SURFACE CHARACTERIZATION OF OLIGONUCLEOTIDES IMMOBILIZED ON POLYMER SURFACES

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ABSTRACT

The immobilization and hybridization of amino-terminated oligonucleotide strands to cyclo-olefin-copolymer (COC) and polycarbonate (PC) surfaces have been investigated for potential application in micro-PCR devices. The oligonucleotides were covalently bound to the plasma-treated COC and PC surfaces via an N-hydroxy-sulfo succinimide (NHSS) intermediate. Analysis by AFM showed that the oligonucleotides were present on the surfaces as lumps, and that the size, both vertically and laterally, of these lumps on the COC surface was larger compared to the PC surface. The immobilization efficiency of the former was also higher (15.8 x 10^{12} molecules / cm^2) compared to the latter (3.3 x 10^{12} molecules / cm^2). The higher efficiency of the COC surface is attributed to the more effective NHSS-functionalization and its higher surface roughness. Subsequent hybridization doubled the height of the lumps, while the lateral dimensions remained essentially unchanged. This is explained in terms of organization of the long probe strands used on the surface as flexible, coil-like polymer chains, which allow the complementary oligonucleotides to bind and increase the height of the lumps. The AFM frictional images showed that the hybridization had the effect of reversing hydrophilicity of the oligonucleotide lumps from being more hydrophilic to more hydrophobic, consistent with the hydrophilic bases of the probe strands being shielded as a result of hybridization.

Keywords: oligonucleotide, immobilization, polycarbonate, cyclo-olefin copolymer, AFM

1. INTRODUCTION

The immobilization of DNA/oligonucleotide on solid supports is important in the design of biosensors to detect microbial pathogens and genetic defects and to identify DNA in many other applications. There are various immobilization methods including: (a) cross-linking to poly-L-lysine-coated microscope slide 1; (b) photochemical immobilization, via photoreactive species, to polymer surfaces 2; (c) attachment of thiol-terminated oligonucleotides on thiol-functionalized surfaces 3-6; (d) attachment to a amino-functionalized surfaces 7; (e) attachment of biotin-labeled oligonucleotides to a streptavidin-functionalised surface 8. The underlying principle of these methods is the covalent binding between DNA/oligonucleotide and a solid support, which allows hybridization with complementary target sequences.

One of the most widespread applications of DNA immobilization technologies is the polymerase chain reaction (PCR). PCR amplification is the most powerful technique, by which a DNA fragment is amplified exponentially for applications such as DNA fingerprinting, genomic cloning and genotyping for disease diagnosis 9. The development
of miniaturised PCR system has received significant interest in recent years, particularly after Northup and co-workers successfully amplified DNA in microfabricated reaction chambers \(^{10,11}\). The microfabricated PCR devices have the advantages of small sample volume, rapid thermal cycle and low cost. As the PCR process involves temperatures up to 95 °C, the solid support for DNA immobilization must have suitable thermo-mechanical properties. Other key parameters for the solid support include density of the binding sites, intrinsic fluorescence, processability and surface roughness. While the macroscopic devices for PCR are mainly polystyrene thin-walled tubes, many microscopic devices to date have been made out of silicon or silicon/Pyrx \(^{12-15}\).

We report here an investigation of immobilization and hybridization of oligonucleotide strands to cyclo-olefin-copolymer (COC) and polycarbonate (PC) surfaces for potential application in micro-PCR devices. Both of these polymers have glass transition temperature Tg in excess of 150°C. While PC is a common polymeric material, COC is a relatively new class of polymers, based on ethylene and norbornene, which has important properties like moisture barrier characteristics, excellent thermal properties and bio-compatibility \(^{16-18}\). In this study, amino-terminated oligonucleotides were covalently bound to the COC and PC surfaces, which were pre-treated in oxygen plasma, via an N-hydroxy-sulfosuccinimide (NHSS) intermediate. Relevant chemistry for covalent binding of amino groups with carboxylic acid groups using NHSS intermediate was described elsewhere \(^{19}\). Atomic force microscopy (AFM) is used as a major tool to monitor the changes in surface characteristics following each experimental stage.

2. METHODOLOGY

2.1 Polymeric surface preparation The treatment of COC and PC samples in oxygen plasma and subsequently with NHSS was carried out at the Microfluidics Laboratory, Motorola (Phoenix, Arizona, USA). The plasma treatment was carried out at a power of 100 W for various periods ranging from 20 sec up to 5 min. Method for NHSS-functionalization of the plasma-treated polymeric surfaces was similar to that described elsewhere \(^{19,20}\).

2.2 Immobilization of oligonucleotides. A 26 base-pair oligonucleotide primer 5’ GTG GAT CAC CTG AGG TCA GGA GTT TC 3’ corresponding to the \(alu\) gene \(^{21}\) was used for covalent attachment to functionalised polymeric surfaces. The primer was amino-modified at 5’-terminus as purchased from GeneWorks. For oligonucleotide immobilization on the polymeric surfaces, approximately 10 \(\mu\)l of the oligonucleotide of a final concentration of 20 nmole/ml suspended in 150mM sodium phosphate, pH 8.5 was placed on the functionalised surface. The solution was incubated in a humid chamber for approximately 6 hours at room temperature (ca. 22 °C). The samples were then washed 3 times with a solution consisting of 0.4 M NaOH and 0.25 % SDS, soaked for 5 min in the washing solution, before being washed again 3 times.

2.3 Hybridization of Oligonucleotides. Prehybridization solution, containing 0.15 M NaCl, 0.015 M sodium citrate, 5x Denhardt’s solution, pH 7.0, was applied to the sample for at least 10 min. A5’-labeled complementary strand Cy5-CA CCT GGT GGA CTC CAG TCC TCA AAGG was dissolved in 5x SSC buffer (750 mM NaCl, 75 mM sodium citrate, pH 7.0) to make 2.2 ng of DNA in 20 \(\mu\)l, which was applied to the sample containing the immobilized primers. After incubation at 42 °C for 4 hours the slides were washed three times with 6x SSC buffer (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1% SDS.

2.4 Surface Characterization. AFM characterisation was carried out on a TopoMetrix Explorer (ThermoMicroscopes) in both the non-contact and normal contact modes. Several scanners were used and the field-of-view ranged from 100x100 down to 2x2 \(\mu\)m². Pyramidal-tipped, silicon nitride cantilevers with a spring constant of 0.032 N/m were used in the contact mode, whereas silicon cantilevers with a spring constant of 42 N/m and resonant frequency of 320 KHz were used in the non-contact mode. The analyses were carried out under air-ambient conditions (temperature of 23°C and 45% relative humidity). More than 25 AFM analyses were carried out on different locations for each sample, and the results presented here are average and/or typical of these analyses. As the tip is scanned across the surface in the contact mode, the frictional force acting on the tip manifests itself through a torsional deformation of the lever, which is sensed by the difference signal on the Left-Right signal on the quadrant detector. It is important to note that both friction and topography contribute to the lateral forces. The contrast due to
friction is inversed upon reversal of scan directions from forward to reverse, whereas the topographical contrast is independent of the scan direction. The contrast inversive in the lateral force image has been used as an important device for deciding whether the contrast is caused by topography or friction. In principle, the topographical contribution to the lateral force image may be removed by subtracting images recorded in the forward and reverse directions; and the difference is the frictional force acting between the tip and the sample.

In the present study, the lateral force imaging was performed simultaneously with topographical imaging in both forward and reverse scan directions. In the interest of brevity and conciseness, only subtracted lateral force images of features of interest which have contrast inversive upon reversal of scan directions are presented. Image subtraction and root-mean-square (RMS) surface roughness calculation were carried out using relevant Explorer softwares.

Elemental analyses of plasma-treated polymeric surfaces were carried out on a Kratos Ultra Imaging X-Ray Photoelectron Spectrometer (XPS), using monochromatised Al Kα (photon energy = 1486.6 eV) radiation at a source power of 150 W. The analysis areas were nominally ~ 700x300µm². Wide scan and region scan spectra were acquired using 160eV and 20eV pass energies, respectively. Electron binding energies were calibrated against the C1s emission at 284.6 eV.

3. RESULTS AND DISCUSSION

3.1 Analysis of plasma-treated and NHSS-functionalized polymeric surfaces. AFM imaging (Figure 1) shows that both COC and PC samples had relatively smooth textured surfaces. The plasma treatment, which is an essential step in the NHSS-functionalization process, did not change the surface morphology significantly until after treatment for 60 sec or more. In particular, these surfaces exhibited “moon-like” features, possibly due to the arching in the plasma reactor, a common problem for dielectric materials processed in plasma. The NHSS-functionalization step appeared to smooth out the PC surface, whereas it did not change the surface roughness of the plasma-treated COC samples significantly. The RMS morphological roughness of the various COC and PC samples are shown in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Control COC</th>
<th>Plasma-treated COC</th>
<th>NHSS-COC</th>
<th>Control PC</th>
<th>Plasma-treated PC</th>
<th>NHSS-PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology (nm)</td>
<td>5.96</td>
<td>6.35</td>
<td>8.55</td>
<td>8.62</td>
<td>3.66</td>
<td>4.17</td>
</tr>
<tr>
<td>Friction (nA)</td>
<td>0.85</td>
<td>1.20</td>
<td>4.04</td>
<td>3.49</td>
<td>1.29</td>
<td>1.50</td>
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When imaging under ambient conditions, the capillary condensation between the tip and sample surfaces reflects the relative degree of hydrophilicity and can be used as a basis for discriminating between hydrophobic and hydrophilic groups. It has been shown that when imaging in air, the frictional force is higher as the tip is scanned across a more hydrophilic region. The image contrast in a frictional force map is a measure of tip-to-surface friction, which effectively reflects the surface chemical heterogeneity. Frictional force images of COC and PC samples before and after the plasma treatment and NHSS-functionalization are shown in Figure 2 and the RMS frictional roughness are tabulated in Table 1. For both COC and PC surfaces, the plasma treatment increased the extent of surface chemical heterogeneity, with the COC surface becoming much more heterogeneous compared to the PC surface, as indicated by the respective increase in RMS frictional roughness. While the subsequent NHSS-functionalization step appeared to smooth out the chemical contrast of the PC surface, it did not reduce the chemical heterogeneity of the plasma-treated COC surface significantly.
While the AFM data showed that the surface morphology and chemical heterogeneity did not change substantially until after treatment in plasma for 60 sec or more, XPS results indicated that the concentration of surface oxygen increased significantly after only very short plasma treatment (20 sec). Increasing the duration of plasma treatment did not result in further increase in surface oxygen level. This is illustrated in Table 2, which shows the dependence of XPS O/C ratio on plasma treatment duration for both COC and PC samples. It is noted that the increase in surface oxygen for COC samples was higher than that for PC samples.

The data obtained suggested that a short plasma treatment was sufficient to achieve the surface chemical modification required for the NHSS-functionalization. Prolonged plasma treatment had the effect of sputtering surface materials, thereby increasing the surface roughness and chemical heterogeneity.

Figure 1: AFM topographical images of COC (left column) and PC (right column) samples: unmodified (a, d); 60 sec plasma treatment (b, e); functionalized with NHSS (c, f). Image size is 25 x 25 µm.
Table 2: The dependence of XPS O/C ratio on duration of plasma treatment for COC and PC samples.

<table>
<thead>
<tr>
<th>Plasma-treated Sample</th>
<th>Control</th>
<th>20 sec</th>
<th>40 sec</th>
<th>60 sec</th>
<th>2 min</th>
<th>5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>COC</td>
<td>PC</td>
<td>COC</td>
<td>PC</td>
<td>COC</td>
<td>PC</td>
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<tr>
<td>O/C</td>
<td>0.14</td>
<td>0.14</td>
<td>0.26</td>
<td>0.21</td>
<td>0.24</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Figure 2: AFM frictional force images of COC (left column) and PC (right column) samples: unmodified (a, d); 60 sec plasma treatment (b, e); functionalized with NHSS (c, f). Image size is 25 x 25 µm. These lateral force images correspond to the respective topographical images in Figure 1.
3.2 Immobilization and hybridization of oligonucleotides. Both the non-contact and contact AFM imaging showed that the covalently bound oligonucleotides were present as “lumps” on the NHSS-functionalized polymer surfaces. The similar surface morphology obtained by the two imaging modes suggests that the oligonucleotides were strongly bound to the polymer surfaces. The frictional force data indicated that the oligonucleotide lumps had higher frictional force, hence were more hydrophilic, than the surrounding region. Figures 4 and 5 show typical AFM topographical and corresponding frictional images of oligonucleotides immobilized on the COC and PC surfaces, respectively.

Analysis of the images showed that the oligonucleotide lumps on the PC surface had size in the range 0.2-0.8 µm and height of about 30 nm, with a surface coverage of 16.94 % and an estimated density of 3.3 x 10^{12} molecules / cm². Compared to the PC surface, the oligonucleotide lumps on the COC surface had larger size ranging from 0.8 to 2.0 µm and height of about 60 nm, with a much larger surface coverage of 41.25 % and a density estimated to be 15.8 x 10^{12} molecules / cm². Oligonucleotide coverage is influenced by a number of factors including the amount of surface NHSS group and the surface roughness. XPS data in this study indicated that the increase in surface oxygen following the plasma treatment is higher for the COC samples, suggesting that the NHSS-functionalization, hence oligonucleotide binding, is more effective. The higher surface roughness of the NHSS-functionalized COC surface may also increase the binding rate of the oligonucleotides. The binding kinetics of analyte in solution to receptor immobilized on a surface has been shown to increase with surface heterogeneity and roughness 28. In addition, surface density and surface diffusion coefficient of biomolecules including DNA oligonucleotides 29 and proteins such as BSA 30 and prothrombin fragment 1 31 have been reported to have an inversely proportional relationship. It is possible that the higher roughness of the COC

![Image](a) ![Image](b)

**Figure 4:** AFM topographical (a) and corresponding frictional (b) images of oligonucleotides immobilized on the COC surfaces. Image size is 20 x 20 µm.

![Image](a) ![Image](b)

**Figure 5:** AFM topographical (a) and corresponding frictional (b) images of oligonucleotides immobilized on the PC surfaces. Image size is 20 x 20 µm.
surface may decrease the surface diffusion of the oligonucleotides and enhance the adsorption / binding events. The increased number of immobilized molecules will act as obstacles to diffusing molecules, thereby increasing the interaction between oligonucleotides. This is consistent with the larger size, both laterally and vertically, of the oligonucleotide lumps on the COC surface compared with those on the PC surface. The interaction between oligonucleotides is also expected to increase with the length of the strands. The rough surfaces and the long strands (26 bases) used in the present study are likely to be major factors responsible for the larger size of the oligonucleotide lumps compared to those reported in literature, for example, reference 6, where shorter oligonucleotides (16 bases) were immobilized on flat mica surfaces.

The COC surface with immobilized oligonucleotides was selected for hybridization experiment. Figure 6 shows typical AFM topographical and corresponding frictional images of hybridized oligonucleotides on the COC surface. The average height of the oligonucleotide lumps effectively doubled following the hybridization, while the lateral dimensions of the lumps remained essentially the same. This is in contrast to previous study 6 using shorter oligonucleotides which showed an increase in lateral dimensions of oligonucleotide clusters as a result of hybridization. Reason for the difference may be related to the way the probe strands organized on the surface. Length of oligonucleotide strands has been suggested to be a key factor influencing their organization on surfaces with shorter strands (<24 bases) tend to align normal to the surface in rod-like configuration (Model I), whereas longer strands (>24 bases) behave as flexible, coil-like polymer chains (Model II) 32.

It may be that in the present case where long strands were used, the probe strands organized on the surface according to Model II. AFM frictional data (Figure 4) indicated that the outer surfaces of the oligonucleotide lumps were more hydrophilic than the surrounding region, suggesting that the hydrophilic bases of the probe strands were exposed for hybridization. The complementary oligonucleotides could then bind to the probe strands at the “top” positions (extending into the aqueous phase), rather than the “side” positions (extending along the solid surface), thus increasing the vertical dimension of the probe strands. In contrast, shorter strands, like those in reference 6, were perhaps immobilized on the surface according to Model I. The effect of hybridization would be to increase the lateral dimensions, rather than vertical, of the oligonucleotide clusters. Further insight into the orientation of the oligonucleotides can be obtained from AFM frictional data after hybridization. From being more hydrophilic, the surfaces of the oligonucleotide lumps became more hydrophobic following hybridization (Figure 6). These results suggest that the interactions of the base pairs between the probe and complementary strands effectively shielded the bases, and exposing more hydrophobic regions.
4. CONCLUSIONS

We have investigated the immobilization and hybridization of 26-base amino-terminated oligonucleotides on COC and PC surfaces, which were functionalized with NHSS groups following treatments in oxygen plasma. The results showed that a short plasma treatment (20 sec) was sufficient to achieve the surface oxygen required for the NHSS-functionalization. The increase in surface oxygen on the COC surface was higher compared to that on the PC surface, suggesting more effective NHSS-functionalization for the former. Analysis by AFM showed that the COC surface had higher coverage of the oligonucleotide lumps and that the size, both vertically and laterally, of these lumps on the COC surface was larger compared to those on the PC surface. While the more effective NHSS-functionalization of the COC surface is a likely reason for the difference in oligonucleotide immobilization, we suggested that the surface roughness of the substrate also had a critical role. In particular, the higher roughness of the COC surface may decrease the surface diffusion of the oligonucleotides, and increase the adsorption / binding events. The increased number of immobilized oligonucleotides will also act as obstacles to diffusing molecules. This would facilitate more interactions between oligonucleotides, resulting in larger oligonucleotide lumps.

Hybridization of the immobilized oligonucleotides effectively doubled the height of the lumps, while the lateral dimensions remained essentially unchanged. We suggested that because the probe oligonucleotide strands used in the present study were long, they would organize on the surface as flexible, coil-like polymer chains. The complementary oligonucleotides could then bind to the probe strands in such a way that facilitated an increase in the vertical dimension of the lumps. The AFM frictional images showed that the oligonucleotide lumps were more hydrophilic than the surrounding surface, consistent with the hydrophilic bases of the probe strands being exposed to facilitate hybridization. Following hybridization, the lumps became more hydrophobic, suggesting that the interactions between the base pairs of the probe and complementary strands effectively shielded the bases, and exposing more hydrophobic regions.

More work is necessary to further the understanding of the way the oligonucleotides organize on the COC and PC surfaces. However, the relatively high immobilization efficiency (3-15 x 10^{12} molecules / cm²), coupled with the stable thermal-mechanical properties, make these polymers, and the associated immobilization strategy via NHSS functionalization, promising candidates for DNA-DNA hybridization assays and PCR applications.

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REFERENCES


