Title :

Submicron patterning of DNA oligonucleotides on silicon

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ABSTRACT

The covalent attachment of DNA oligonucleotides onto crystalline silicon (100) surfaces, in patterns with submicron features, in a straight-forward two-step process is presented. UV light exposure of a hydrogen-terminated silicon (100) surface coated with alkenes functionalized with N-hydroxysuccinimide ester groups resulted in the covalent attachment of the alkene as a monolayer on the surface. Submicron scale patterning of surfaces was achieved by illumination with an interference pattern obtained by transmission of 248 nm excimer laser light through a phase mask. The N-hydroxysuccinimide ester surface acted as a template for the subsequent covalent attachment of aminohexyl modified DNA oligonucleotides. Oligonucleotide patterns, with feature sizes of 500 nm, were reliably produced over large areas. The patterned surfaces were characterised with atomic force microscopy, scanning electron microscopy, epifluorescence microscopy and ellipsometry. Complementary oligonucleotides were hybridised to the surface attached oligonucleotides with a density of 7 x 10^{12} DNA oligonucleotides/cm^2. The method will offer much potential for the creation of nano and micro-scale DNA biosensor devices in silicon.

INTRODUCTION

Recent advances in the methodology used for the immobilisation of biomolecules in patterns on surfaces have enabled the rapid development of biosensors for the analysis of DNA [1-2]. Numerous DNA sensors with patterned biomolecule motifs, of feature sizes from tens to hundreds of microns, have been fabricated [1-6]. An increasing need for miniaturised tools for pharmaceutical and diagnostic applications [7], as well as the newly emerging areas of DNA-based nano-assembly of electronic devices [8] has resulted in the need to develop methods for the high-resolution patterning of DNA on surfaces at the nanometre scale.

Scanning probe lithography or dip-pen nanolithography offer attractive possibilities for precisely anchoring nanoscale patterns of DNA on gold [9-10]. Feature sizes of the order of tens to a few hundred nanometres are achievable, although, unfortunately, with slow 'write speeds' and small patterning areas. In contrast, the advanced lithographic techniques used in the microelectronics industry achieve high-throughput fabrication of lateral dimensions in a range of to a few nanometres to hundreds of microns [11], and would have much potential for the construction of miniaturised biosensors. There have already been a number of approaches derived from photolithographic methods for the attachment of biomolecules to surfaces, which can be categorised as follows: i) Photo-ablation: laser induced photo-ablation offers a one-step fabrication method whereby exposed regions of a homogeneous anti-adhesive surface coated substrate are removed to leave behind well-defined openings for physical adsorption of DNA or proteins [12-14]. Submicron features have been achieved using this approach but the biomolecules are not covalently attached [14]. ii) Covalent photoattachment of photobiotin to organic polymer materials by exposure to UV light has been used as a template for the association of biomolecules in patterns [15]. The association of the biomolecules to these photo-attached biotin patterns is achieved by treatment of the surface with avidin (which binds to the attached biotin) followed by association of biotin-biomolecule conjugates to the associated avidin. Thus, this method does not yield covalently attached biomolecules in patterns *per se*, but does provide a versatile

method for surface patterning with micrometer dimensions, to which avidin-labelled biomolecules can be associated. [16-17]. iii) Photo-de-protection or photo-activation of a surface through a photolithographic mask has been used to generate precise patterns for the subsequent immobilization of biomolecules [18-20]. The majority of the reported cases of this type were achieved using glass, oxidised silicon or other amorphous surfaces. Even though stable DNA features of 1 micron have been achieved on glass substrates [20], the creation of even smaller patterns on glass is expected to be problematic due to the amorphous structure, thus the surface will be non-uniform with respect to chemical reactivity. In addition, the fabrication of integrated electronic devices using glass as a substrate is not possible.

Crystalline silicon has an excellent surface chemical purity and flatness, and it is ubiquitous in the semiconductor industry. Although largely unexplored to date, the use of bulk silicon as a substrate for biosensor construction is anticipated to enable further miniaturization of existing device technologies as well as to provide a platform for novel biosensor creation, such as practical devices that are actuated by DNA-mediated charge transfer [21] and nano-wires [8]. The possibility for the direct immobilisation of DNA on crystalline silicon was facilitated by the discovery of the photo- and thermal reaction of hydrogen-terminated silicon surfaces with alkenes [22]. The first examples of the immobilisation of DNA on silicon have recently been demonstrated [23-26]. For all the methods presented so far the whole silicon surface was modified with functionalised alkenes and the DNA oligonucleotide solutions spotted onto the modified silicon surface; oligonucleotide patterns on the millimetre scale have been achieved [25-26].

Although nanometre sized motifs have been fabricated on gold surfaces [9-10], gold is incompatible with microelectronics fabrication because of contamination issues (gold easily diffuses into silicon, thus affecting the electrical properties of the subsequent microelectronic devices). We were motivated to explore the potential of creating nanometre-sized motifs of covalently attached DNA oligonucleotides on surfaces for the creation of silicon-based biosensors. It was important to provide a highly reproducible method for the synthesis of motifs with high oligonucleotide density and accessibility toward selective hybridisation with the complementary

sequence. Chemical modification of the silicon surface has been tailored for optimisation of the photoreaction and to facilitate the immobilisation of the oligonucleotide [27].

In this paper, we present the first report of the fabrication of DNA oligonucleotide patterns on silicon on a submicron scale. The attachment of the DNA to the surface is achieved in a straightforward two-step process. The immobilised oligonucleotide patterns show excellent hybridisation properties and high attachment densities. The method will offer the potential for the creation of a range of novel silicon-based biosensing devices in the future.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Aldrich Chemical Company and used without further purification. Deionised water (ELGA genetics system) was used for all the aqueous solutions. Undecylenic acid N-hydroxysuccinimide ester (UANHS) was synthesized in-house as described elsewhere [27].

Scanning Electron Microscopy (SEM)

SEM (LEO 430 SEM, Oxford Instruments) was used to image the alkene-patterned silicon surfaces, which were scanned at low voltage without gold coating. Alkene-patterned surfaces, once evaluated by SEM, were not used further; identically prepared samples were used for the attachment of DNA oligonucleotides.

Epifluorescence Microscopy

The fluorescein-labelled oligonucleotide-patterned surfaces were visualised using a Zeiss Axiovert 200 inverted microscope, coupled with epifluorescence illumination and fitted with filter sets. For low-resolution imaging, all samples were placed face

down on a drop of Tris-HCl buffer over a microscope slice toward an Epiplan Neofluar 10X or 20X objective lenses. The fluorescence images were captured under the same imaging conditions for each experiment. The fluorescence signal to noise ratio (SNR) was determined by comparing the fluorescence intensity of single pixel points within the patterned area to the surrounding background using AxioVision Viewer software (Carl Zeiss). At least 20 random points within a single scan were determined. The average value of 3-6 scans for one wafer was given.

The fluorescence images of sub-micron oligonucleotide patterned silicon surfaces were obtained using a confocal laser microscope (Carl Zeiss, LSM510 Meta) with an Epiplan Neofluar 63X (water immersion) objective lens. The excitation laser wavelength was fixed at 488 nm and the emission filter was 505nm long pass. A laser power of 6 mW and a pixel dwell time of 3.2 μsec were used. All samples were covered in Tris-HCl buffer (20 mM Tris-HCl, 100 mM $MgCl_2$, pH 8.0) during the imaging process.

Atomic force microscopy (AFM)

An Explorer™ AFM from Veeco Instruments was used in the tapping mode with high resonance frequency non-contact tips for scanning the surface of the oligonucleotide patterned silicon. All measurements were carried out under air-ambient conditions (temperature of 20°C, and relative humidity of 45%).

Ellipsometry

Ellipsometric measurements were carried out with an imaging ellipsometer (I-Elli2000, Nanofilm Technology) as described elsewhere [27].

Photopatterning of alkene layers

A single-polished silicon wafer (100) was pre-cleaned as described in [27], etched in 2% aqueous hydrofluoric acid for 2 minutes, washed thoroughly with water, dried

under pressurised nitrogen and immediately spin-coated with 40 µl of 10% UANHS solution in CH_2Cl_2 at 2000 rpm for 20 seconds.

The wafer was exposed using a KrF excimer laser (IPEX^{TM}-800, GSI Lumonics) at a wavelength of 248 nm through a photolithographic mask or a phase mask. The photolithography masks were prepared with chrome-coated UV grade fused silica (Suprasil) using the wet etching method. A +1/-1 phase mask, having a periodicity of 2 µm was fabricated by the Ibsen Photonics Company. The masks were placed above a silicon wafer. After photoreaction, the wafer was thoroughly washed with dichloromethane and rinsed with acetone.

Immobilisation of DNA oligonucleotides

Oligonucleotides were synthesized in-house using conventional phosphoramidite chemistry and purified by reverse phase HPLC. Oligonucleotide concentrations were determined using extinction coefficients at a wavelength of 260 nm. The sequences used are 5'-FAM-TGC AGA TAG ATA GCA GT-3'-aminomodifier C7 CpG (Glen Research) (oligonucleotide A), 5'-TGC AGA TAG ATA GCA GT-3'-$(CH_2)_6$-NH_2) (oligonucleotide B), and 5'-TGC AGA TAG ATA GCA GTTTTTTTTTTTTTTT-3'-$(CH_2)_6$-NH_2) (oligonucleotide BT). Sequence A was labelled with the fluorescein dye at the 5' end, which allows for the direct visualisation of the fluorescent pattern of the attached single strands of the oligonucleotide. The oligonucleotides were dissolved in a 0.1 M sodium carbonate solution to a concentration of 10 µM (pH=8.4). 8 µl of the oligonucleotide solution was deposited on the alkene patterned silicon wafer and covered with a cover slip (2cm×2cm). The wafer was incubated overnight at room temperature in a humid chamber. After incubation, the wafer was washed thoroughly by rinsing with 0.1 M MES (2-(N-Morpholino)ethanesulfonic acid) buffer (pH 6.4) and deionised water. Samples immobilised with the oligonucleotides were kept in deionised water at 4 °C prior to characterisation or further hybridisation.

Hybridisation and denaturation experiments

To explore the hybridisation properties of the immobilised oligonucleotides (DNA oligonucleotides B and BT), three 5'-fluorescein-labelled oligonucleotides were used.

Oligonucleotide b (5'-FAM- CTG CTA TCT ATC TGC A-3'-aminomodifier C7 CpG) is complementary to oligonucleotide B and BT; whereas oligonucleotide b1 (5'-FAM-CTGCTTTCTATCTGCA-3'-aminomodifier C7 CpG) has one thymine base mismatch, and oligonucleotide c (5'-FAM-CTTACCGATCGTATTC-3') is not complementary. The hybridisation solutions were prepared with the fluorescein-labelled oligonucleotides (10 μM) in Tris-HCl buffer (20 mM Tris-HCl, 100 mM $MgCl_2$, pH 8.0). A wafer patterned with oligonucleotides B or BT was pre-soaked in the Tris-HCl buffer for 20 minutes and then excess buffer was removed. Immediately afterwards, 8 μl of the oligonucleotide hybridisation solution was deposited on the wafer and covered with a cover slip (2cm×2cm). The wafer was incubated in a humid chamber for 3 hours at room temperature. After incubation, the wafer was rinsed thoroughly with the Tris-HCl buffer and kept in a Tris-HCl buffer prior to characterisation.

Denaturation was accomplished by placing the samples in an 8.3 M urea solution for 15 minutes at 40 °C followed by rinsing with water [25]. The surfaces were then evaluated by using epifluorescence microscopy to make sure that the denaturation was complete. Subsequent hybridisation was repeated using the same procedure.

Density of hybridisation with the surface-attached oligonucleotides

A similar method to that reported by Strother *et al.* [25] was employed. Oligonucleotides B were patterned on silicon surfaces using a photolithographic mask as described. Complementary fluorescent oligonucleotides b were hybridised and washed thoroughly with Tris-HCl buffer. The fluorescent images were acquired

to calculate the exact surface area of the oligonucleotide patterns. The wafer was then placed in 800 µl water at 90 °C for 15 minutes to allow for the denaturation of the duplex. The wafer was removed and rinsed twice with 200 µL water; the denaturation solution 800 µl was mixed with the rinsing solutions (2 x 200 µL). The surfaces were then observed with the epifluorescence microscope to ensure that the denaturation of the duplex was complete. The water containing the denatured fluorescent DNA strands was freeze dried and the resulting dried oligonucleotides were re-dissolved in 50 µL of 0.05 M Tris buffer (pH 9.0). The fluorescence yield from the sample was measured in a 50 µL cuvette in a fluorimeter (Cary Ellipse, Varian Inc., λ_{exc} = 488nm, λ_{em} = 525nm). The concentration of DNA strands present in the sample was determined by reference to a standardisation curve prepared from a series of fluorescently labelled DNA oligonucleotide samples of known concentration.

RESULTS AND DISCUSSION

Surface attachment chemistry

Undecylenic acid N-hydroxysuccinimide ester (UANHS) attachment onto hydride terminated silicon surfaces has been demonstrated [27]. This method has been exploited, in the current study, for submicron patterning. Briefly, a hydride-terminated silicon surface was spin–coated with a dichloromethane solution of UANHS and irradiated with an excimer laser of wavelength 248 nm. Patterns of the surface attached UAHNS were achieved by illumination through either a photolithographic mask (Suprasil) or a phase mask. Typically, the laser pulse (20 ns) intensity was 15 kW, delivered with a repetition rate of 10 Hz, with illumination times of up to 2 mins. Significantly shorter illumination times were necessary than for previously reported photo-initiated alkene reactions with silicon [23, 25-26, 28], where long time exposures (several hours or a day) and illumination under nitrogen gas was necessary. The N-hydroxysuccinimide ester group is a robust coupling agent for amino-modified biomolecules [29]. Coupling the N-hydroxysuccinimide ester

terminated silicon surface in an aqueous solution with an amino-modified oligonucleotide provides a method for the creation of DNA oligonucleotide surfaces without further processing (Figure 1).

Submicron patterning of oligonucleotides

A reduction in the dimensions of oligonucleotide patterns below a micron is desirable for the creation of small scale sensors. In practice, a creation of a motif by illumination of dimensions approaching 1 μm is difficult to achieve by standard photolithographic mask techniques, even with the mask in close contact [11]. Interference lithography, which has been widely used to generate sub-100 nm features [30] or even sub-50 nm features when ultraviolet light has been used for other types of surface patterning [31-32], and appeared a promising approach for our aims.

Figure 2 shows the scheme adopted for the submicron patterning of oligonucleotides used here. A +1/-1 phase mask (2 μm period) was placed above a silicon wafer coated with UANHS. On one of the flat surfaces of the phase mask, a one dimensional periodic surface relief structure had been etched. The laser light, incident normal to the phase mask, was diffracted by the periodic corrugations of the phase mask diffract light into the +1 and -1 orders. Interference between these two orders created a light intensity profile of alternate maximum peak and minimum valleys of 1 μm period. After the exposure the un-reacted UANHS was washed off the silicon wafer, an amino-modified DNA oligonucleotide was added to the patterned surface to couple the oligonucleotide onto the UANHS motifs on the surface.

A wide range of exposure conditions were tested at a fixed repetition rate of 10 Hz, between 0.3 mJ/pulse.cm^2 and 2 mJ/pulse.cm^2, and exposure times from 20 seconds to 2 minutes). The resulting alkene motifs were evaluated by SEM and ellipsometry to enable the optimal exposure conditions to be determined. No reaction was detected when the sample was irradiated at 0.3 mJ/pulse.cm^2 for 30 seconds, but an increase in the exposure time to 60 seconds yielded a grating pattern that could be

detected by SEM as shown in Figure 3, where a periodic pattern of ~1μm is observable. Irradiation at higher intensity with a shorter exposure time yielded a very similar result (i.e. 1.5 mJ/cm^2 for 15 seconds). Patterns of 1 μm periodicity could be produced using a range of light fluences (0.17 to 0.85 J/cm^2). Ellipsometry was used to evaluate the film thickness. Because the ellipsometer could not be used to resolve nanometre sized patterns the UANHS films were irradiated without the phase mask (the phase mask only reduces the transmitted light by 5%.). The thickness of the formed UANHS layers, determined by ellipsometry, obtained with the fluences of 0.17 J/cm^2 were 0.85 J/cm^2, are 8.6 Å and 11.2 Å respectively, which is in the approximate range for the formation of a monolayer of the alkene containing less than 18 carbons [22]. However, the difference between the thickness values is only 3 Å, almost at the margin of the experimental error (1~2Å). We suggest that there may be a denser film of surface attached UANHS molecules obtained with the higher fluences; as demonstrated previously by ellipsometry for other films [22].

Subsequent coupling with fluorescein-labelled oligonucleotides onto the patterned UANHS leads to an oligonucleotide pattern on the submicron scale and this was visualised by high resolution atomic force microscopy as shown in Figure 4. The oligonucleotides (bright spots) are mainly located on the raised uniform parallel lines.

Hybridisation of complementary and mismatched DNA oligonucleotides with the surface patterned DNA surfaces

To evaluate the submicron patterned DNA oligonucleotides with respect to hybridisation with the complementary sequence, an unlabelled oligonucleotide B-pattern was prepared on the silicon surface. A series of identical samples were tested towards hybridisation to the fluorescein-labelled oligonucleotides b, b1, and c; these oligonucleotides are complementary (b), contain a one base mismatch (b1) and are non-complementary (c) to oligonucleotide B. After stringent washing, the epifluorescence images of the hybridised samples were obtained (Figure 5); only the

sample hybridised with the complementary oligonucleotide b revealed a highly fluorescent pattern.

The fluorescence signal after treatment of the patterned surfaces with each of the oligonucleotides was quantified as the normalised fluorescent signal-to-noise ratio N-SNR (fluorescent signal to noise ratio SNR of the sample after hybridisation experiment /SNR of the same sample before hybridisation) in Figure 6a. The SNR for samples hybridised with one-mismatch (sequence B-b1) was 1.25 ± 0.24 and non-complementary sequence was 1.09 ± 0.05 (sequence B-c), indicating the presence of a very small amount of non-specifically bound oligonucleotide to the silicon attached oligonucleotides. The SNR for the complementary sequences was evaluated as 2.99 ± 0.45 (sequence B-b).

As a test to prove that there was no variation between the hybridisation efficiency between different DNA oligonucleotide patterned silicon surfaces, i) a silicon sample with surface attached oligonucleotides was treated under hybridisation conditions with the complementary oligonucleotides b. ii) Following this the sample was evaluated and the hybridised oligonucleotides removed under denaturation conditions, then iii) the same surface patterned silicon sample was treated with the oligonucleotide b1, the mis-match sequence under hybridisation conditions, (a sample where steps i and iii are exchanged was also tested). In all cases, fluorescent patterns were obtained if the complementary oligonucleotide sequence (b) was used; the normalised fluorescent signal to noise ratios were comparable to those obtained for the freshly prepared oligonucleotide-patterned silicon samples. Thus, hybridisation with the patterned DNA oligonucleotide surfaces is specific and is stable.

The length of the alkane linking chain between the oligonucleotide and the silicon surface was estimated to be ~12 Å. It was therefore considered that there may be some surface effects which could influence the hybridisation of the complementary sequence. In order to investigate this, oligonucleotide BT was attached to the silicon surface in patterns; the oligonucleotide BT consists of sequence B and fifteen thymine bases to which the 3'-end is attached to the silicon surface. The hybridisation tests, described above, were carried out and the SNR

obtained, 3.20 $\pm$ 0.21 (sequence BT-b), was similar to that obtained previously for oligonucleotide B (without the spacer group) (2.99 $\pm$ 0.45 (sequence B-b)), suggesting that there was little detectable difference on the attachment distance of the surface attached oligonucleotides toward specific hybridisation, under the conditions used here.

The density of fluorescein-labelled oligonucleotides that hybridised to the surface was determined using a modified version of a previously reported method (see experiment section) [25]. The surface density of complementary strands hybridised onto oligonucleotide B patterned surface was found to be 6.7×10^{12} oligonucleotides per cm^2 (one oligonucleotide per 15 nm^2) which is slightly higher than that obtained previously for covalently attached DNA oligonucleotides on glass (2.3×10^{12} oligonucleotides per cm^2) [26]. Thus the method presented here provides excellent surface coverage of the attached DNA oligonucleotides that are accessible towards hybridisation with complementary sequences.

If oligonucleotide patterns are reduced toward the nanometre scale for the creation of biosensors, highly effective hybridisation of the immobilised oligonucleotide with complementary sequences is vital. A silicon wafer with the non-fluorescent oligonucleotide B, attached onto the silicon surface with a submicron-scale periodic pattern, (fabricated as described above) was prepared. Fluorescently labelled oligonucleotides b were hybridised with the patterned oligonucleotide surface and then evaluated with a laser confocal microscope. A fluorescence pattern of 500 nm lines separated by 500 nm spaces was observed over the whole region (Figure 6). The results demonstrated that the immobilised oligonucleotides, in patterns of submicron dimensions, were accessible towards specific hybridisation.

CONCLUSIONS

DNA oligonucleotides have been successfully attached onto bulk silicon semiconductor with motifs with submicron feature sizes. This has been achieved by a two-step process by i) photo-exposure using UV light in interference patterns onto

films of UANHS on hydrogen-terminated silicon surfaces followed by ii) treatment of the surface motifs with amine-functionalised DNA oligonucleotides. The immobilised oligonucleotide patterns on the silicon surfaces show excellent specific hybridisation with the complementary sequence in high densities.

The method is straight-forward method and should offer much potential for the creation of self-assembled nano-bioelectronic devices and for the miniaturisation of emerging bio-analytical tools, such as integrated silicon BioMEMS devices.

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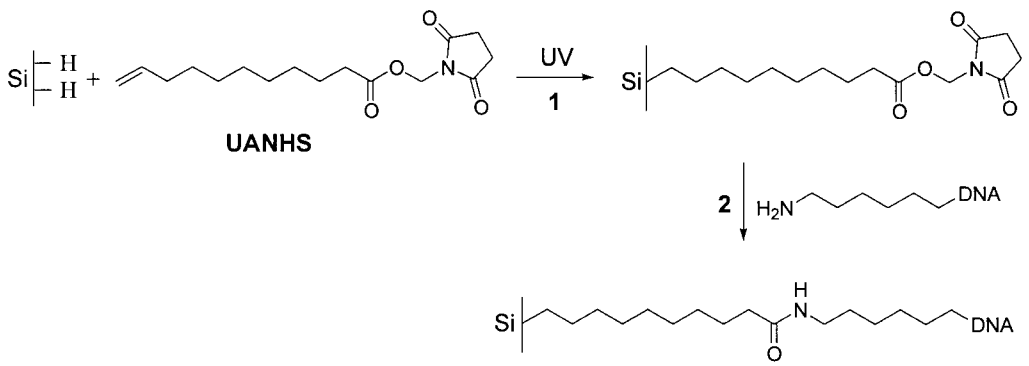

Figure 1 Schematic diagram for the covalent attachment of oligonucleotides to a silicon surface. A layer of the N-hydroxysuccinimide ester of undecylenic acid (UANHS) was covalently attached to a hydride-silicon surface by UV irradiation to generate the succinimide ester functionalised surface (step 1). A 3'aminolinking-DNA oligonucleotide was then added to displace the succinimide ester group to yield covalently attached oligonucleotides (step 2).

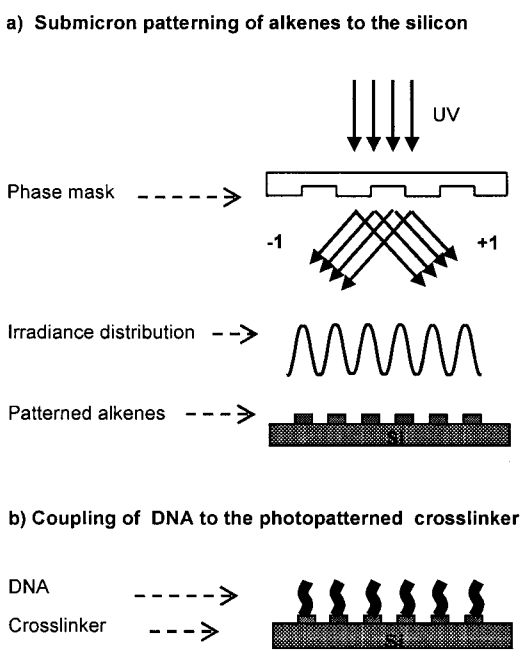

Figure 2 Schematic diagrams showing the method used for submicron patterning of the oligonucleotides on silicon surfaces. a) A +1/-1 phase mask was placed above a silicon wafer coated with the UANHS. Interference between the light diffracted from the phase mask yields a grating pattern with a 1 μm period; photoreaction of the silicon surface with the UANHS yields the pattern of surface attached alkene. b) Coupling of DNA to the photo-attached functionalised alkenes on the surface.

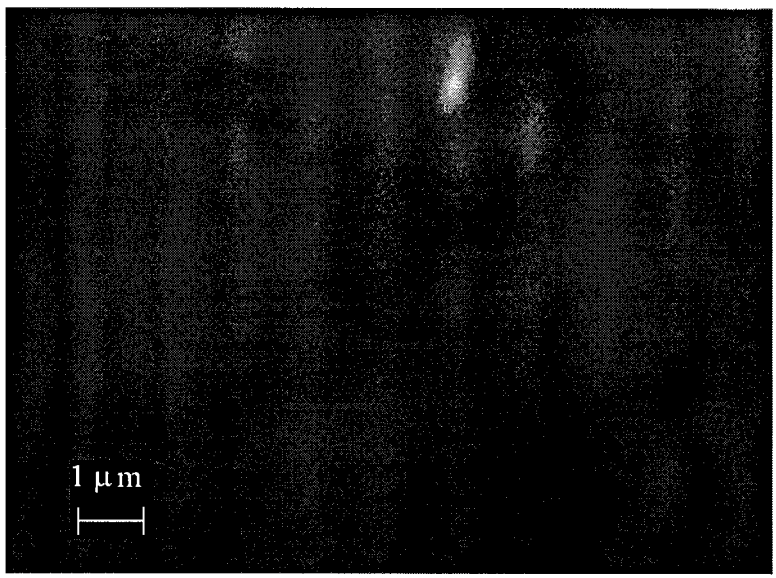

Figure 3 SEM image of a submicron patterned monolayer of UANHS on silicon. The patterns of UANHS attached to silicon can be observed as bright lines of 500nm. The periodicity of the pattern is 1 μm.

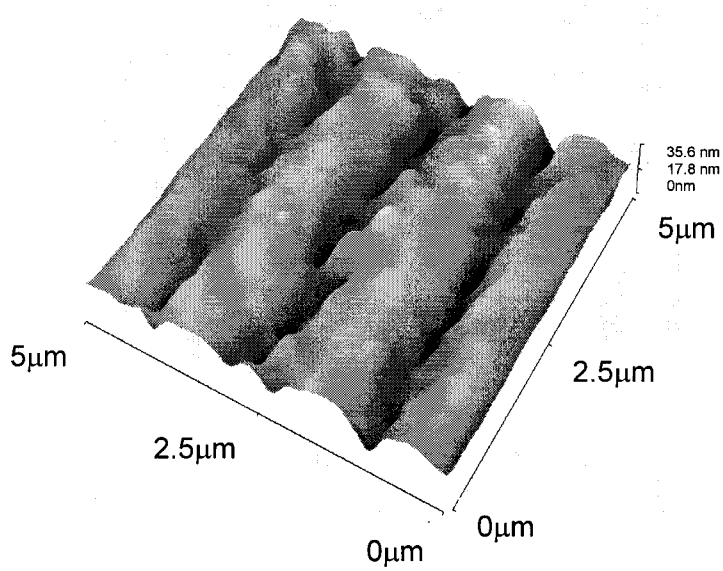

Figure 4 AFM image of oligonucleotides labelled with fluorescein dye on silicon surfaces in sub-micron patterns. The oligonucleotides (bright spots) were mainly located on the uniform parallel lines with a period of ~1.5 μm.

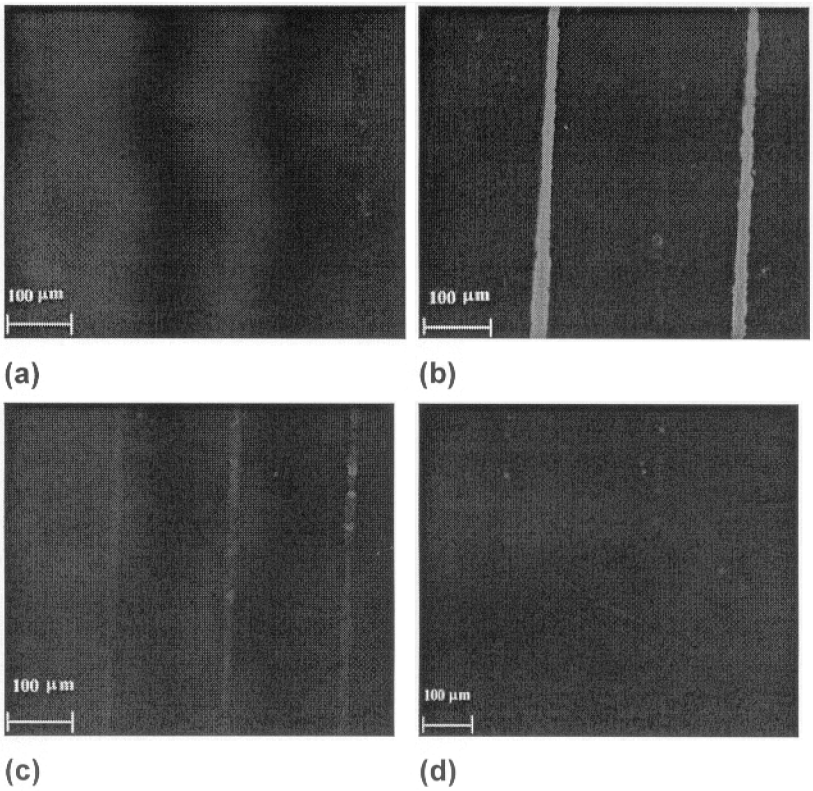

Figure 5 Epifluorescence images of the oligonucleotide B patterned surfaces before hybridisation and after hybridisation with fluorescein-labelled oligonucleotides. All the images were taken with identical settings (100x magnification). (I) before hybridisation (II) hybridised with its complementary oligonucleotide **b**. (III) hybridised with a one-mismatch oligonucleotide **b1**; (IV) hybridised with the non-complementary oligonucleotide **c**. A strong fluorescent signal was only observed on the samples hybridised with the complementary oligonucleotide **b**. The scale bar represents 100 μm.

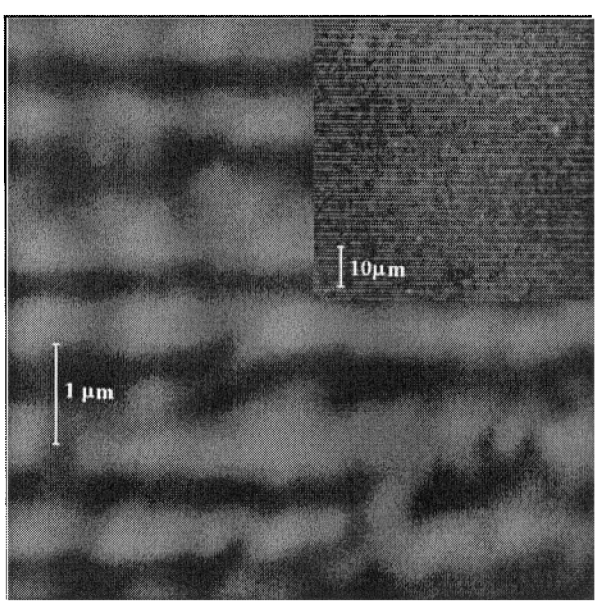

Figure 6 Confocal epifluorescence image of the sample with submicron patterned oligonucleotides hybridisation with the complementary sequence with a fluorescein label. The scale bar represents 1 μm and the scale bar in the inserted image is 10 μm.