Application of nanostructured biochips for efficient cell transfection microarrays

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ABSTRACT

Microarrays, high-throughput devices for genomic analysis, can be further improved by developing materials that are able to manipulate the interfacial behaviour of biomolecules. This is achieved both spatially and temporally by smart materials possessing both switchable and patterned surface properties. A system had been developed to spatially manipulate both DNA and cell growth based upon the surface modification of highly doped silicon by plasma polymerisation and polyethylene grafting followed by masked laser ablation for formation of a pattered surface with both bioactive and non-fouling regions. This platform has been successfully applied to transfected cell microarray applications with the parallel expression of genes by utilising its ability to direct and limit both DNA and cell attachment to specific sites. One of the greatest advantages of this system is its application to reverse transfection, whereupon by utilising the switchable adsorption and desorption of DNA using a voltage bias, the efficiency of cell transfection can be enhanced. However, it was shown that application of a voltage also reduces the viability of neuroblastoma cells grown on a plasma polymer surface, but not human embryonic kidney cells. This suggests that the application of a voltage may not only result in the desorption of bound DNA but may also affect attached cells. The characterisation of a DNA microarray by contact printing has also been investigated.

Keywords: transfection cell microarray, cell viability, plasma polymerisation, transfection efficiency, contact printing

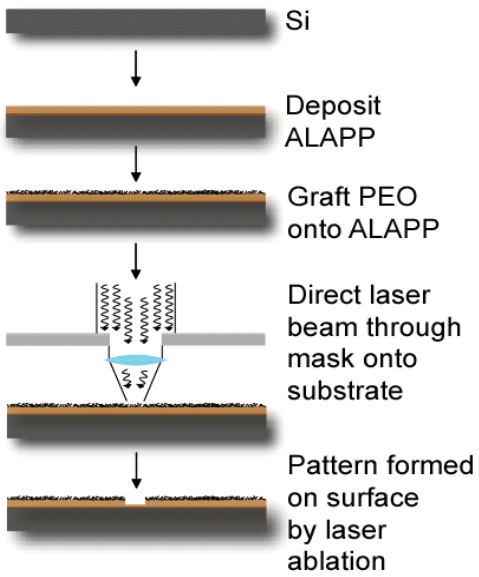
1. INTRODUCTION

Increased throughput of gene sequencing, which has enabled the complete sequencing of the human genome and the formation of cDNA libraries covering entire genomes, demands the development of systematic and high-throughput approaches to characterising gene function. The development of microarray technology had enabled analysis of gene expression in a high-throughput, cost effective method[1]. Microarrays are defined as high-density arrays of compounds of interest in defined, addressable locations that enable high-throughput screening and analysis of the interactions of a particular arrayed molecule with an analyte of interest. Libraries of DNA or proteins arranged in microarrays can be used to analyse the information contained in a genome or proteome. Microarrays are formed by the spotting of nucleic acids or proteins onto a substrate, typically a glass slide, a silica, titanium or gold microchip or an array of beads[2, 3].

Advances in DNA and protein microarray technology have revolutionised the way scientists have studied gene expression by providing high throughput analysis methods where an entire genome can be studied in a single array[4]. Recently, genomic analysis using microarrays has been broadened by the development of cell microarrays[1]. There are two predominant types of cell microarrays[1]: Substrate based cell arrays, where cells are seeded onto microarrayed biomolecules[5, 6] and genuine cell microarrays where the cells themselves are microarrayed[7]. Substrate based microarrays have been of particular interest and have included cells seeding onto arrays of DNA plasmids[8], small interfering RNA's (siRNA)[9], carbohydrates[10] and extracellular matrix (ECM) proteins[11].

Transfected cell microarrays (TCMs), are substrate based cell microarrays which enable genomic analysis in living cells where all the machinery is present for the correct expression of proteins. This in turn allows phenotypic changes to be observed as a result of a genotypic alteration. TCMs were initially formed by spotting defined cDNAs onto a glass slide in an array format at addressable locations and a lawn of Human Embryonic Kidney cells (HEK) being grown over this

Smart Materials IV, edited by Nicolas H. Voelcker, Proc. of SPIE Vol. 6413, 64130R, (2006)
0277-786X/06/$15 · doi: 10.1117/12.695954



improved transfection efficiency observed by the controlled desorption of DNA from a surface may relate to the improved availability of this DNA for uptake by nearby cells. However, the exact mechanism of this improvement remains unclear.

In the present study, the improved solid-phase transfection efficiency due to the application of voltage was further investigated. The focus here was on the effect of voltages on the viability of living cells. The application of the patterned ALAPP/PEG to a microarray format was also of interest, with the methodology required for microarray formation being investigated.

2. METHODOLOGY

2.1 Substrate preparation

Glass slides and boron doped p^{++} silicon wafers (Virginia Semiconductors, Inc.) were cut into 15 mm x 15 mm pieces. All substrates were cleaned by sonication for 30 min in a 5% surfactant (RBS 35, Pierce USA), with subsequent washing in ultra pure water (Labconco Water Pro PS) and drying under a stream of nitrogen. Silicon wafers were further oxidised under UV light for 10 min.

2.2 Plasma polymerisation

Plasma polymerisation reactions were performed in a custom built reactor described elsewhere[24]. Briefly, 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% pure) 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 and a deposition time of 25 sec.

2.3 X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) was conducted on an AXIS HSi spectrometer (Kratos Analytical Ltd.) equipped with a monochromatised Al K_α source. Pressure at analysis was typically $5x10^{-8}$ 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.

2.4 Plasmid isolation and quantification

Plasmids pEGFP-N1 (4.7 kb) (Clontech), encoding the green fluorescing protein (GFP) (excitation 488 nm, emission 509 nm), were propagated in JM109 *Escherichia coli* (*E.coli)* strain. Cells were transformed with plasmid by the heat shock method. Plasmids were isolated using the QIAprep® Mini prep kit (Qiagen) according to the manufacturer's specifications.

2.5 Sterilisation of substrate and materials for cell growth

All substrates for transfection and cell growth were sterilized by incubation in ethanol (70%) for 15 min and transferred to sterile water after which they were allowed to air dry in a sterile laminar flow hood. Other materials were sterilised by autoclaving.

2.6 Cell lines and cell culture

Human embryonic kidney cells (HEK 293) and neuroblastoma cells (SK-N-SH) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (DMEM powdered media, 6.7g/L; $NaHCO_3$, 1.85g/L) supplemented with antibiotics penicillin and streptomycin and 10% turbo calf serum. Cells were maintained at 80% confluency before use. They were incubated at 37^0C, 5% CO_2 and 60-70% humidity.

2.7 Cell viability

Cell viability was studied on Si coated ALAPP and pure Si surfaces. These experiments were conducted in a custom built electrochemical cell described previously[25] using Echem v1.5 software (AD Instruments), ALAPP coated Si sample as the working electrode, a AgCl/Ag saturated KCl reference electrode and a platinum auxillary electrode. HEK 293 or SK-N-SH were seeded onto ALAPP or bare Si samples for 60 min (HEK 293) and 90 min (SK-N-SH) giving time for the cells to attach. After attachment -1 V was applied for 0 min, 1 min, 2 min and 5 min, mimicking the electro-stimulated DNA adsorption previously described[19]. Cells were incubated for a further 24 hrs, after which fluorescein diacetate (FDA) (excitation 495 nm, emission 520 nm) was added. FDA stains only living cells that are actively metabolising. Hoechst 33342 dye (excitation 355 nm, emission 465 nm) (10 mg/ml) was added, staining the nucleus of all cells, both dead and alive. Images were captured using a Leitz fluorescence microscope Laborlux D and a Nikon Digital Sight DS-L1 camera. FDA fluorescence images were captured through a 450-490 nm band pass excitation filter and a 515 nm low pass suppression filter. Hoechst fluorescence images were captured through a 355-425 nm band pass excitation filter and a 580 nm long pass suppression filter. The number of FDA stained cells was compared to the total cells, determined from the Hoechst fluorescence, to determine the cell viability percentage.

2.8 Microarray formation and characterisation

A BioOdyssey^{TM} Calligrapher^{TM} MiniArrayer, BioRad was used microarray formation. Initially, contact printing of DNA was characterised using a 6-carboxyfluorescein (6-FAM)-labelled (excitation 497 nm, emission 514 nm) 16-mer oligonucleotide (5'-FAM-GCCAGAAGCCAGTACT-3') (Geneworks). Contact printing was conducted using a quilled pin (SMP3, ArrayIt, 100 μm diameter). In order to improve the binding of DNA to the ALAPP surface physical entrapment within gelatin was investigated. A 0.2% (w/v) aqueous gelatin solution was prepared in MilliQ water by heating and in a 60^{o}C water bath for 15 min. Oligonucleotide solution was added to the gelatin solution to a final DNA concentration of 0.050 μg/μl. Gelatin was labelled with Texas Red succinimidyl ester (Molecular Probes) (excitation 596 nm, emission 610 nm) in order to visualise gelatin spots. 6-FAM fluorescence images were captured through 450-490 nm band pass excitation filter and a 515 nm low pass supression filter. Texas Red fluorescence images were captured through 530-560 nm band pass excitation filter and a 580 nm low pass suppression filter.

2.9 Solid phase transfection

Solid phase transfection was carried out on an ALAPP coated Si sample. 1 μl of 300 ng/μl pEGFP-N1 solution was spotted onto the sample and allowed to air dry. After drying, 1 μl of Effectene transfection reagent (Qiagen) was spotted onto the plasmid and allowed to air dry. The transfection reagent was prepared according to the manufacturer's specifications. Alternatively, DNA and Effectene transfection reagent was spotted onto the ALAPP surface by contact printing for formation of a microarray as described above. After the spots were completely dried, HEK 293 cells were seeded onto the sample and incubated for 24-48 hrs and then were characterised microscopically. Cells were counterstained with Hoechst 33342.

3. RESULTS AND DISCUSSION

3.1 Surface characterisation

Bare silicon and freshly deposited ALAPP and PEG films were characterised by XPS. The elemental composition of each is shown in Tab. 1. Bare silicon shows a high Si content, as expected, but still shows a significant carbon surface contamination (12.9%) even after repeated washing. The deposition of ALAPP was confirmed by the introduction of a nitrogen signal (11.0%) as compared with bare silicon. The absence of a silicon signal for ALAPP suggests that a pin-hole free film with a thickness greater than 10 nm was formed, exceeding the penetration depth of XPS. The presence of oxygen in the ALAPP signal is due to post-deposition reactions of long-lived reactive species within the ALAPP film such as radicals with atmospheric oxygen[26, 27].

Table 1: Elemental composition (in atomic %) of the Si substrate and Si coated with ALAPP as determined by XPS.

	C	O	N	Si	O/C	N/C
Si	12.9	34.2	0.0	50.8	2.66	0.00
Si - ALAPP	80.0	9.0	11.0	0.0	0.11	0.14

3.2 Cell viability

Of interest was the mechanism for improved transfection efficiency observed for solid-phase transfection experiments where a negative voltage was applied after cell attachment to DNA spots[19]. We hypothesise that the application of a voltage may affect the cells attached to the electrode, resulting in a compromise in the cellular membrane, increasing the ability for plasmid to enter the cell. In order to investigate this HEK 293 and SK-N-SH cells were seeded onto both bare silicon and freshly deposited ALAPP samples and -1 V was applied after cell attachment. The viability of cells after voltage application was determined. Results were standardised to a control where zero volts were applied. The results for cells grown on silicon are shown in Fig. 2 and the results for cells grown on the ALAPP surface are shown in Fig. 3.

On bare silicon, HEK 293 showed no significant change in viability when -1 V was applied for up to 5 min (Fig. 2(a)). Similarly, no increasing cell mortality was observed for SK-N-SH cells when -1 V was applied for less than 3 mins (Fig. 2(b)). However, when -1 V was applied to SK-N-SH cells for 5 min cells were significantly less viable than for shorter exposure times (40 %). This large change is presumably the result of the voltage application and suggests that the voltage can adversely affect the cells in contact with the electrode. Potentially cells are also affected by the voltage when applied for shorter lengths of time, however, if so any alterations made to cells seem to be entirely reversible. Fig. 2 also suggests that SK-N-SH cells are more susceptible to voltage induced mortality than HEK 293 cells.

On ALAPP films, HEK 293 cells again showed no change in viability as a result of the application of -1 V for up to 5 min (Fig. 3(a)). However, SK-N-SH cells again showed a decrease in viability as a result of the application of -1 V (Fig. 3(b)). In this case cell viability was seen to drop as the length of voltage application increased up to 2 mins, after which viability stabilised up to 5 mins at 70% (Fig. 3(b)). This value is higher than the viability observed on bare silicon after application of -1 V for 5 min (40%), suggesting that ALAPP may help protect the cells from the cell destructive effect of the applied voltage as a result of ALAPP being non-conductive. Again, the greater resilience of HEK 293 cells was demonstrated and may suggest that SK-N-SH cells are more susceptible to improved transfection efficiency by voltage application as opposed to HEK 293 cells.

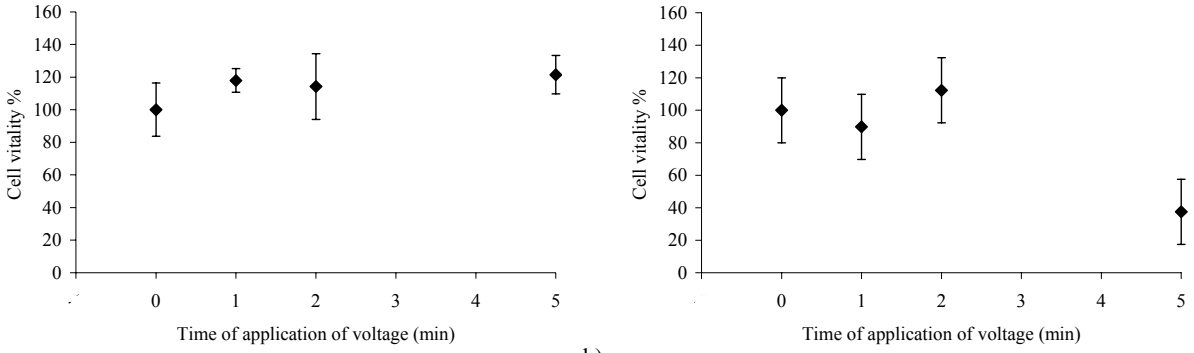


Figure 2: Cell viability of a) HEK293 cells and b) SK-N-SH cells on bare silicon after the application of -1 V from 0-5 min. Results were normalised to a control experiment where no voltage was applied.

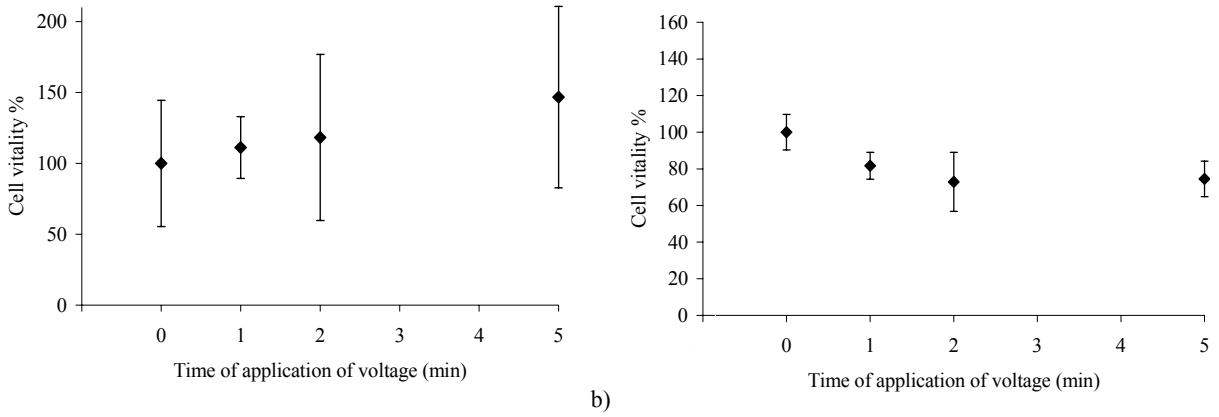


Figure 3: Cell viability of a) HEK293 cells and b) SK-N-SH cells on ALAPP after the application of -1 V from 0-5 min. Results were normalised to control experiment where no voltage was applied.

3.3 Characterisation of microarrays on ALAPP/PEG surfaces

In order to prepare for the application of patterned ALAPP/PEG surfaces for TCM applications the formation of DNA microarrays on ALAPP surfaces first needed to be characterised. This was achieved by formation of a 6-FAM labelled oligonucleotide microarray and subsequent microscopic characterisation of the printed spots . For contact printing, the approach speed to the surface and the dwell time of the square quilled pin in contact with the surface are important variables for formation of uniform, reproducible spots. Thus, arrays of oligonucleotides were formed to investigate the effects of changing these variables. Fluorescence microscope images of arrays formed at dwell times of 1, 25 and 36 ms are shown as Fig. 4(a), (b) and (c), respectively. Fluorescence microscope images of arrays formed at approach speeds of 1, 10 and 16 m/s are shown in Fig. 5(a), (b) and (c), respectively.

It was observed that the morphology of spots varied with changes to the approach speed and dwell time. The optimised conditions for formation of uniform, reproducible spots of regular shape were a dwell time of 25 ms and an approach speed of 10 m/s. These conditions were used for subsequent array formation.

3.3 Solid-phase transfection

Initially, transfection experiments were conducted with large spots of pEGFP-N1 with a typical diameter of 1.5 mm in order to confirm the successful transfection of HEK 293 cells. A typical fluorescence image is shown as Fig. 6. Cells counterstained with Hoechst 33342 are shown as Fig 6(a), whilst cells expressing the plasmid, as indicated by the green fluorescence from EGFP expression are shown in Fig 6(b).

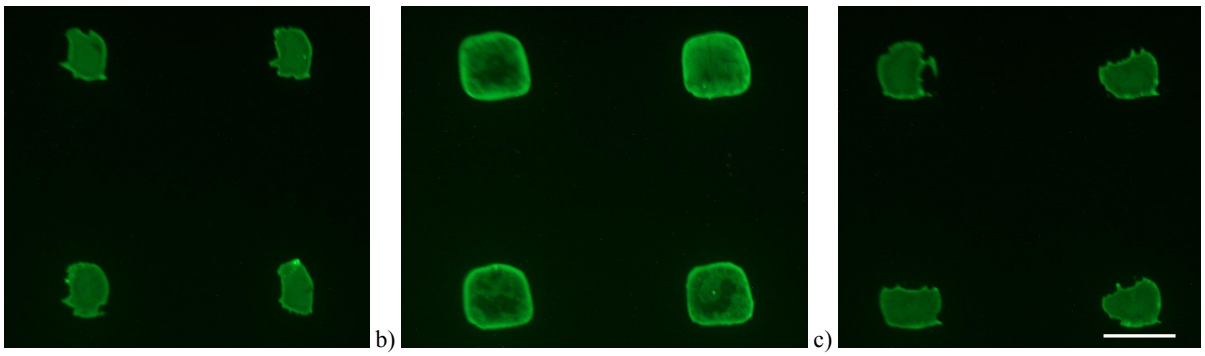


Figure 4: Fluorescence microscopy image of microarrays of 6-FAM labelled oligonucleotide formed by contact printing with a varied dwell time of the pin in contact with the surface; (a) 1 ms (b) 25 ms (c) 36 ms on an ALAPP coated silicon sample. Images were taken through a 450-490 nm excitation filter and a 515 nm suppression filter. Scale bar equals 100 μm.

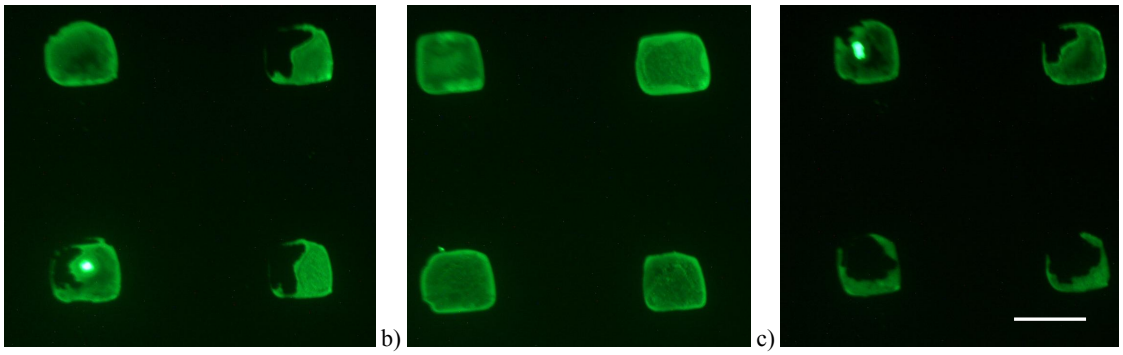


Figure 5: Fluorescence microscopy image of microarrays of 6-FAM labelled oligonucleotide formed by contact printing with a varied approach speed of the pin to the surface; (a) 1 m/s (b) 10 m/s (c) 16 m/s on an ALAPP coated silicon sample. Images were taken through a 450-490 nm excitation filter and a 515 nm suppression filter. Scale bar equals 100 μm.

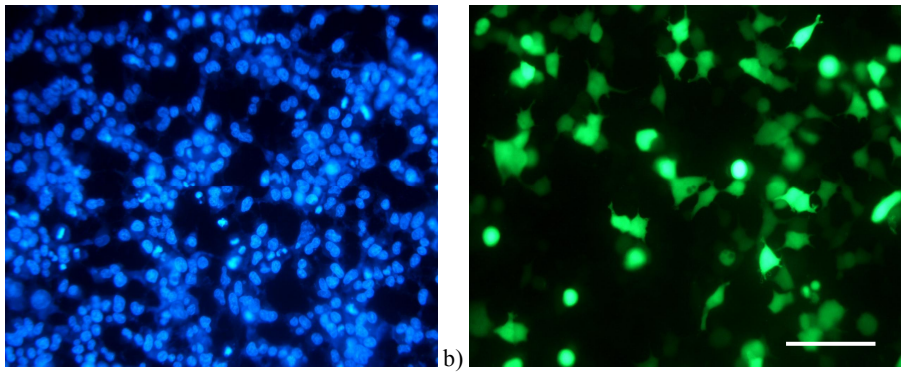


Figure 6: Fluorescence microscopy images of solid phase transfection experiment using HEK 293 cells on an ALAPP film. a) Cells stained with Hoechst 33342. Image taken through a 355-425 nm excitation filter and a 410-580 nm suppression filter. b) Cells expressing EGFP. Image taken through a 450-490 nm excitation filter and a 515 nm suppression filter. The scale bar equals 150μm.

Once the successful transfection of HEK 293 cells by pEGFP-N1 was achieved, a similar transfection experiment was performed using a plasmid DNA array formed by contact printing. The resulting fluorescence image is shown as Fig. 7.

Fig. 7 clearly shows that cells were not transfected at the regions where DNA was deposited. In fact, an array of transfected cells was not observed. This suggests that the DNA spotted to the surface is not effectively retained by the surface and is able to either undergo surface diffusion or diffuse into solution upon addition of cells where it is able to be uptaken by cells from the liquid phase. This result demonstrates the need to develop patterned surfaces that are able to control the behaviour of DNA at the surface and prevent unwanted diffusion.

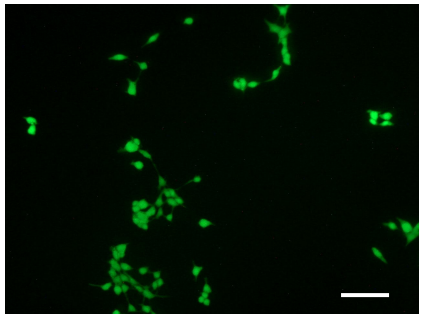


Figure 7: Fluorescence microscopy images of HEK 293 cells expressing pEGFP-N1, seeded onto a DNA array formed by contact printing onto an ALAPP film. Image taken through a 450-490 nm excitation filter and a 515 nm suppression filter. The scale bar equals 150 μm.

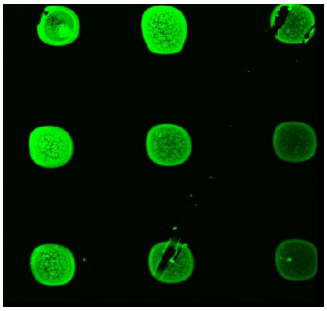
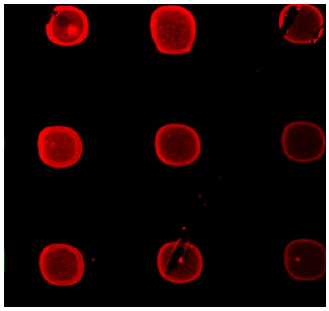
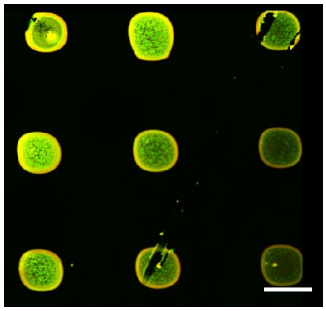
a)  b)  c) 

Figure 8: Fluorescence microscopy image of a 9x9 array of 6-FAM labelled oligomer entrapped within Texas Red labelled gelatin. (a) Image of 6-FAM fluorescence taken through a 450-490 nm excitation filter and a 515 nm suppression filter. (b) Image of Texas Red fluorescence taken through a 530-560 nm excitation filter and a 580 nm suppression filter. (c) Overlay of images (a) and (b). Scale bar equals 100 μm.

3.4 Formation of DNA/gelatin microarray

One method previously used to minimise DNA diffusion is the inclusion of gelatin in the DNA solution[6]. We explored this option by attempting to form a gelatin/DNA microarray. This was achieved by spotting a 6-FAM labelled oligomer solution in 0.2% (w/v) aqueous gelatin labelled with Texas Red succinimidyl ester. Fluorescence images of an array formed in this manner is shown as Fig. 8. Fig. 8(a) shows the fluorescence of the oligonucleotide spots, whilst Fig. 6(b) shows the red fluorescence due to the dye-labeled gelatin. Fig. 6(c) shows an overlay of Fig. 6(a) and (b). An array containing a DNA/gelatin complex can be formed where both constituents are evenly distributed. Further studies investigating the diffusion of DNA from the DNA/gelatin complex and its use for solid-phase transfection applications are currently being undertaken.

4. CONCLUSION

The viability of HEK 293 and SK-N-SH cells grown on ALAPP and bare silicon samples after application of -1 V up to 5 min was investigated as a potential method for improving solid-phase transfection. Significantly, the viability of SK-N-SH cells was reduced after the application of -1 V, however, HEK 293 cells were not affected. The decrease in viability of SK-N-SH suggests that -1 V does alter the properties of attached cells and warrants further investigation for transfection applications. The reduction in viability of SK-N-SH cells was greater for cells grown on bare silicon than on ALAPP samples suggesting that the non-conductive properties of ALAPP help to insulate the cells from the active electrode.

Microarray formation by contact printing was successfully characterised and optimised for further research. Transfection was attempted on a pEGFP-N1 microarray, however, transfection was observed at regions on the surface where DNA was not spotted, suggestion DNA migration. Thus, the physical entrapment of DNA in gelatin was demonstrated. Further studies of transfection on DNA/gelatin microarrays are underway.

ACKNOWLEDGEMENTS

Support from Flinders University, the Australian Research Council and CSIRO is kindly acknowledged.

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