA stamps consisting of 800 nm tall posts with diameters of 5 μm (aspect ratio = 6.25) and 2.5 μm (aspect ratio = 3.13) are shown. 20X fluorescence micrographs of Texas Red-labelled streptavidin patterns printed on a PMMA substrate are also given, using these stamps, at (a) 3×10^6 Pa and (b) 7.5×10^5 Pa pressure.

Repetition of the printing using Neutravidin (c) and (d) followed by staining with biotin-4-fluorescein. Functionality was only observed in the Neutravidin transferred at 7.5×10^5 Pa pressure (d).

Examination of the whole transferred surface showed, in both cases, that the protein seemed to transfer to the PMMA surface with excellent fidelity over large areas (1cm^2). However, when the

neutravidin protein activity was tested after printing, by staining with biotin-4-fluorescein (Figure 2c and d), fluorescence was only observed on the surface patterned at 7.5×10^5 Pa. Similar fluorescence intensity profiles of neutravidin patterns stained with biotin-4-fluorescein were obtained by conventional μ CP (Figure 3).

Figure 3. Line profiles of fluorescence intensity after biotin-4-fluorescein staining of neutravidin (1) transferred at 7.5×10^5 Pa using a PMMA stamp and (2) using conventional micro-contact printing.

PDMS and PMMA stamps with 20 μ m circular features and aspect ratios of 25 were used to pattern streptavidin-Texas Red applying the respective methodologies (Figure 4a and b). The result shows that, when using an aspect ratio unsuitable for conventional μ CP, and roof collapse occurs, the new methodology produces successful protein patterning.

Figure 4. Comparison of (a) conventional micro-contact printing and (b) nanoimprint assisted contact printing, of proteins on PMMA substrates, using stamps with aspect ratios of 25. (a) During conventional micro-contact printing using a PDMS stamp, roof collapse occurs and consequently protein is transferred to the area between the required circular features. (b) No roof collapse occurs when using PMMA stamps. Scale bar = 20 μ m.

In order to determine the aspect ratio threshold of the new contact printing technique, streptavidin-Texas Red patterns were obtained using stamps with aspect ratios of 100, 200 and 300 and applied pressures of 7.5×10^5 , 1.5×10^6 , 2.25×10^6 and 3×10^6 Pa, on PMMA substrates. Figure 5 shows the fluorescent images, where we can see that for pressures larger than 7.5×10^5 Pa, some stamps collapsed.

Figure 5. Patterning of Texas-Red labelled Streptavidin using high aspect ratio PMMA stamps with aspect ratios of 100, 200, and 300, at 3×10^6 , 2.25×10^6 , 1.5×10^6 , 7.5×10^5 Pa. No collapse was observed using 7.5×10^5 Pa pressure for any of the moulds. In each image for the stamp with aspect ratios of 300, the magnified areas show that, as the applied pressure is decreased, the extent of stamp collapse is reduced (the penumbra of fluorescence molecules surrounding the stamped square pattern disappears as the pressure is reduced).

To prove that the new methodology is able to work at the nano-range, AFM measurements show nanopatterning (Figure 6b) of 150 nm protein lines on flat glass using PMMA stamps (Figure 6a) containing 100 nm tall, 200 nm wide lines of 400 nm periodicity (aspect ratio = 2).

Figure 6. Nanopatterning of Streptavidin using PMMA stamps, containing 100 nm tall, 200 nm wide lines of 400 nm periodicity (aspect ratio = 2), on flat glass. (a) AFM measurements reveal that no collapse of the stamp occurred. (b) The printed protein lines are 150 nm wide due to the slightly rounded shape of the structures in the PMMA stamp.

DISCUSSION The possibility of printing proteins onto polymer surfaces using “hard” thermoplastic (non-elastomeric) polymeric stamps has been demonstrated. These stamps allow us to print protein patterns using aspect ratios that are unobtainable by conventional μCP methods using elastomeric polymers, such as PDMS [15]. One of the problems commonly associated with elastomeric stamps is that high aspect ratio structures are unable to support the weight of the stamp on the substrate, and consequently the structures collapse [13]. Here, the rigidity of the polymer means that the structures,

both with high aspect ratios and nanometric dimensions, are able to support the stamp, even under the applied pressure with the nanoimprinter.

Using PMMA stamps within a commercial nanoimprinting system allows us to precisely control the contact pressure. Initially, although the stamp is positioned over the substrate, non-conformal contact occurs due to the rigidity of the stamp (Figure 1a). Pressure is applied to produce the conformal contact and this allows the protein to be transferred (Figure 1b). The use of the nanoimprinter minimises problems due to double-printing, which are occasionally observed in μ CP due to movement of the stamp over the substrate when applying pressure manually. Here, double-printing patterns do not occur because at atmospheric pressure there is non-conformal contact between the stamp and substrate; this only occurs when the nanoimprinter homogeneously applies the pressure to the stamp. The pressure for the transferring of proteins has to be large enough to ensure conformal contact between the polymer stamp and the polymer substrate but low enough to maintain the activity of the protein after printing. Staining of neutravidin patterns with biotin-4-fluorescein (Figure 2d) shows that, even when the applied pressure is seven times the atmospheric pressure (7.5×10^5 Pa), the protein transferred from the stamp to the substrate retains its activity. Comparison of the fluorescence profiles with those for micro-contact printed neutravidin (Figure 3) shows a similar fluorescence value, which suggests that the amount of active protein transferred with the new technique is comparable to that obtained using the well-established μ CP methodology. At higher pressures, the protein is also successfully transferred to the substrate (Figure 2a), but after staining with biotin-4-fluorescein no fluorescence is detected (Figure 2c). This may be due to the protein molecules being denatured by the printing pressure, which causes them to lose their biological activity.

Although the requirement for the nanoimprint apparatus does increase the complexity of the method, compared to conventional μ CP, the technique has some advantages. The main advantage of the new technique comes from the fact that high aspect ratio stamps can be used in order to transfer the proteins. Using a PDMS stamp with an aspect ratio of 25, conventional μ CP is not possible, due to collapse of the PDMS stamp in the space between features (Figure 4a). With the PDMS stamp, it

is possible to see that only a thin ring remains without deposited protein, around where the stamp structure has contacted the surface. This is a typical feature observed in μ CP when collapse of the stamp occurs during transfer (assuming there is no ink diffusion). Using the new technique, with a PMMA stamp with the same aspect ratio, the printing is successful (Figure 4b). This does however tell us that protein transfer to the PMMA substrate is rapid, and occurs in less than 1 minute. The ability to use high aspect ratio structures allows the stamp structures to be well spaced without roof collapse occurring; indeed we have been able to print structures using stamps with aspect ratios of 300 and 7.5×10^5 Pa without this problem arising (Figure 5). Using this technique it is possible to pattern $20 \mu\text{m}$ features using stamps with only 70 nm high structures, or protein patterns separated by $3000 \mu\text{m}$ using stamps only $10 \mu\text{m}$ tall. The achievable limit to the aspect ratio at low pressures remains to be explored although we can assume that aspect ratios slightly higher than 300 could be used for transfer, taking in to account that at double the pressure ($1.5 \times 10^6 \text{ Pa}$), the stamp with the aspect ratio of 300 is only slightly collapsed and aspect ratios of 200 and 100 can still be patterned.

It is worth noting that the PMMA stamp does collapse in the areas surrounding the stamp structures, as evidenced by the increased amount of fluorescent protein in these areas. At pressures causing the stamp to collapse, these areas border the printed pattern; at lower pressures, this penumbra is more remote. This effect also occurs in conventional micro-contact printing with PDMS stamps. A tentative analysis of the distance from the printed structures at which this penumbra occurs gives an idea of the maximum aspect ratio that may be available for use. The distances from the printed structures to the penumbra range from $15 \mu\text{m}$ at $3 \times 10^6 \text{ Pa}$ to $80\text{-}100 \mu\text{m}$ at $7.5 \times 10^5 \text{ Pa}$. This latter result suggests that it may be possible to achieve aspect ratios in excess of 400 at this pressure. One possible use of the technique could be the integration of a protein transfer step during the microfabrication of microelectrodes. A PMMA stamp with structures aligned to the working electrodes of a number of well-spaced biosensors could be used to functionalise, in parallel, the gold surface with thiol-modified antibodies.

To examine the size resolution of the technique and to prove that it is suitable for use in the nanometer range, protein transfer using nanostructured stamps was carried out. Figure 6 shows a

topographical AFM image of the stamp containing nanometric structures and the streptavidin printed on a glass substrate using this PMMA stamp. The printed protein lines are 150 nm wide due to the slightly rounded shape of the structures in the PMMA stamp. AFM measurements reveal that no collapse of the stamp occurred. The lines of streptavidin produced appear to be discontinuous, which may suggest that the protein transfer was not entirely uniform, and optimisation of the printing parameters, such as stamp inking, may be required. The nanoscale surface structure of the PMMA stamp is also important and any inhomogeneity in the structures will be evidenced by discontinuities in the printed pattern. However, the patterned lines can clearly be seen (Figure 4b), and can be expected to interact with cells at these dimensions in the case of cell culture studies. The discontinuities may also arise from protein agglomeration formation over the structures during the inking procedure. Nanometric line patterns of rabbit antibodies, micro-contact printed on a glass substrate, show the patterned proteins as agglomerations 50 to 100 nm in diameter [16]. Similarly, poly (lysine) micro-contact printed over large areas on glass produced a discontinuous monolayer, which suggests a granular or agglomerated structure [22]. In our results, the agglomerations of protein are ~4 nm high and in places they spread across the whole of the 150 nm width of the printed lines. The high aspect ratios allow us to produce printed protein lines with a high density as, using PMMA, there are no problems of structures pairing; the lines produced in figure 6 have a period comparable with those produced by elastomeric stamps [16].

CONCLUSIONS. Protein patterning on surfaces is a technique essential for a number of applications, such as tissue engineering and the mimicking of biological surfaces. The new contact printing technique allows the patterning using stamps with aspect ratios unsuitable for μ CP. Stamps with aspect ratios up to 300 can be used to pattern proteins on surfaces without problems arising from stamp collapse. Low pressures (7.5×10^5 Pa) are sufficient to achieve conformal contact between the stamp and the substrate, while being low enough to avoid destroying the protein activity during the transfer. At this moment, we can state that at these pressures the protein is seen to be active. At the higher pressures used in the paper, the neutravidin is seen to be inactivated. To examine why the protein becomes inactivated at the higher pressures will require further experimentation into the

structure of the protein before and after the printing. At this moment, we can only speculate that the higher pressures cause irreversible damage to the protein function, possibly by altering the 3D structure. Nanopatterning resolution can be achieved by this technique, and the high aspect ratios of the stamps allow us to produce printed protein lines with a high density; on a par with those produced by elastomeric stamps [16]. Work is ongoing to study the patterning of proteins over a variety of substrates, such as polymer surfaces with active groups that react with the proteins (activated polymer surfaces) or protein adsorption resistant surfaces, such as agarose [23], PEG-DMA [24] or PEG functionalised glass [25] surfaces. In principle there is no limitation on the type of substrates that will work with this technique, as long as the transfer dynamics are favourable towards the printing of the ink molecules on the substrate surface.

ACKNOWLEDGEMENTS The authors thank Dr. A. Errachid, Dr. J. Bausells and G. Villanueva for providing the microstructured masters used here to produce the stamps and the Nanotechnology Platform of the Barcelona Scientific Park for providing the nanoimprint equipment and for the production of the FIB structured moulds. We acknowledge financial support of the Spanish Ministry of Science and Technology via the Ramon y Cajal program. This paper and the work it concerns were generated in the context of the CellPROM project, funded by the European Community as contract No. NMP4-CT-2004-500039 and it reflects only the authors' views.

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