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Ultrathin and smooth poly(methyl methacrylate) (PMMA) films for label-free biomolecule detection with total internal reflection ellipsometry (TIRE)

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ABSTRACT

Ultrathin poly(methyl methacrylate) PMMA films were prepared on gold substrates by spin coating PMMA dissolved in toluene. By varying the concentration of PMMA, spin coating speed and curing condition, we obtained very smooth and ultrathin PMMA films. The PMMA films were transformed into highly reactive film containing carboxylic functionalities using UV/O_3 irradiation. These films were shown to remain stable and reactive for at least one week. We then demonstrated the application of the UV/O_3 treated PMMA films for the detection of microRNAs using a label-free detection method called total internal reflection ellipsometry (TIRE). A limit of detection of 10 pM was established. The technique proposed here is a simple and quick method for generating carboxylic functional films for label-free bioanalytical detection techniques.

1. Introduction

Poly(methyl methacrylate) (PMMA) is an attractive material for the fabrication of low-cost micro-total analysis systems (μ-TAS) since it possesses excellent optical, thermal, chemical and biocompatible properties (Brown et al., 2006; Castaño-Álvarez et al., 2005; Chen et al., 2003; Galloway et al., 2002; Klank et al., 2002; Koesdjojo et al., 2008; Lee et al., 2001; Muck et al., 2004; Yao et al., 2005). PMMA can be used to fabricate microchips or microchannels either by injection molding or hot embossing/imprinting (Brown et al., 2006; Galloway et al., 2002; Lee et al., 2001) or laser ablation (Cheng et al., 2004; Klank et al., 2002; Yao et al., 2005). In addition, through appropriate treatment of its surface, PMMA can be functionalised to enable the covalent attachment of biomolecules for bioassay development (Choi et al., 2010; Fixe et al., 2004; Kimura, 2006; Liu and Rauch, 2003; Nugen et al,. 2009; Situma et al., 2007; Situma et al., 2005; Soper et al., 2005; Welle and Gottwald, 2002). The ability to integrate both structure and chemical functionality on a single PMMA monolithic device reduces its complexity resulting in substantial savings during device fabrication (Nugen et al., 2009; Situma et al., 2005; Soper et al., 2005; Wang et al., 2003).

Pristine PMMA is a relatively inert and hydrophobic material. Consequently, without surface treatment, bio-recognition elements such as oligonucleotides and antibodies can only be non-covalently adsorbed on the methyl ester surface, resulting in poor device performance. Thus, surface treatment of PMMA is necessary in order to generate reactive surfaces for the attachment of biomolecules. Reactive functional groups are typically introduced onto the surface of PMMA either by chemical or photophysical modification routes. A popular procedure for the chemical functionalization of PMMA involves amination in *N*-lithioethylenediamine solution followed by addition of a homo bifunctional cross-linker molecule such as glutaraldehyde to enable attachment of aminated biomolecules and then capping of the unreacted aldehyde functional groups with a reducing agent (Fixe et al., 2004; Wang et al., 2003). Alternatively, NaOH can be used to hydrolyze the methyl ester moieties of native PMMA to produce carboxylic acids (Choi et al., 2010). The photophysical modification of PMMA by UV/O_3 treatment is also a very

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attractive method for providing carboxylic functionality which has been extensively used in fluorescence-based biomolecular detection techniques (Liu and Rauch, 2003; Situma et al., 2007; Situma et al., 2005; Soper et al., 2005; Tsao et al., 2007; Welle and Gottwald, 2002). Once the carboxylic groups have been generated on the PMMA surface, other chemical groups such as thiols or amines can be further introduced (Ligaj et al., 2006; Wei et al., 2005). However, UV/O_3 oxidation is an aggressive surface activation method since ozone is directly photolyzed with UV light, producing molecular oxygen and an oxygen atom which can result in high background fluorescence of the polymer substrates. Consequently, UV/O_3 treatment is unsuitable for the generation of substrates for fluorescence-based detection applications (Diaz-Quijada et al., 2007; Gubala et al., 2010).

MicroRNAs (miRNAs) are small non-coding (~ 22nt in length) RNAs. miRNAs have been shown to play a role in many critical biological processes and as a result have been extensively studied for their role in disease. miRNAs have been found circulating in the blood stream, in urine and saliva in a stable form (Dobbins et al., 2008; Heneghan et al., 2010; Weber et al., 2010), opening up the possibility of their use as minimally invasive biomarkers. Their short length and the high level of sequence homology between closely related miRNAs make them difficult to distinguish. In this work, we demonstrate the use of modified PMMA thin films for the label-free detection of two closely related miRNAs using a surface plasmon resonance (SPR)-based method called total internal reflection ellipsometry (TIRE) (Fig. 1). To this end, PMMA was spin coated onto a Au-coated glass slide. By carefully controlling the concentration of PMMA, the spin speed and the curing condition, we generated ultrathin and smooth PMMA films which could be readily converted into a reactive carboxylic film by UV/O_3 treatment, enabling oligonucleotide probe immobilization. We have successfully detected the target miRNAs with the lowest detection concentration at 10 pM.

The technique demonstrated here is label-free, thus eliminating the problems associated with using fluorescence based methods when UV/O_3 treatment is used. In addition, the surface chemistry approach presented here offers a simple way of generating reactive surfaces for other label-free optical detection methods such as surface plasmon resonance (SPR), dual-polarization interferometry (DPI), quartz crystal microbalance (QCM) or microcantilever-based biosensors by spin coating PMMA onto sensor surfaces.

2. Materials and methods

2.1. Materials

Poly(methyl methacrylate) (PMMA) sheets (0.25 mm thick, impact modified, MW = 120,000) were supplied by Goodfellow Cambridge Limited (Huntingdon, England). Gold-coated standard glass slides (Ti/Au = 2 nm/30 nm, 26 mm × 76 mm, 1 mm thick) were purchased from PhasisSarl (Geneva, Switzerland). N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and toluene were purchased from Sigma Aldrich (Arklow, Ireland). All chemicals were used as received without further purification. 5′ amino modified oligonucleotide DNA probes (5′-*CGC-CAA-TAT-TTA-CGT-GCT-GCT-A*-3′) (*miR*-16 *probe*, 22-mer) and synthetic oligonucleotide target RNAs (5′-*U-AGC-AGC-ACG-UAA-AUA-UUG-GCG*-3′) (*miR*-16 *target*, 22-mer) and (5′-*UAG-CAG-CAC-AGA-AAU-AUU-GGC*-3′) (*miR*-195 *target*, 21-mer) were purchased from Eurofins MWG Operon (Ebersberg, Germany).

2.2. Preparation of PMMA thin films

A PMMA sheet was cut into small pieces and dissolved in toluene at concentrations ranging from 0.1 to 0.5% w/v to make the raw PMMA solution. The solution was sonicated for 15 min to completely dissolve the PMMA pieces. The PMMA solution was then filtered through a PTFE filter (pore size 0.2 μm) (Chroma-filXtra PTFE-20/25 Macherey-Nagel, Duren, Germany) to eliminate precipitates and dust particles. The PMMA solution was then spin-coated onto the clean Au-coated glass slide at three spin speeds (1000, 2000 and 3000 rpm) for 45 s. The acceleration and deceleration to and from the desired spin speed took place in 5 s.

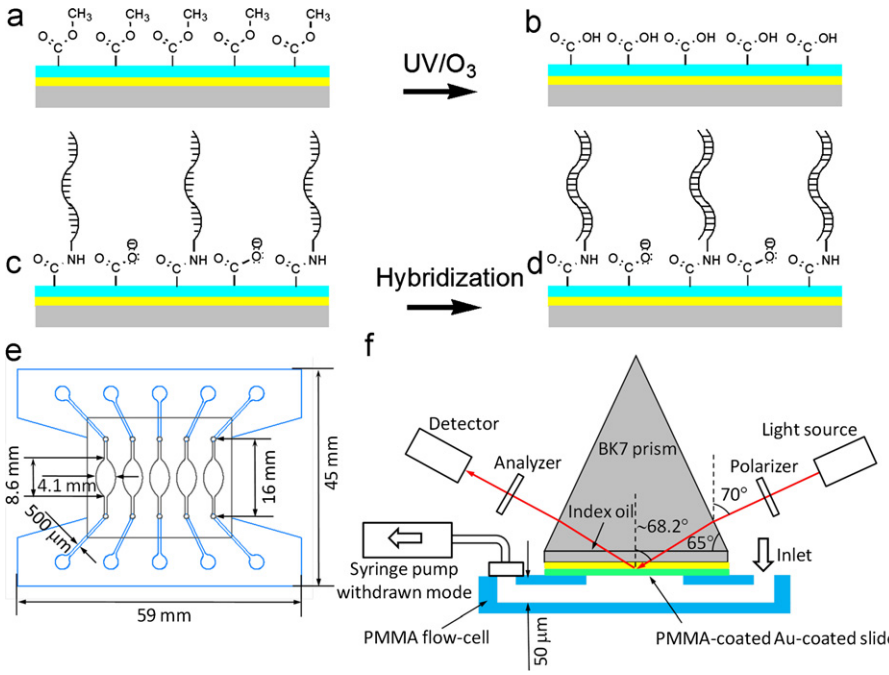

Fig. 1. Steps in preparation and experiment with PMMA thin films (a) PMMA spin-coating onto Au-coated glass slides; (b) PMMA thin films undergone UV/O_3 treatment (50% of 50 W power at 254 nm peak and 5% of 50 W power at 185 nm peak, treatment time 8 min); (c) DNA probe immobilization with N-(3-dimethylaminopropyl)-N′-ethylcarbondiimide hydrochloride (EDC) activation; (d) oligonucleotide hybridization assays to detect microRNAs (e) PMMA flow-cell fabricated by CO_2 laser machining; (f) total internal reflection ellipsometry (TIRE) setup based on UVISEL spectroscopic ellipsometry (Horiba Jobin Yvon).

Initially, a fast curing method was employed in which the PMMA spin-coated Au-coated glass slide was cured in ambient air for 2 h followed by 1h at 140 °C in an oven (Walsh and Franses 1999, 2003). However, we found that this method resulted in very rough PMMA surfaces due to the rapid evaporation of toluene leaving a wrinkled PMMA film (Fig. 1S, supplementary information). We modified this method by first curing at room temperature overnight and then in an oven for 2 h at 80 °C. This method resulted in very smooth and uniform PMMA films on Au-coated glass slides.

2.3. Modification of PMMA surface by UV/O_3 treatment

UV/O_3 treatment was performed using a commercial ozone cleaning and activation system (PSD-UV, Novascan Technologies, Ames, IA, USA). According to the manufacturer specifications, at the 50 W power setting, approximately 50% of the total lamp output power is delivered around the 254 nm peak and 5% around the 185 nm peak. The optical power was kept constant but the treatment time was varied. We found that an 8 min UV/O_3 treatment was optimal for thin PMMA films spin-coated onto the Au surfaces. When the treatment time is too short the carboxylic functionality might not be generated sufficiently while if the treatment time is too long, the thin PMMA film will be etched away (data not shown).

2.4. Water contact angle

The wettability of the different surfaces was analyzed by measuring the water contact angle (WCA) of the surfaces using the First Ten Angstroms FTA200 (Portsmouth, VA, USA) contact angle analyzer. High purity HPLC grade water (Sigma Aldrich, Arklow, Ireland) was used for the measurement. The water contact angle of each surface was measured three times at three different locations.

2.5. Atomic force microscopy

Surface morphology and roughness of Au coated, PMMA coated, UV/O_3 treated PMMA was measured with a Digital Instruments (DI) BioScope™ II (Veeco Instruments Inc., Plainview, NY, USA) atomic force microscope (AFM) in tapping mode in air. Silicon cantilevers with integrated tips (TESP, Veeco Probes, Camarillo, CA, USA) and with resonant frequencies between 327 and 349 kHz, and with $\approx$30 N/m spring constant were used for measurements. For each surface, three locations with a surface area of $2 \times 2\ \mu m^2$ each were imaged at a rate of 0.5 Hz and at a resolution of 512×512. Research NanoScope 7.30 software (Veeco Instruments Inc., Plainview, NY, USA) was used to analyze the data and estimate the RMS roughness.

2.6. Spectroscopic ellipsometry and total internal reflection ellipsometry

The thicknesses of the PMMA films and the subsequently treated PMMA films deposited on the Au substrate were measured by an UVISEL spectroscopic ellipsometer (JobinYvon Horiba, France). Measurements were performed in external mode at angle of incidence of 70° with wavelengths ranging from 350 to 800 nm with a resolution of 5 nm. A three-layer model (BK7 glass, Au and organic layer) was used in the fitting with PsiDelta 2 software (JobinYvon Horiba, France) to obtain the thickness of the Au and PMMA films from the measured Ψ and Δ spectra. The Ψ and Δ values are defined as the ratio ρ of the reflection coefficients R_p and R_s for components of light polarized parallel-*p* and perpendicular-*s* to the plane of incidence, respectively, following the ellipsometry equation (Azzam and Bashara, 1987). The refractive indices of BK7, Au and organic layer (i.e. PMMA) are detailed elsewhere (Le et al., 2010).

The total internal reflection ellipsometry (TIRE) experimental setup was also based on the same UVISEL spectroscopic ellipsometer (JobinYvon Horiba, France) (Fig. 1(e–f)) (Le et al., 2010; Le et al., 2011). The PMMA modified Au-coated sensing substrate was first glued to a PMMA flow-cell. A BK7 prism was placed on top of the sensing substrate with a layer of refractive index matching oil and secured by Scotch® tape. A syringe pump (Harvard Apparatus, Boston, USA) was connected to the outlet of one microwell of the flow-cell through silicone tubing and a PDMS connector for conducting one assay at a time. The flow rate from the pump was controlled to 1–2 μl/min. Two modes of measurements were carried out with TIRE: spectral and kinetic measurement, which also recorded Ψ and Δ values (Nabok et al., 2007; Nabok et al., 2009; Poksinski and Arwin, 2004). In the spectral mode, the angle of incidence was approximately 68.3° and the wavelength was scanned over the range between 450 nm and 1100 nm. The integration time was 100 ms and the spectral resolution was 2 nm. After a set of Ψ and Δ spectra were recorded, the kinetic mode was switched on at the same incident angle and at a fixed wavelength, i.e. around 2–3 nm shift from the surface plasmon resonance (SPR) wavelength obtained from the first Ψ spectrum, to detect maximal change in either Ψ or Δ during the introduction of the analyte. The integration time and interval in kinetic mode were set at 100 ms and 100 ms, respectively. Even though the kinetic mode could not provide quantitative information about the thickness of the bound biomolecule layer, it helps to monitor the reactions in the microwells.

The miRNA hybridization assay was conducted in a fresh microwell after the baseline Ψ and Δ spectra of the UV/O_3 treated PMMA surface in 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 6.5) buffer were recorded. 80 μl of aminated *miR-16probe* at a concentration of 1 μM in 100 mM EDC in MES buffer (pH 6.5) was then pumped into the microwell and allowed to react for 60 min with a continuous flow rate of 1.3 μl/min. A second set of Ψ and Δ spectra, which corresponded to the immobilization of *miR-16 probe*, was measured after the microwell was rinsed with 80 μl of phosphate buffered saline (PBS, pH 7.0). Next, 80 μl of complementary *miR*-16 *target* at concentrations ranging from 1 pM to 1 μM in hybridization buffer (150 mM NaCl, 150 mM saline-sodium citrate (SSC) buffer, pH 7.0) was pumped into the microwell and also allowed to react for 60 min before rinsing with another 80 μl of PBS buffer. A third set of Ψ and Δ spectra was then recorded corresponding to the hybridization of the complementary *miR*-16 *target* to the capture *miR*-16 *probe*. A fresh microwell was used every time a new assay was started. Two control experiments were conducted. The first control experiment was performed to assess the ability of the UV/O_3 treated PMMA surface to bind the aminated *miR*-16 *probe* in the absence of EDC. The second control experiment investigated the specificity of the capture *miR*-16 *probe* by using *miR*-195 *target* at a concentration of 1 μM in hybridization buffer.

PsiDelta 2 software (JobinYvon Horiba, France) was used for fitting the data from the measured Ψ and Δ spectra from TIRE. A four-layer model similar to the model used in (Le et al. 2010; Le et al. 2011; Nabok et al. 2007; Nabok et al. 2009; Poksinski and Arwin 2004) was used in the fitting program to estimate the thickness of oligonucleotide probe and target layers bound onto the modified PMMA surfaces, subsequently.

3. Results and discussion

3.1. PMMA thin films preparation

The curing of PMMA films prepared on Au substrates requires careful consideration to obtain the level of smoothness and uniformity required for bioanalytical sensing using

TIRE. The curing process consisted of two steps: (1) the as-prepared PMMA films were first cured overnight in ambient air in a closed chamber to prevent contamination, and (2) the PMMA films were then cured in an oven at 80 °C for 2 h. With this curing process, very thin, smooth and uniform PMMA surfaces were achieved. The film thickness was measured by ellipsometry and the film roughness was measured by AFM. The affect of varying the spin speeds and concentrations of PMMA is summarized in Table S1, supplementary information. From this table, it can be seen that by varying spin speed and (or) PMMA concentration, film thickness and roughness can be controlled. Low speed and high concentration resulted in thick films with high roughness while high speed and low concentration resulted in thin films with low roughness. These data are in good agreement with results reported previously on Si substrates (Walsh and Franses 1999, 2003). Films prepared at 0.25% w/v PMMA and 2000 rpm were chosen for all further experiments. This PMMA film was then subjected to UV/O_3 treatment for 8 min to introduce carboxylic functionality. After this treatment, the apparent thickness was reduced by approximately 3 nm (Fig. 2(a)), as measured by ellipsometric fitting, whilst the water contact angle was substantially reduced to about 25° (Fig. 2(b)). The AFM results from bare Au, PMMA coated Au and UV/O_3 treated PMMA films are summarized in Fig. 2(c–e) confirming that smooth and uniform films were obtained. The oxidized PMMA coated Au slides were stored under vacuum until used.

3.2. Aging study of UV/O_3 treated PMMA surface

In order to assess how long the oxidized film could be kept whilst retaining its carboxylate reactivity, an aging study of the UV/O_3 treated surfaces stored in a vacuum desiccator was performed. The results are summarized in Fig. 3. Changes in water contact angle and the thicknesses of the immobilized oligonucleotide layer after the reaction with constant concentration of 1 μM of *miR*-16 *probe* over a period of 8 day were measured sequentially on five pairs of samples prepared on the same day. We found that while the contact angles increased and saturated after 4 day, the signal corresponding to the change in the refractive index and hence to the amount of *miR*-16 *probe* remained constant over the time period investigated. It is speculated that the carboxylic groups are still abundant for the DNA probe to bind to even though the water contact angle has increased to around 40° which is still much lower than that of pristine PMMA film. This relative stability of the oxidized PMMA treated by UV/O_3 is in contrast to a very short lifetime of plasma oxidized cyclo-olefin polymer (COP) substrates, which have been used for capturing biomolecules (Gubala et al. 2010).

3.3. Oligonucleotide hybridization assays for detection of microRNAs

Prior to the oligo hybridization experiment, two control reactions were performed. Ψ and Δ spectra of the two control experiments

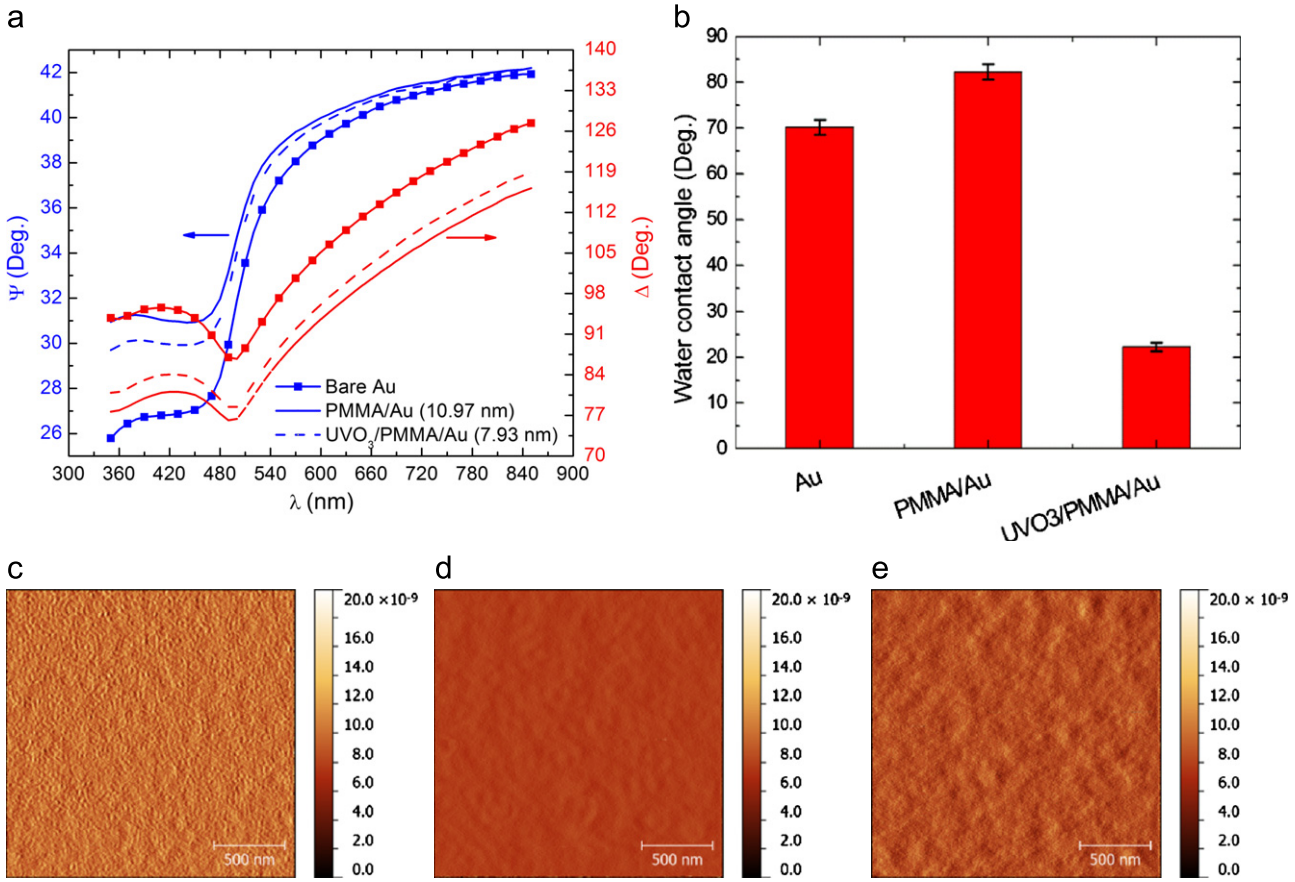

Fig. 2. (a) Spectroscopic ellipsometry measurements on bare Au substrate, PMMA coated Au substrate and UV/O_3 treated PMMA film, the thicknesses were obtained from ellipsometric fitting. Here, the thicknesses are the total thicknesses measured from the gold surface (b) water contact angle change from bare Au substrate to PMMA coating and then UV/O_3 treatment; tapping mode AFM images measured in air of surfaces of (c) bare Au, RMS roughness = 0.39 ± 0.01 nm, (d) PMMA coated Au substrate, RMS roughness = 0.22 ± 0.07 nm (e) UV/O_3 treated PMMA, RMS roughness = 0.32 ± 0.03 nm.

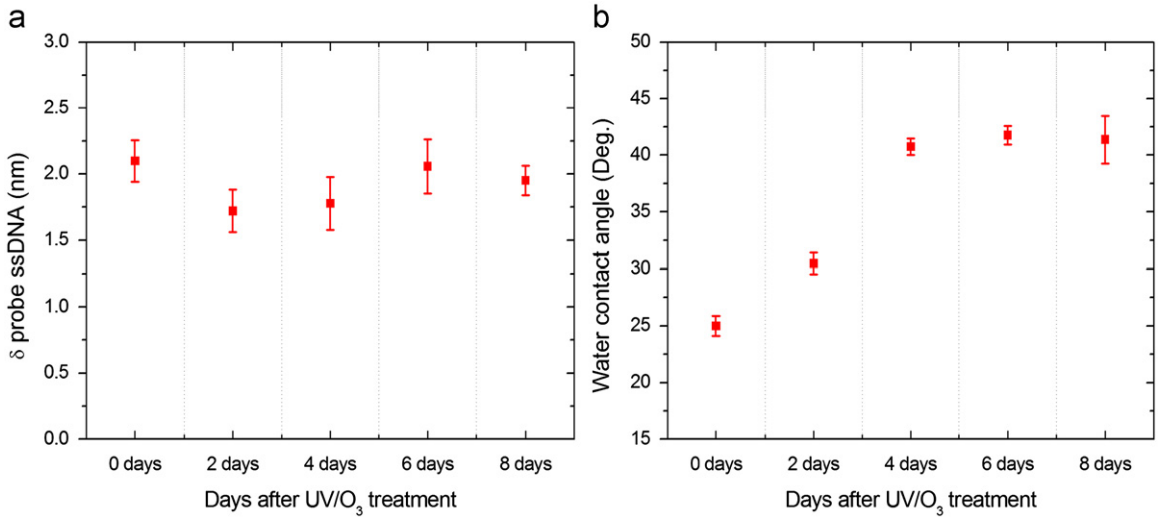

Fig. 3. (a) Aging study on binding of 1 μM of *miR*-16 *probe* for 0, 2, 4, 6 and 8 day, (b) water contact angle change after UV/O_3 treatment for 0, 2, 4, 6 and 8 day. Five sets of samples were used in these measurements. On each sample, measurements on three locations were obtained.

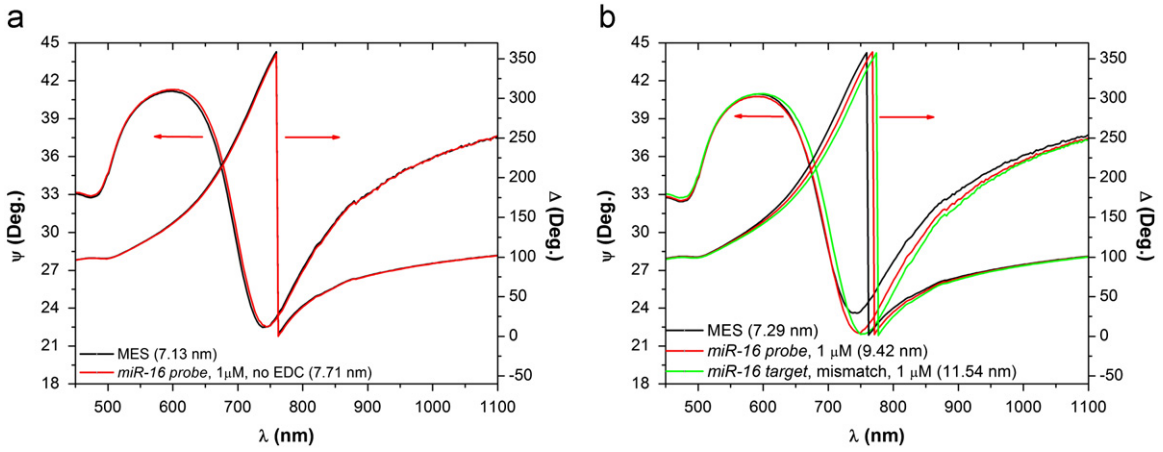

Fig. 4. Two control experiments with DNA on UV/O_3 treated PMMA films (a) When *miR*-16 *probe* (22-mer) at 1 μM was introduced to the UV/O_3 treated PMMA surface without EDC activation, the increase in thickness was minimal due to non-specific binding (b) When *miR*-195 *target* (non-complementary) at 1 μM was incubated with the capture *miR*-16 *probe* at 1 μM concentration, there was some level of non-specific hybridization, resulting in an increase in thickness of about 2.1 nm. In these figures, the thicknesses indicated are the total thicknesses measured from the gold surface.

are shown in Fig. 4(a) and (b). In the first control experiment, an NH_2-modified *miR*-16 *probe* (22-mer) was allowed to react with the COOH groups of the UV/O_3 treated PMMA film in the absence of EDC. As expected, and illustrated in Fig. 4(a), there is only a very small shift in Ψ and Δ spectra corresponding to a very small amount of physically adsorbed *miR*-16 *probe* after 60 min. In the second control experiment, the same amino-modified *miR*-16 *probe* (22-mer) was covalently immobilized to the COOH surface using EDC and then incubated with the non-complementary *miR*-195 *target* (21-mer). The length of the immobilized *miR*-16 *probe* (22-mer) is close to 7 nm, but the observed thickness increase was only 2.13 nm, suggesting that the immobilized probe was lying 'flat' on the surface. As seen in Fig. 4(b), the introduction of the non-complementary *miR*-195 *target* solution resulted in small shift in the Ψ and Δ spectra, corresponding to a further 2.1 nm increase in thickness. This additional increase in thickness was attributed to non-specific hybridization of the target to the immobilized probe. Given that *miR*-195 and *miR*-16 *targets* differ by only three bases (Fig. S2, Supplementary information), this result is not surprising. This non-specific hybridization could potentially be controlled by optimizing hybridization conditions such as surface blocking and temperature and salt concentration of the buffer and by probes design. To summarize both control experiments, physical adsorption of the probe leads to a small, 0.58 nm increase in thickness of the deposited material. Covalent attachment of the probe resulted in 2.13 nm thickness increase, which was further extended by 2.1 nm upon reaction with the non-complementary target.

For the hybridization experiment involving a perfectly complementary probe and target, we covalently immobilized the capture *miR*-16 *probe* (22-mer) on the surface and then allowed it to hybridize to its complementary *miR*-16 *target* (22-mer). While the thickness increase of 1.83 nm after the first capture reaction was consistent with the results in the second control experiment, the specific hybridization reaction and formation of a RNA–DNA hybrid helix resulted in large shifts in both Ψ and Δ spectra (Fig. 5(a)). The kinetic measurement mode (Fig. S3, supplementary information) clearly shows the change in Ψ signal when *miR*-16 *probe* and *miR*-16 *target* were sequentially introduced to the UV/O_3 treated PMMA surface. Fitting both Ψ and Δ spectra revealed a large increase in thickness corresponding to 6.5 nm for the hybrid helix. We speculate that initially the purine and pyrimidine bases of the single-stranded probe have a

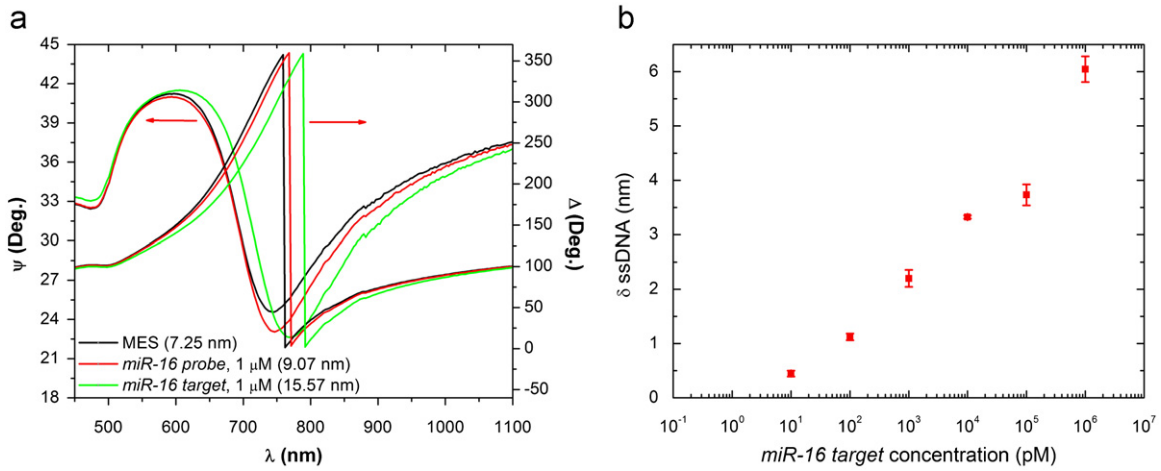

Fig. 5. (a) Complementary probe:target hybridization assay on UV/O_3 treated PMMA films, *miR-16 target* (complementary) at 1 μM was incubated with capture *miR*-16 *probe* at 1 μM concentration, there was a significant increase in thickness of about 6.5 nm due to the possibility that the hybrid duplexes were stretched out during the hybridization process to its physical length. In this figure, the thicknesses are the total thicknesses measured from the gold surface. (b) A standard curve plotting the thickness changes when increasing the concentration of *miR*-16 *target*. The *miR*-16 *probe* concentration was fixed at 1 μM. The concentration of *miR*-16 *target* was shown on logarithmic scale, while the error bars represent the standard deviations of three measurements at three locations in the same reaction microwell. A lowest detectable signal was obtained at target concentration of 10 pM.

tendency to maximize some energetically favorable, non-covalent interactions with the oxidized PMMA surface and hence the probe lies 'flat' on the surface. However, the formation of a hybrid helix renders the supramolecule significantly negatively charged with exposed phosphate groups on the duplex backbone. Therefore, the dominant factor for the hybrid helix orientation is electrostatic repulsions between the negative charge of the double strand and the surface $-COO^-$ groups. The small difference in the thickness of the captured probe for experiments shown in Fig. 4(b) and Fig. 5(a) could be explained by effects related to aging and some degree of non-uniformity of the COOH groups. In order to assess the sensitivity of the TIRE technique, we decreased the concentration of the *miR*-16 *target* to 1 pM. When the immobilized probe concentration was kept constant at 1 μM, 10 pM of target miRNA was detected (Fig. 5(b)). However, the 1 pM concentration of *miR*-16 *target* did not result in any detectable shift in the Ψ and Δ spectra. The typical, complete Ψ and Δ spectra at different target concentrations is summarized in Fig. S4 (supplementary information). Previously, TIRE has been used to study the adsorption and the interaction of DNA molecules on polyethylenimine (PEI) films deposited on Au surfaces (Nabok et al. 2007; Nabok et al. 2009). However, in these two studies the DNA probes were physically adsorbed onto the PEI surfaces and the DNA molecules were very long. Here, we have successfully demonstrated the specific detection of miRNAs on UV/O_3 treated PMMA surfaces using TIRE. These results are in good agreement with previous work where other surface chemistries have been used to probe DNA hybridization (Lao et al. 2009; Pollet et al. 2009).

4. Conclusions

A method to prepare carboxylic functional film on a Au sensing substrate for use in a label free TIRE detection technique is reported. By tuning the condition of spin coating of PMMA and the curing condition, ultrathin, smooth and uniform PMMA films were achieved. UV/O_3 treatment was then used to generate carboxylic functionalities readily for binding of biomolecules. We have successfully demonstrated the detection of miRNAs on UV/O_3 treated PMMA surfaces and confirmed a 10 pM limit of detection for the miRNA, *miR*-16 *target*. Furthermore, the UV/O_3 treated surface show excellent stability of reactivity for a period of up to 8 day. Our results could be useful when a quick method to prepare carboxylic functionality on spin coated PMMA surfaces is required. This technique is particularly well suited to other label-free SPR-based optical detection techniques by spin coating PMMA onto Au substrates. Alternatively, it could also be used in other label-free sensing technique such as DPI, QCM or micro-cantilever-based systems by spin coating PMMA on to quartz or silicon surface, respectively.

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Appendix A. supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2012.04.032.

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