Plasma processes and applications in NanoBiotechnology

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Abstract: Nanostructured surfaces presenting chemical or topographical patterns are now being increasingly developed in nanobiotechnology. Major applications are related to cell culture models and biodetection. We show that plasma technologies, in particular the combination plasma polymers deposition and etching, together with colloidal lithography, e-beam lithography and microcontact printing, are essential tools to produce nanostructured surfaces. We show that chemical and topographical patterns can be obtained on different substrates, with dimensions down to some 10 nm. The applications of these nanostructured surfaces in biology and bio-detection are reviewed and the advantages and limitation of the techniques underlined.

1. Introduction
In NanoBiotechnology, the development of bio/non-bio interfaces has been the focus of attention from the research community. It has been demonstrated that patterned surfaces with contrasted physico-chemical functionalities –e.g. adhesive/non-adhesive, hydrophilic/hydrophobic, positive/negative- are particularly interesting in tissue engineering [1,2,3,4,5], cell behavior investigations [6,7,8,9,10,11], or microfluidics [12,13]. The micro or nanostructures lead to special properties related either to the kinetics of biochemical reactions at the interface [14,15], or control of biological responses from living cells [1,5,10,16,17]. Numerous methods have been developed for the production of surfaces with controlled chemical functions using Self Assembled Monolayers (SAMs) [18,19] or electrodeposition of functional polymers [20]. The patterns can be produced by different types of lithography as well as microcontact printing [16,21,22,23]. The surface chemistries obtained are well controlled but have several limitations particularly in relation to the substrate that has to be used, for instance Gold or Silicon in the case of SAM. This is why other possibilities have been investigated, in particular in the use of functional plasma polymers that can be deposited on virtually any substrate and at low temperatures. The plasma processes allow, by the choice of the right precursors, to deposit materials a very large contrast of properties and chemical functionalities, such as –COOH [24], -NH2 [25,26] and their combination [27] or anti fouling polymers based for instance on polyethylene oxide (PEO) [28,29]. An excellent review of the different functionalities produced up to now can be found in the article published by Shyong Siow et al. [30]. Due to their variable degree of cross-linking related to the discharge conditions, these coatings are resistant to classical polar solvents and can be used in standard lift-off processes [32,39], but can also be selectively dissolved by the appropriate solvent. Plasma polymers can be then assembled in structures with sub-micron dimensions by

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combination of colloidal lithography deposition and etching [31,32], and by direct modifications by electron beam irradiation [56]. We present here some examples of the work we performed, based on the use of plasma assisted deposition and plasma etching of functional polymers and colloidal and electron beam lithography together with microcontact printing. Application of micro-contact printing onto non-fouling PEO-like plasma polymer is also presented.

2. Experimental details
In this work, two different types of plasma polymers were used: acrylic acid plasma polymer (pAA) containing COOH moieties and plasma polymerized Polyethylene Oxide (PEO-like). The PEO-like polymer is resistant to protein adsorption in liquid solution.

2.1. Films processing
The RF plasma deposition on Si substrates of the plasma polymerized PEO-like and pAA films are described in details elsewhere [24,32,33,34]. Briefly, the systems used are capacitively coupled reactors with two symmetrical parallel-plate electrodes. For PEO-like coating, plasma polymerization is carried out by using a radio frequency generator (13.56 MHz) in pulsed mode (time on = 10 ms, time off = 100 ms, nominal power = 5 watts) of pure Diethylene Glycol Dimethyl Ether (Di-glyme), (CH₃OCH₂CH₂)₂O) vapors (Sigma Aldrich, used as received). For pAA, acrylic acid vapours are mixed with Ar in pulsed RF mode (13.56 MHz; pulsed plasma: time on = 4 ms; time off = 36 ms). The films obtained are uniform and their thickness can be adjusted between 20 and 200 nm. Characterisation of the pAA and PEO-like films can be found in [28]. Typically, the pAA films contain varying degree of carboxylic functions, depending on the power applied during the discharge. As discharge power is increased, the O/C ration as measured by XPS decreases from 50% down to a few percent, corresponding to an increase of the films cross-linking and stability. For PEO-like films, similar results are obtained and the antifouling properties of the films are directly related to the importance of the C-O-C peak in the C1S XPS spectrum, which decreases as the discharge power increases [28,35]. So the composition of the films, as well as the degree of cross-linking, vary continuously as the discharge power is increased.

2.2. Surface patterning using colloidal lithography
The patterning of a surface by colloidal lithography consists in creating a 2-D crystalline structure of polystyrene (PS) beads on the pAA, those beads being used as masks during subsequent etching and deposition operation [36,37]. First, the pAA layer (thickness = 200 nm) is deposited on a Si substrate as described above. Then, a micro-drop (5 μl) of PS beads (typically of 200 to 500nm diameter) suspension is deposited on the samples surface. The sample is then spun to produce a slow evaporation of the liquid. It is thus possible to create macroscopic homogeneous areas covered by mono-layered nano-beads of few mm² with a surface coverage ranging from 70% to 100%. The next operation consists in a O₂ plasma etching which removes the pAA between the beads (Figure 1a). Once the polymeric nano-structures are created on the surface, the chemical contrast is formed by the plasma deposition of the PEO-like coating deposited on the surface through the nano-masks (Figure 1c). The deposition is isotropic and the regions unprotected by the masks are coated.
The nano-sphere mask is then removed by ultrasonication in ultra-pure water. The final surface is composed of carboxylic domains of a few hundreds of nm embedded in the anti-fouling background (Figure 1d). The SEM images of Figure 2 show the surface of the sample before lift off of the beads, for different etching times. It can be seen that the patterns have an hexagonal distribution, the dimensions of which (typically a few hundreds of nm) depend on the initial size of the beads (here 500nm).

Moreover, the distance between active areas can be adjusted by the duration of etching. Structures of 200nm up to 1000nm and more can be obtained with this process, with coverage of about 90% over a few mm². Details of the experiments are given in [54,55].
2.3. Direct surface patterning using electron-beam lithography

It has been shown that plasma polymers can be patterned by direct photolithography using photoresists and PMMA [38,41]. Sub-micron nanopatterns of typically 200nm could be obtained by this approach. Lower dimensions necessitate electron-beam lithography, which can offer a high-resolution definition [39,40,41,42]. In our work, we used the possibility of cross-linking water-soluble pAA directly by the electron beam. The deposition of this layer is made with a low power (nominal power of 10 watts, operating in pulsed mode with a t_on of 4ms and a duty cycle of 10 %). The film thus obtained is not cross-linked and dissolves readily in water. The surface is then exposed to the electron-beam with an energy of 20 keV and incident dose of 1600 mC/cm^2, which produces a local cross linking of the polymer. After immersion in water and drying, the cross-linked domains remain on the surface. The chemistry of the film produced is similar to plasma polymerized acrylic acid layer produced at optimum plasma density and used for bio specific applications [43] with a percentage of retention of COOH groups around 7-10 %.

Figure 3 shows a SEM image of pAA-structures on a PEO-like background (right) and fluorescence image of microscale structure after incubation with BSA-FTIC. Well-defined features sizes down to 200 nm in lateral resolution can be fabricated with several heights (from 80 nm to 500 nm). More experimental details can be found in [56].

![Figure 3](image)

Figure 3. SEM and fluorescence picture of nanostructures obtained by e-beam patterning of an unstable pAA film and after incubation with BSA-FTIC.

2.4. Microcontact printing

On bio/non-bio interfaces, the interactions with cells or tissues are always mediated by protein adsorption on the surface [44,45,46,47]. In our work, we have optimized a platform for the patterning of human umbilical cord blood - derived neural stem cells in order to study cellular maintenance and differentiation when interacting with polypeptide micropatterns [48,49,50,51,52]. To produce these surfaces, microcontact printing has been used as a mean to deposit controlled, geometric patterns of Poly-L-lysine (PLL) and fibronectin (FN) on glass substrates previously coated with a protein resistant, cell repellent PEO-like film. The PEO-like layer has the important characteristic of being cell repellent in solution but protein adhesive under the dry conditions used during the microcontact printing operation [53]. It must be underlined that microcontact printing can be used to produce patterns down to 100nm scale.

3. Applications and results

3.1. Protein adsorption on nanopatterns

The nanostructures produced by colloidal and electron-beam lithography have been extensively studied with different biological tests and protein adsorption experiments [15,54,55,56,57,58].
particular, ELISA tests and Quartz Crystal Microbalance with dissipation (QCM-D) (Qsense, Sweden) has been used for quantitatively compare the mass of proteins absorbed on uniformly functionalized and nano-structured surfaces. Both the probe (human IgG) immobilisation and the antibody/antigen (human IgG/Anti-human IgG) reaction have been monitored on-line (IgG concentrations ranging from 1 to 15 µg/ml in PBS solution). Figure 4 shows that the nano-patterned ND platform presents much higher reaction efficiency than all the other cases, evidencing the capability of the adhesives/anti-adhesives nanopatterned surface to immobilize the molecules in a reactive state, thus increasing their possibility to form complexes. This result has been further confirmed by other techniques (SPRi, QCM) [54,55,58,59,60,61] and we found that the reaction enhancement was increased as the nanostructure dimensions decrease (see Figure 5).

Figure 4. ELISA test performed on differently functionalized surfaces with an Ova-c-HRP concentration of 10 µg/ml

Figure 5: Calibration Curves of the immuno-reaction IgG/Ab-IgG) for the flat ppAA and the nanostructured surfaces (200, 500 and 1000nm)

A possible explanation is linked to a better orientation of the antibodies on a nanostructured surfaces and a reduction of the steric hindrance linked to the antibodies adsorption on the edges of the structures, making binding reactions more efficient on the nanostructures as compared to uniform surfaces [58].

3.2. Cell culture experiments

Microcontact printing of Fibronectin (FN) and poly-L-Lysine (PLL) was used to study the growth of neural stem cells and as a model to study different processes regarding stem cell neural lineage commitment. Human umbilical cord blood derived neural stem cells (HUCB-NSC) [62] have been incubated on the polypeptide functionalized surfaces. Previous studies on non-patterned surfaces [63] showed that the density of plating and the type of polypeptide used for attachment can change the HUCB-NSC commitment to a specific lineage (neuronal, astrocytic, olegodendroglial). In this work, the cells attached to the FN and PLL domains grow following the pattern but show also a behaviour that is sensitive to both geometry and cell density. It was also noticed that cells on densely crowded islands stayed in non-differentiated mode longer than low populated samples where cell commitment to a differentiated mode is favoured. Figure 6 shows that by varying the conditions of culture (with and without serum, with and without growth factor, on FN and PLL patches), we found that the differentiation of HUCB-NSC could be modulated to a large extent, the population of neurons, astrocytes and oligodendrocytes varying between 10% and 80% depending on the conditions [9,16,62,63].
Figure 6: Immuno-images of HUCB-NSC grown on PLL (A-D) and fibronectin (E-H) microarrays for 7 days in serum (A, B, E, F) and serum free conditions (C, D, G, H) differentiating in the presence of dBCAMP (B, F, D, H) or growing as a control cultures (A, C, E, G). Neuronal (β-tubulin III) and astrocytic markers indicate the changes in differentiation depending on the PLL or FN markers. The scale bar is 100 μm for all images. Reproduced with permission from [9]

4. Conclusions
We have shown that plasma technologies, and in particular deposition, lithography and etching can be combined with colloidal and electron-beam lithography as well as microcontact printing for production of high quality micrometric and sub-micrometric patterned surfaces with arbitrary geometries and controlled chemistry. The major advantage of plasma polymers is related to the large range of properties that can be obtained, and their relative stability with time and against different solvents and on the fact that they can be applied on virtually any substrate. A major challenge of these processes will be optimization of the robustness and control of surface properties to make them applicable at a large scale at low cost.

5. References
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