Development of an electro-responsive platform for the controlled transfection of mammalian cells

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ABSTRACT

The recent development of living microarrays as novel tools for the analysis of gene expression in an in-situ environment promises to unravel gene function within living organisms. In order to significantly enhance microarray performance, we are working towards electro-responsive DNA transfection chips. This study focuses on the control of DNA adsorption and desorption by appropriate surface modification of highly doped p++ silicon. Silicon was modified by plasma polymerisation of allylamine (ALAPP), a non-toxic surface that sustains cell growth. Subsequent high surface density grafting of poly(ethylene oxide) formed a layer resistant to biomolecule adsorption and cell attachment. Spatially controlled excimer laser ablation of the surface produced micron resolution patterns of re-exposed plasma polymer whilst the rest of the surface remained non-fouling. We observed electro-stimulated preferential adsorption of DNA to the ALAPP surface and subsequent desorption by the application of a negative bias. Cell culture experiments with HEK 293 cells demonstrated efficient and controlled transfection of cells using the expression of green fluorescent protein as a reporter. Thus, these chemically patterned surfaces are promising platforms for use as living microarrays.

Keywords: Surface modification, plasma polymerisation, PEO, non-fouling, DNA, adsorption, desorption, transfection

1. INTRODUCTION

Rapid developments in DNA sequencing techniques and the complete sequencing of the human genome have put increasing pressure on the development of techniques and analytical tools for the study of DNA function within cells1-2. The development of DNA and protein microarray technology has revolutionised the way scientists have been able to study gene expression by providing a high throughput method of analysis where entire genomes can be studied on a single array3-4. In recent years, the scope of microarray technology has been broadened by the development of a living microarray5 consisting of cDNAs arrays spotted onto a glass slide at addressable locations Human embryonic kidney cell line (HEK293) grown over this DNA array were observed to take up DNA creating regions of localised transfection within a lawn of non-transfected cells5. However, a successful mechanism for the prevention of cross-contamination of cells and DNA between adjacent colonies or DNA spots has not been successfully implemented. Szili et al.6 recently developed a platform for a transfection chip using the modification of a substrate material by plasma polymerisation. Plasma polymerisation refers to the formation of polymerised material by use of a monomer in the plasma state7 and can be used to effectively modify surfaces with a thin, well adherent polymer layer that, by the choice of the monomer, contains the desired functional groups without altering the substrates bulk properties. Plasma polymerisation has been achieved with monomers containing alcohol, amine and carbonyl functional groups to produce plasma polymers with equivalent functionality8-11. We have previously used allylamine as a monomer to produce an amine-rich allylamine plasma polymer (ALAPP) with subsequent grafting of poly(ethylene oxide) (PEO)12. Excimer laser ablation was then used to produce regions of ALAPP, which sustains cell growth, and PEO, which has been shown to resist cell attachment12. We observed that this chemical pattern exerted spatial control over cell attachment. Yet, the amine functionalities of ALAPP, in their protonated state, are of additional use as they undergo electrostatic interactions with the negatively charged phosphate backbone of DNA.13-14. ALAPP coated surfaces should therefore lend themselves to...
the surface-retention of DNA. Investigation of spatially controlling DNA adsorption on this substrate, and the optimisation of transfection conditions would aid in the development of a highly useful substrate surface with application for biochips in general and living microarrays in particular.

![Figure 1: Depiction of the method used to pattern the transfection chip surface. The steps of the process are, A) cleaning of the Si substrate, B) the deposition of an ALAPP film, C) grafting of a PEO brush to the ALAPP film, D) masked excimer laser ablates which results in, E) formation of a ALAPP/PEO pattern. Not drawn to scale.](image1)

The effectiveness of living microarrays is hampered somewhat by the lower transfection efficiencies\(^6,15\) seen when transfection occurs on the solid phase. The reasons for lowered solid phase transfection efficiency include the ‘stickiness’ of DNA on the surface. However, transfection efficiency could be enhanced by initiating the controlled release of DNA from a suitable substrate surface once a cell lawn has formed to stop released DNA from diffusing into the bulk solution. The use of a voltage bias as the stimulus for controlled adsorption and desorption of DNA has been extensively studied on metal electrodes\(^16-17\).

![Figure 2: Depiction of the spatially controlled transfection of mammalian cells on a patterned ALAPP/PEO substrate. Cells expressing the plasmid pEGFP-N1 are green.](image2)

In the present study, the production and characterisation of a chemically patterned surface that allowed spatially controlled cell attachment and DNA adsorption was performed. The method is based on our previous work\(^6,7\) as depicted in Fig. 1. In this case however, we chose highly doped p++ (low resistivity) silicon wafer as a substrate surface.
After plasma polymerisation of ALAPP, an aldehyde terminated PEO polymer was grafted onto the ALAPP layer using reductive amination. Subsequent use of masked excimer laser ablation produced the desired patterned surface.

The adsorption of oligonucleotides and plasmid DNA to ALAPP was manipulated by the application of a positive voltage to the semi-conducting silicon substrate and the growth and transfection of mammalian cells on this substrate was studied as depicted in Fig. 2.

2. METHODOLOGY

All chemicals were purchased from Aldrich and used as received except where otherwise specified.

2.1 Substrate preparation
Boron doped p++ silicon wafers (Virginia Semiconductors, Inc.) were cut into approximately 10x10 mm pieces. All substrate materials were cleaned by sonication for 30 min in a 5% (detergent concentration) surfactant (RBS 35, Pierce USA) wash.

2.2 Plasma polymerisation
Plasma polymerisation reactions were preformed in a custom-built reactor described elsewhere. In short, the plasma reactor consisted of two circular electrodes separated by 12.5 cm in a cylindrical reactor being 35 cm high with a diameter of 17 cm. Allylamine (Aldrich, 98% purity) was used as a monomer. Polymerisation conditions used were a frequency of 200 kHz, a power of 20 W and an initial monomer pressure of 0.188 mbar. Deposition time was 25 s.

2.3 Poly(ethylene oxide) grafting
Poly(ethylene oxide) (PEO) monoaldehyde (Shearwater Polymers, Huntsville AL, USA) with molecular weight 5000 g/mol was grafted onto freshly deposited ALAPP layers by reductive amination. Grafting was performed under ‘cloud point’ conditions in 20 ml of a 0.1 M sodium phosphate buffer, containing NaCNBH₃ at pH 6.2.

2.4 Excimer laser ablation
Ablation experiments were conducted using a 248 nm KrF excimer laser Series 8000 (Exitech Limited, UK) equipped with a Lambda Physik LPX2 10i laser source. The beam delivery system contained beam shaping and homogenisation optics to create a uniform, square beam at the plane of a mask held on an open frame CNC controlled X-Y stage set. The square beam was passed through a chrome-on-quartz mask pattern. The beam was then passed through a 1:10 demagnification lens, NA of 0.3, a 1.5 mm diameter field and a theoretical resolution of 0.8 µm. PEO grafted ALAPP samples on silicon substrate were ablated at an energy density of 60 mJ/cm² and 4 pulses of 20 ns duration per area to form a spatially patterned substrate.

2.5 X-ray photoelectron spectroscopy
X-ray photoelectron spectroscopy (XPS) was conducted on an AXIX His spectrometer (Kratos Analytical Ltd.) equipped with a monochromatised Al Kα source. Pressure at analysis was typically 5 x 10⁻⁸ mbar. Elemental composition of surfaces was determined from survey spectra, collected at a pass energy of 320 eV. High-resolution spectra were obtained at a pass energy of 40 eV. Binding energies were referenced to the aliphatic carbon peak at 285.0 eV. Peak fitting of high-resolution spectra was conducted with Vision 1.5 software, Kratos analytical Ltd.

2.6 Atomic force microscopy
Surface topography and RMS roughness of ALAPP and PEO samples was analysed by atomic force microscopy (AFM) using a Nanoscope 4 Multimode microscope (Digital instruments) in Tapping Mode. Commercial Si FESP cantilevers (Digital Instruments, Santa Barbara, USA) were used for all experiments. Image analysis was done using Nanoscope 5.12 software (Digital Instruments).

2.7 Plasmid propagation
Plasmids pEGFP-N1 (4.7 kb) (Clontech), encoding the green fluorescing protein, were propagated in the JM109 Escherichia coli (E-coli) strain (Promega). Cells were transfected with plasmid by the heat shock method. Plasmid was
isolated using the QIAprep® Miniprep Kit (Qiagen) according to the manufacturer’s specifications.

2.8 DNA adsorption and desorption studies
DNA adsorption and desorption studies were initially conducted in a custom-built flow cell used in conjunction with a Leitz Fluorescence microscope. Patterned ALAPP modified PEO grafted samples were clamped into the flow cell and 200 µl of ultra pure water was injected over the sample. After washing, 10 µl of 290 ng/µl 6-FAM labelled 16-mer oligonucleotide (5'-GCCAGAAGCCAGTACT-3') (Geneworks) solution was injected over the sample and adsorption was observed. Positive voltage (0–2 V) was applied to the surface with a platinum wire counter electrode to investigate electro-induced DNA adsorption. Likewise, negative voltage (0–2 V) was applied to the surface with a platinum wire counter electrode to investigate electro-induced DNA desorption. Ultra pure water was injected to remove unbound DNA at various stages and for final washing.

2.9 Solid phase transfection
All substrates used for transfections and cell growth were sterilised by incubation in ethanol (70%) for 15 min after which they were transferred to sterile water and allowed to air dry in a sterile laminar flow hood. Other materials and solvents were sterilised by autoclaving for 15 min at 120°C and 100 kPa. A Human Embryonic Kidney cell line (HEK 293) was used. Cells were cultured in Dulbecco’s modified eagle media (DMEM) containing penicillin and streptomycin and incubated at 37°C, 5% CO₂ and 60-70% humidity. Transfections were conducted on ALAPP substrates. 1 µl of 300 ng/µl pEGFP-N1 solution was spotted onto the substrate surface and allowed to air dry in a sterile Laminar Flow hood. Samples were washed three times with 1xPBS, pH=7.4. Effectene Transfection Kit was used in some cases to enhance transfection efficiency. 100 µl of HEK 293 cells (7.1x10⁵ cells/ml) dislodged from a growing flask (confluency ~ 80%) were added on top of each substrate and cells were allowed to attach for 2 hrs before further DMEM media was added. Cells were incubated for another 24 hrs before being characterised microscopically. HEK 293 cells were counterstained using Hoechst 33352 dye. Cells were incubated in 10 µg/ml Hoechst solution for 10 min, after which cells were washed with 1xPBS.

3. RESULTS AND DISCUSSION

3.1 Characterisation of polymer films
Chemical characterisation of untreated and surface modified p⁺⁺ Si wafers was carried out by XPS analysis. The elemental composition of each surface was determined from survey spectra (Tab. 1). High-resolution XPS spectra of the C1s peak are shown in Fig. 3. Both these C 1s spectra could be de-convoluted into four peaks. In order of ascending binding energy, the four peaks correspond to aliphatic (C-H and C-C) carbon at 285.0 eV, C-O and C-N groups at 286.5 eV, amide and carbonyl groups at 288.0 eV, and finally ester and carboxylic acid groups at 289.0 eV. The quantification of the C 1s high-resolution spectra is summarised in Tab. 2.

The absence of a Si signal in the Si-ALAPP surfaces (Tab. 1) suggests that pinhole-free films with a thickness of more than 10 nm (the approximate penetration depth of XPS) were formed. The oxygen content in this film is due to the uptake of oxygen once it is re-exposed to air by an oxidative reaction with long-lived reactive species in the polymer film such as free radicals. Upon subsequent grafting of PEO to the amino groups of the ALAPP coating, a significant decrease in the nitrogen content (11% to 5.7%) and an accompanying rise in the oxygen content (8.9% to 20.9%) was observed. Furthermore, the C 1s spectrum in Fig. 4B shows a large increase in the C-O, C-N peak and a relative decrease in the aliphatic carbon peak and secondary oxidised carbon peaks as compared with the ALAPP film (Fig. 3; Tab. 2). These results suggest not only that PEO was successfully grafted, but also that amino groups were indeed present on the ALAPP surface. The presence of a nitrogen signal for the PEO grafted surface suggests that this layer is less than 10 nm thick, as XPS is able to probe the underlying ALAPP layer. The theoretical length of a fully extended PEO chain of molecular weight 5000 g/mol is approximately 42 nm. The large difference between this theoretical value and the observed value is partly due to the XPS measurement being taken dry where the grafted polymer collapses. A recent study by Zdryko et al. reports a greater than 3 fold swelling of the PEO layer in aqueous environment as compared with a dry sample.
Table 1: Elemental composition (in Atomic %) of Si, Si modified with ALAPP and PEO grafted to ALAPP as determined by XPS.

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>O</th>
<th>N</th>
<th>Si</th>
<th>O:C</th>
<th>N:C</th>
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<tr>
<td>Si</td>
<td>12.88</td>
<td>34.22</td>
<td>0.0</td>
<td>50.76</td>
<td>2.66</td>
<td>0.0</td>
</tr>
<tr>
<td>Si - ALAPP</td>
<td>79.99</td>
<td>8.98</td>
<td>11.03</td>
<td>0.0</td>
<td>0.11</td>
<td>0.14</td>
</tr>
<tr>
<td>Si - ALAPP - PEO</td>
<td>73.05</td>
<td>20.96</td>
<td>5.70</td>
<td>0.0</td>
<td>0.29</td>
<td>0.08</td>
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Figure 3: High-resolution C 1s spectra for Si modified with A) ALAPP, B) PEO grafted onto ALAPP. Curves fitted with four peaks.

Table 2: Quantification of high resolution C 1s spectra in Figure 3.

<table>
<thead>
<tr>
<th>Peak Binding Energy (eV)</th>
<th>285.0</th>
<th>286.5</th>
<th>288.0</th>
<th>289.0</th>
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<tr>
<td>% peak area</td>
<td>Si-ALAPP</td>
<td>68.35</td>
<td>23.84</td>
<td>6.12</td>
</tr>
<tr>
<td></td>
<td>Si-ALAPP-PEO</td>
<td>36.34</td>
<td>61.75</td>
<td>1.60</td>
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Si-ALAPP surfaces were further characterised by AFM. Typical AFM height images for ALAPP and PEO are shown in Fig. 4 and RMS roughness measurements are shown in Tab. 3. ALAPP films were shown to be smooth and free of pinholes. The RMS roughness was 0.35 nm. This surface was marginally flatter than bare Si (0.42 nm). At high image resolution, a grain structure can be distinguished with average lateral dimensions of 23.6 nm and average height of 1.4 nm. PEO films were shown to exhibit a distinctively different topography to ALAPP films and also exhibited a 1-fold increase in roughness in comparison to ALAPP.

Figure 4: AFM (Tapping Mode) height images of A) Si-ALAPP, B) Si-ALAPP-PEO. Lateral image scale 500x500 nm.
Table 3: Roughness (RMS) values determined by AFM of modified Si surfaces. Range given is one standard deviation unit in magnitude.

<table>
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<tr>
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<th>RMS roughness (nm)</th>
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<tr>
<td>Bare Si</td>
<td>0.42 ± 0.12</td>
</tr>
<tr>
<td>Si-ALAPP</td>
<td>0.35 ± 0.05</td>
</tr>
<tr>
<td>Si-ALAPP-PEO</td>
<td>0.72 ± 0.08</td>
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3.2 DNA adsorption and desorption studies

DNA adsorption and desorption from the Si-ALAPP-PEO ablated surfaces was initially studied using a custom-built flow cell. Initially, short (16mer) oligonucleotides were used labelled with 6-FAM (ex 497 nm, em 514 nm) at the 5’ end. Fig. 5 shows typical fluorescence microscopy images of the spatially controlled adsorption of oligonucleotides to a Si-ALAPP-PEO surface patterned by excimer laser ablation. 6-FAM labelled oligonucleotide solution, injected over the patterned ALAPP and PEO surface, preferentially adsorbed to the ALAPP regions as opposed to the PEO regions. The mechanism for this adsorption has been proposed to be due mainly to electrostatic interactions of protonated amino groups with the negatively charged backbone of DNA. The presence of amino groups in the ALAPP film was demonstrated by the nitrogen signal in the XPS elemental composition data (Tab. 1), the presence of a C-N peak as identified in the high resolution C 1s spectrum for ALAPP (Fig. 3.A), and indirectly by the successful grafting of PEO to the ALAPP film as confirmed by XPS (Tab. 1; Fig. 3.B). As experiments were conducted at a pH7.4, and given the typical isoelectric point of amines (for n-Bu groups attached to amines, 1° – pI 10.7, 2° – pI 11.3, 3° – pI 9.9)\(^\text{23}\), the amino groups present on the ALAPP film were expected to be protonated. However, it cannot be said conclusively from the fluorescent images as to whether the PEO coating quantitatively prevents DNA adsorption. Fig. 5.A and Fig. 5.B both show a green background on the PEO regions that could be due to oligonucleotide adsorbing to the PEO surface, to free oligonucleotide in solution or to autofluorescence effects of the coating. The flow cell design allowed the interrogation of the pattern while applying an electrical bias. After the application of a positive voltage (Fig. 5.B), and subsequent washing (Fig. 5.C) the pattern persisted, whilst the pattern disappeared after applying a negative bias (Fig. 5.D) suggesting the complete removal of oligonucleotide. The application of a positive or negative voltage indeed appeared to stimulate the adsorption or desorption of DNA. Admittedly, additional factors such as the stringency of the washings and background adsorption at 0V bias needed to be considered. We observed a fading of the fluorescence upon successive rinsing even at +1.5V bias (data not shown), suggesting that the washing protocol could influence the results of the fluorescence studies. Therefore, to independently investigate voltage bias induced DNA adsorption, an experiment was conducted without washing. Oligonucleotide was injected over the patterned surface (Fig. 5.E) and higher fluorescence intensity is observed on the ALAPP regions. Once +1.5 V was applied to the surface, increased fluorescence intensity was observed on the chip surface due to bias-enforced adsorption of the oligonucleotide to the surface (Fig. 5.F). However, adsorption in this case was not discriminative between ALAPP and the PEO surfaces, suggesting that prevention of DNA adsorption by the PEO layer was overcome by the application of a positive voltage. Furthermore, the application of -1.5 V resulted in the release of oligonucleotide from the surface as witnessed by a decrease in fluorescence (Fig. 5.G) both on ALAPP and PEO regions, suggesting that the attractive interactions between the DNA and ALAPP as witnessed in Fig. 5.E are overcome by the application of the negative voltage. However, a faint contrast remains in Fig. 5.G indicating that some DNA remains adsorbed to the surface despite the application of -1.5 V.

3.3 Transfection experiments

The ultimate test of the thin plasma polymer films investigated here was their ability to sustain solid phase transfection with reasonable efficiencies. HEK293 cells were grown on ALAPP films after adsorption of plasmid DNA in the presence or absence of a transfection agent (Effectene). Transfection efficiency was determined by counting the number of green-fluorescent cells expressing the plasmid pEGFP-N1. The total cell population was visualised and counted using the Hoechst 33352 dye, which is incorporated into the nucleus of all cells, non-transfected and transfected. Transfection efficiencies were determined by dividing the number of transfected cells by the total number of cells present. Results are shown in Tab. 4.
Figure 5: Fluorescence microscopy images of 6-FAM labelled oligonucleotide adsorption on a patterned ALAPP-PEO surface detected through a 450-490 nm excitation filter and a 515 nm suppression filter. Images A) - D) and E) - G) represent two separate experiments. A) 10 µl of 290 ng/µl oligonucleotide solution injected over patterned surface and incubated for 5 min. B) +2 V applied to substrate and incubated for 5 min. C) Surface washed with 100 µl ultra pure water whilst still applying +2 V. D) Surface washed whilst 100 µl ultra pure water whilst applying -2 V. E) 10 µl of 290 ng/µl oligonucleotide solution injected over patterned surface and incubated for 5 min. F) +1.5 V applied to substrate and incubated for 5 min. G) -1.5 V applied to substrate and incubated for 5 min. Images A) and B) have identical exposure times. Images C) and D) have identical exposure times. Images E), F) and G) have identical exposure times. The scale bars equal 100 µm.

Table 4: Transfection efficiencies for ALAPP surface with adsorbed pEGFP-N1. Transfection experiments were carried out with or without Effectene transfection agent.

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<th>Transfection efficiency (%)</th>
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<tr>
<td>No agent</td>
<td>0.56</td>
</tr>
<tr>
<td>With agent</td>
<td>25.70</td>
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Tab. 4 clearly shows that transfection efficiencies were greatly enhanced by the use of the Effectene transfection agent. Efficiencies for transfection on the solid phase up to 25% were measured in the presence of transfection agent. These values although still below the reported efficiency in the liquid phase (40-60% according to the manufacturer) are an improvement over previous solid phase transfection studies of 5-15% \( 6,15 \). In the light of these results, the controlled release of plasmid DNA from the surface using a negative voltage bias is suspected to further increase the effective concentration of plasmid available for cellular uptake.
4. CONCLUSION

The formation and characterisation of two-dimensional chemical patterns on silicon substrates using plasma polymerisation of allylamine in conjunction with PEO surface grafting and subsequent patterning by mask-assisted excimer laser ablation was demonstrated. The preferential adsorption of DNA onto the ALAPP regions on an ALAPP-PEO patterned surface was noted, showing that the PEO film repels not only proteins, as shown before, but also nucleic acid and therefore provides a general non-fouling coating. We demonstrated voltage bias-stimulated adsorption and desorption of DNA by fluorescence measurements. Solid phase transfection was shown to be feasible on the ALAPP regions with an efficiency of up to 25%, which compares favourably with literature values.

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REFERENCES


