

A self-contained fluorescent fiber optic DNA biosensor

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Single-stranded DNA (ssDNA) sequences can be used as probes to detect complementary targets, and represent useful analytical reagents for the detection and identification of bacteria, viruses and mutations. The hybridization process between probe sequences immobilized at a surface and complementary nucleic acid targets in a sample solution can, under optimal conditions, be complete in several minutes with a high degree of selectivity. Fluorescent dyes such as thiazole orange (TO) have been used extensively to quantify DNA by measuring the differential spectroscopic properties of free dye and the dye that associates with double-stranded DNA by intercalation. In an effort to develop a reagentless biosensor, TO has been covalently tethered by various poly(ether) strands at the 5' end of ssDNA probes, in a detection system where the oligonucleotide probes are immobilized onto the surfaces of fused silica optical fibers. Characterization of the surface immobilization has been completed using X-ray photoelectron spectroscopy. The biosensors provided changes in steady-state fluorescence intensity signals upon hybridization, that reached saturation in seconds to minutes, and were able to provide a quantitative determination of hybridization at nanomolar detection limits. Aspects such as ionic strength, length of the tether that was used to attach TO to ssDNA, and the packing density of the probe molecules were examined to determine the influence of these parameters on the thermodynamic and kinetic performance of the biosensor. In a preliminary investigation of this application, the biosensor was used to detect PCR products from *Erwinia herbicola*.

1. Introduction

Although DNA has only 4 bases, the conserved physical structure of oligonucleotides provides for a high level of selectivity from the process of hybridization. The selectivity of DNA for a target analyte can be controlled more easily than that of other biological recognition elements.¹ The relative simplicity and selectivity of analysis of nucleotide sequences provides for a wide range of applications such as detection of diseases and pathogens, genome sequencing, forensic DNA profiling and ecological control.^{2–4} With the completion of the human genome sequencing project,⁵ the focus has changed from the collection and archiving of genomic data, to analysis and use in prediction and discovery. Nucleic acids can be used as selective biomolecular reagents. Single-stranded nucleic acid oligomers (ssDNA), can be isolated or synthesized and then used as probes for target identification.⁶ Depending on the desired recognition events, nucleic acid probes can range from chemically synthesized oligonucleotides of between 12–50 bases to cloned polynucleotides consisting of hundreds or even thousands of bases.⁷ Techniques that are based on the principle of hybridization of labelled target nucleic acids with surface-immobilized complementary probe molecules as found in applications of microarrays^{8,9} and DNA biosensors have become popular tools in molecular biology. However, there has been only limited effort towards the development of fluorescence strategies associated with signals that are directly derived from the probe molecules immobilized onto surfaces,

where the surfaces are ideal for optical excitation and signal collection.

A DNA biosensor is a device that incorporates a deliberate combination of a biologically active element (that provides for a recognition event) and a physical element (that transduces the recognition event into a measurable analytical signal).¹⁰ Immobilized single-stranded oligonucleotide probes are placed on a transducer surface to recognize the complementary sequence by hybridization. Electrochemical,^{11,12} surface acoustic wave^{13,14} and optical^{15–17} methods represent the most commonly used transducer platforms. We have been interested in developing DNA biosensors based on detection of fluorescence from DNA duplexes in an evanescent field using a total internal reflection fluorescence configuration. Such detection using an evanescent field restricts excitation to materials that are in close proximity of the waveguide.¹⁸ Optical fibers can serve as platforms for the immobilization of ssDNA. Fused silica is particularly attractive because of the relatively low cost, a relatively flat and homogeneous surface, and versatility in terms of chemical modifications.¹⁹ Optical fibers are coated with short ssDNA probes that are attached to the surface using hydrophilic linkers, such as hexaethylenglycol (HEG), and hybridization with the complementary target sequences in a sample solution can be monitored by detection of changes of fluorescence intensity using structurally sensitive dyes, such as the intercalating dye thiazole orange (TO). The linker provides mobility to the covalently anchored oligonucleotides.²⁰ Such DNA biosensors have been used to detect short oligonucleotides, PCR products and genomic materials cut by ultrasound, and provide detection

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systems that can be indicative of specific diseases, pathogens, or events that are related to the natural function of DNA such as transcription.^{21–23}

The detection of double-stranded DNA (dsDNA) formation can be done by use of free intercalating dye in solution, but this necessitates use of extra reagents in the analytical protocol and extra steps in the methodology. A different approach is to anchor the dye near the ssDNA so that it is readily available for intercalation upon hybridization. This provides potential for improvement of the speed of response and for reversibility of the biosensor. There are several procedures for attaching fluorescent dyes to the 5' end of the oligonucleotides. Such a location for the fluorophore causes little or no destabilization of the hybridized oligonucleotide compared to the unlabelled form,²⁴ while leaving the 3' end free for immobilization to the surface. A number of reagents with a protected amine, thiol, or carboxyl groups have been developed for coupling reactions at the 5'-end of an oligonucleotide during solid phase synthesis.²⁵ Our previous work indicated that immobilized oligonucleotides that were modified with TO exhibited significantly enhanced fluorescence intensity upon hybridization.²⁶ The change of fluorescence intensity from the thiazole orange was attributed to the restriction of rotation around the methine bond between the two heterocyclic systems in the molecule.^{27,28} A homogeneous hybridization assay based on the interaction between TO and dsDNA can provide a significant fluorescence signal enhancement.²⁹

Herein, we describe the optimization of a self-contained DNA biosensor using TO that is tethered to oligonucleotides. Firstly, silane chemistry was used to build a monolayer terminated with epoxy groups on fused silica optical fibers.³⁰ Then, a hexaethyleneglycol linker terminated with dimethoxytrityl (DMT) group was attached, and the system was ready for solid-phase oligonucleotide synthesis. The availability of DMT groups was used to template the density of ssDNA probes. Thiazole orange derivatives were then attached to the 5'-terminus of the immobilized ssDNA using glycol-based tethers of various lengths. The hydrophilic poly(ether) chains were expected to offer adequate flexibility to facilitate intercalation between a TO derivative and dsDNA.³¹ Parameters such as ionic strength, tether length and ssDNA packing density were investigated to determine effects on speed of hybridization and melt temperature of dsDNA. As a first step in application to practical determinations using this biosensor, the system was used to detect PCR products from *Erwinia herbicola* (a non-pathogenic model target).

2. Experimental

2.1 Materials

All solvents were distilled prior to use. Anhydrous solvents were distilled under argon as follows: THF from sodium; pyridine from CaH₂; CH₃CN from CaH₂; toluene/xylenes from sodium. Chemicals were from Sigma-Aldrich (Mississauga ON). Reagents grade salts were from VWR (Toronto, ON). DNA synthesis reagents were from Dalton Chemical Laboratories Inc. (Toronto, ON) and Glen Research Co. (Sterling, VA). Molecular biology grade poly(acrylamide)

gel electrophoresis reagents and apparatus were from Bio-Rad (Hercules, CA). Fused silica optical fibers of 400 μm core diameter (3 M Powercore Series Optical Fiber, FT-400-URT or FP-400-UHT) were from Thor Labs Inc., Newton, NJ. Fused silica fiber segments and controlled pore glass (CPG) (CPG Inc., Lincoln Park, NJ) were used as solid substrates for automated DNA synthesis. Fused silica wafers (5 mm² × 1 mm thick, polished both sides to a 20/10 scr/dig, flat to 1/5 wave) were from Valley Design Corp. (Santa Cruz, CA). Fast deprotecting phosphoramidites Pac-dA (5'-dimethoxytrityl-*N*-phenoxyacetyl-2'-deoxyadenosine) and Ac-dC (5'-dimethoxytrityl-*N*-acetyl-2'-deoxycytidine) (Glen Research, VA) were used in automated DNA synthesis of mixed-base sequences immobilized onto silica substrates.

2.2 Instruments

The XPS analysis was done using a Leybold Max-200 spectrometer (Leybold-Heraeus, Cologne, Germany) at the Surface Interface Ontario (SIO) centre at the University of Toronto. The X-ray source was unmonochromated Mg Kα using a sample size of 1 × 1 cm². All the binding energy peaks were calibrated relative to the C (1S) peak at 285.0 eV. The excitation voltage was 1253.6 eV, using 12.5 kV and an emission current of 20 mA. A pass energy of 192 eV was used for low resolution survey. X-ray angularly-dependent XPS spectra were recorded at take-off angles of 90, 45, 30, and 20°. The processing of raw XPS data enabled the determination of elemental compositions of C (1S), Si (2P), and N (1S) by scanning each corresponding binding energy peak area in the low resolution spectra. The software used to process the raw XPS data was SpecsLab v. 1.8.2 (© 1993–1998) Specs GmbH.

Fluorescence studies of nucleic acid hybridization at the surface of optical fibers were done using an automated spectrofluorimeter instrument that has been described previously.³² A CW argon ion laser operated at 488 nm provided excitation radiation. A dichroic mirror (505 nm cut-off, Omega Optical, Battleboro, VT) oriented at 45° to the incident beam reflected the 488 nm radiation. The fiber was placed in a stainless steel hybridization cell with a Teflon[®] cap and a waterproof compression-type seal. The flow cell allowed a 26 ± 3 μL volume of solution to pass over the sensing fiber. Fluorescence emission wavelengths greater than 505 nm from the fiber sensor were collimated and guided back through the dichroic mirror. Fluorescence emission intensities were then detected by a cooled photomultiplier tube. A glass encapsulated bead thermistor (Fenwal Electronics Inc., Toronto, ON) was used to determine the temperature of the solution of the flow cell to an accuracy within 0.1 °C.

2.3 Procedures

Preparation of optical fiber segments. The polymeric outer cladding of the fused silica fibers was removed mechanically using a fiber-stripping tool and fibers were scored and cleaved.^{22,32} The fused silica fiber segments, CPG and fused silica wafers that were used as solid substrates for automated DNA synthesis were cleaned prior to modification of the surface by the two-stage method of Ken and Puotinen.³³

Functionalization of substrates with 3-glycidoxypropyl-trimethoxysilane (GOPS). The cleaned fused silica substrates were suspended in a solution of xylene-3-glycidoxypropyl-trimethoxysilane (GOPS)-diisopropyl ethylamine (100 : 30 : 1 v/v/v). The mixture was stirred at 80 °C for 24 h under Ar. The substrates were then washed with methanol (2 × 50 mL), CH₃Cl (2 × 50 mL), Et₂O (2 × 50 mL) successively, dried and stored under vacuum and P₂O₅ at room temperature (rt) until required.

Linkage of DMT-HEG onto GOPs functionalized substrates. To 500 mg (20 mmol) of NaH suspended in 10 mL anhydrous pyridine was added 1 g (1.7 mmol) of DMT-HEG dissolved in 10 mL anhydrous pyridine. The mixture was stirred at rt for 2 h under Ar. The reaction mixture was filtered through a sintered glass frit under a positive pressure of Ar into a round bottom flask containing the GOPs functionalized substrates. GOPs functionalized substrates were separated into three batches and then underwent the DMT-HEG coupling reaction. The mixtures were gently shaken on an oscillating platform stirrer under Ar for 1, 4 and 12 h, respectively. Then the substrates were washed with methanol (3 × 20 mL), water (3 × 20 mL), methanol (3 × 20 mL), CH₂Cl₂ (3 × 20 mL), Et₂O (3 × 20 mL), respectively. The DMT-protected HEG functionalized substrates were dried under vacuum and over P₂O₅ until further required.

Capping of unreacted silanol and hydroxyl functionalities with chlorotrimethyl-silane (TMSCl). The dried substrates were suspended in a solution of TMS-pyridine (1 : 10, v/v) for 16 h at rt under Ar. The substrates were subsequently recovered and washed with pyridine (3 × 20 mL), methanol (3 × 20 mL), Et₂O (3 × 20 mL), respectively. The substrates were stored under vacuum and over P₂O₅ at rt until further required.

Synthesis of tethered probes on optical fibers and fused silica wafers. The synthesis of tethered probes on modified optic fiber and fused silica wafer was done using the PE-ABI 392-EP DNA synthesizer as previously described.³² The pre-programmed cycles for the DNA synthesis were modified to adjust the reagent delivery times in order to ensure that the synthesis columns used were completely filled and the reactions were complete. The column used for oligonucleotide synthesis onto optical fiber segments was a custom manufactured Teflon[®] synthesis column (6 mm i.d. × 50 mm) capable of holding 8 fibers without contacting each other. The column used for oligonucleotide synthesis onto fused silica wafers was a custom manufactured Teflon[®] synthesis column (8 mm i.d. × 10 mm) capable of holding 4 wafers.


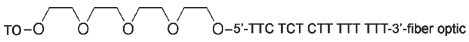
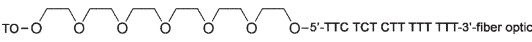
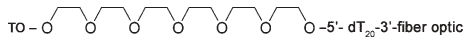
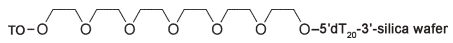
The following step was used for the final coupling to attach TO to the 5' end of the probes molecules (TO-ssDNA) using various poly(ethyleneglycol) linkers. After the synthesis attached the final nucleotide, equal volumes of (2-cyanoethoxy)-bis(diisopropylamino)phosphine (1 M, 1.0 mmol) and tetrazole (0.5 M, 0.5 mmol) in CH₃CN were added to the support by a pre-programmed synthesis cycle. Then, the support was removed from the synthesizer and a mixture of tetrazole (0.5 M in CH₃CN, 1.0 mmol) and TO derivatives³⁴ (0.5 mmol) was added to the support by syringe over a

period of 18 h under Ar. After washing the support with CH₃CN, the oxidant (20 mL, 0.1 M I₂ in a solution of 1 : 10 : 40 H₂O-pyridine-THF) was added to the support over a time of 20 min to finish the oxidation step. The conjugate was then washed with CH₃CN and CH₂Cl₂, treated with 0.05 M K₂CO₃ in anhydrous methanol for 48 h under N₂ in order to deprotect primary amine groups on the nitrogenous bases. The use of the weak base K₂CO₃ minimized the cleavage of immobilized DNA from the substrate surface in the process. The sequences of tethered oligonucleotides immobilized on various surfaces are summarized in Table 1.

Synthesis was done on GOPS-functionalized controlled-pore glass (CPG) with a well-defined surface area, in tandem with the oligonucleotide synthesis on the optical fiber surfaces. The conjugates were cleaved from the CPG by means of exposure to concentrated NH₄OH for 3 h. The wash solution was lyophilized and redissolved in water. An estimate of the efficiency of the final coupling step could be determined by comparing the ratio of the oligonucleotide (failed coupling product) to the conjugated oligonucleotide, as could be quantified by a reverse phase anion exchange HPLC isolation procedure.²² Separation was done on a Series 400 solvent delivery system equipped with a LC-95 UV-vis spectrophotometer detector tuned to 260 nm (Perkin-Elmer, Norwalk, CT, USA). An anion-exchange Gen-Pak Fax column was used (Waters, Milford, MA, USA). The mobile phase was delivered to the column at a flow rate of 0.5 ml min⁻¹. Two main solvent systems were used: Buffer A consisted of 25 mM Tris-HCl (Sigma, St. Louis, MO, USA) and 1 mM EDTA (disodium salt, ACP, MontréAl, PQ, Canada) in 10% aqueous acetonitrile. The pH was adjusted to 8.0 using a 0.5 M sodium hydroxide solution (Sigma). Buffer B had the same composition as buffer A except that it additionally contained 1 M sodium chloride. After keeping the ratio of buffer B at 10% for the first 5 min of the separation, a linear gradient of buffer B over 30 min was used.

Calibration for retention times, signal magnitude and sample loss were established using standard additions of

Table 1 Structures of immobilized oligonucleotides

1b ^a	
2b ^a	
3a-c ^a	
4b ^a	
5 ^b	

^a Compounds a: fiber was reacted with DMT-HEG for 1 h; compounds b: fiber was reacted with DMT-HEG for 4 h; compounds c: fiber was reacted with DMT-HEG for 12 h. ^b Silica wafer was reacted with DMT-HEG for 4 h.

oligonucleotides of known length and concentration. It was assumed that oligonucleotides would covalently attach to the surfaces as unimolecular layers, and the average area of occupancy and centre-to-centre distances were determined by quantitative analysis of the number of oligonucleotide molecules recovered in HPLC experiments from samples of known surface area. All experiments at any one set of reaction conditions were done at least 3 times, and the cumulative results were used to establish an estimate of precision of approximately 15% for the determination of density of immobilized oligonucleotide.

Sample preparation for XPS experiments. Blank silica wafers, GOPS functionalized silica wafers, and TO-dT₂₀ immobilized silica wafers were sonicated for 90 min in ethanol to remove any loosely adsorbed material from the surfaces, followed by rinsing with 3 × 20 mL H₂O and 3 × 20 mL MeOH. The wafers with immobilized oligonucleotide films were incubated with 0.1 μM dA₂₀ (cDNA) in 1 × PBS buffer at rt for 30 min, then heated to 80 °C for 10 min, followed by cooling to rt for re-annealing. Wafers were then rinsed with 3 × 20 mL H₂O at 80 and 25 °C, respectively. This procedure was repeated 3 times to remove any adsorbed contaminants. All samples were stored under vacuum.

Hybridization assays of immobilized nucleic acids. Fluorescence measurements were done in 1 × TE buffer, pH 7.2 or 1 × PBS buffer, pH 7.0 at duplex or triplex concentration of approximately 1 μM. DNA concentration at this range has an absorption of less than 0.05 at 480 nm, making inner filter effects negligible.³⁵

Steady state fluorescence studies at the surfaces of the optical fibers were investigated by using the automated spectrofluorimeter. All sensors were sonicated in ethanol for 30 min (Bransonic[®], 40 W, Branson Ultrasonics Co., Danbury, CT) to remove any adsorbed contaminations from the surface. Stock phosphate buffered saline (PBS) hybridization buffers (1.0/0.5/0.1 M NaCl, 50 mM PO₄ⁿ⁻, pH 7.0) were used to dilute target DNA and as running buffer to wash the sensor before and after each cycle. Sample delivery time was 25 s and the total volume passing the reaction chamber in the stopped flow experiment was 150 ± 20 μL. The hybridization time was 5 min unless specified, after which the sensor was washed with buffer at a flow rate of 1 mL min⁻¹ for 2 min to remove any adsorbed materials from the interface. Chemical denaturation was achieved by delivering 1 mL 90% formamide in TE buffer (10 mM Tris HCl, 5 mM EDTA, pH 8.3) followed by 1 mL of 95% ethanol and 20 mL H₂O. Assay temperature was kept at 30 °C unless specified.

Thermal denaturation studies using optical fibers began with target sequence delivery to the reaction chamber in 25 s. The hybridization time was 5 min unless specified, after which the sensor was washed with buffer at a flow rate of 1 mL min⁻¹ for 2 min to remove any adsorbed materials from the interface. Thermal denaturations were studied by monitoring the intensity of fluorescence emission at 555 nm over the temperature range of ca. 25–75 °C using a temperature ramp rate of 0.3 °C min⁻¹ to ensure that equilibrium conditions were satisfied. All analyses were done in triplicate for each

experimental condition. Removal of complementary oligonucleotide between experiments was achieved by flushing 1 mL 90% formamide in TE buffer through the flow cell.

Amplification of fragments of genomic DNA by PCR. All PCR amplicons used in hybridization assays were prepared by Dr David C. W. Mah from Canada West Bioscience, Inc., and were provided by Dr William Lee, Defence Research and Development Canada. The PCR procedure used for the preparation of amplified fragments of genomic DNA began with a hot start at 95 °C for 15 s, then an amplification cycle of 94 °C min⁻¹, 60 °C min⁻¹, 72 °C 2 min⁻¹, for 45 cycles, and a final extension at 72 °C 7 min⁻¹.

PGI[pA1]. 286 Bp dsDNA segment, containing target sequences to probes: replicate PCR assays were done using 18CA14F (5'-GTT TTG CCG CCT ATT TCC-3') and 18CA14R (5'-TCT CTA GCC CAT ATC GTG-3') PCR primers, and pPGI[pA1]3 plasmid DNA template. The pPGI[pA1]3 plasmid contained a recombinant *Erwinia herbicola* PGI (Phosphoglucose Isomerase) gene fragment that included a dT20-dTTCTCTCTTTTTTTT probe hybridization target sequence. Pooled gel-purified replicate PCR assays were ethanol-precipitated and dried. In addition to the PCR product, dried samples may also have contained trace amounts of sodium acetate.

CA14PGI. 345 Bp dsDNA segment, negative control to the probes: replicate PCR assays were performed using 18CA14F (5'-GTT TTG CCG CCT ATT TCC-3') and 18CA14R (5'-TCT CTA GCC CAT ATC GTG-3') PCR primers, and pPGI-1N plasmid DNA template. The pPGI-1N plasmid contained an *E. herbicola* PGI gene fragment. Pooled gel-purified replicate PCR assays were ethanol-precipitated and dried. In addition to the PCR product, dried samples may also have contained trace amounts of sodium acetate.

Dried ethanol-precipitated PCR-amplified DNA was resuspended in sterile deionized distilled water and the DNA concentration was determined using a SyberGreen[™] II fluorescence-based DNA assay. The assay was performed using HindIII-digested lambda phage DNA (Invitrogen[™]) as a DNA standard and a Turner Fluorimeter[™]. Based upon the calculated DNA concentration, 20 μg of DNA was aliquoted into each microtube and dried.

3. Results and discussion

The mixed base 15-mer conjugates **3a–c** were synthesized on both GOPS-functionalized fused silica fibers and also controlled-pore glass (CPG), in tandem. The oligonucleotides were then cleaved from the CPG by exposure to concentrated NH₄OH for 3 h, and were collected to characterize the density and quality of synthesis of the immobilized tethered conjugates. Quantification of the cleaved HEG–mix15-mer conjugates was done by the reverse phase HPLC isolation procedure with a standard addition method, in which recovered oligonucleotide synthesis products underwent co-injection with a known quantity of TO-HEG–mix15-mer (synthesized from pre-packed CPG columns and purified by

Table 2 Density of immobilized oligonucleotides on GOPS functionalized substrates as determined by reverse phase HPLC

Immobilized sequences	Duration of DMT-HEG coupling reaction/h	Center-to-center separation distance/Å ($\pm 15\%$)	Packing density
3a	1	372	Low
3b	4	134	Medium
3c	12	67	High

reverse phase HPLC²⁶). The labelled oligonucleotide could be quantitatively identified in chromatograms, and this permitted an estimate of 40–50% efficiency for the final coupling step of TO to the oligonucleotide to be determined. The densities of immobilization for tethered conjugates onto GOPS-functionalized substrates are shown in Table 2. The data show that the initial availability of DMT-HEG on the surface can be used to immobilize oligonucleotides at different densities. The three different densities of immobilized conjugates represent three distinctive cases on the optical fiber surfaces. Considering that the TO tethered oligonucleotide-HEG conjugates were *ca.* 120–150 Å in length, interactions between neighboring strands were, on average, unlikely for the low-density samples **3a** (assuming homogeneous oligonucleotide distribution). Medium-density samples **3b** consisted of immobilized conjugates with the center-to-center distance similar to the length of a conjugate, which may have permitted some interaction between neighboring stands. Finally, the high-density samples **3c** consisted of immobilized conjugates where the center-to-center distance was significantly less than the length of a conjugate. This represented a system that was likely to provide for interactions between neighboring strands.

3.1 Characterization of immobilization by X-ray photoelectron spectroscopy (XPS)

It is well known that functional silanes can polymerize at a surface or in solution under certain experimental conditions, such as in the presence of water.³⁶ Through careful control of the reaction conditions it is possible to generate homogeneous films of covalently immobilized oligonucleotides with desired levels of dynamic range and sensitivity. In order to investigate the nature of the immobilized oligonucleotide films, X-ray photoelectron spectroscopy (XPS) was used to study the immobilization of TO-dT₂₀ (**5**) on the surfaces of fused silica wafers that were functionalized with GOPS-HEG-DMT. Fig. 1 shows XPS data for the nitrogen (1S), silicon (2P), and carbon (1S) core levels at different stages of chemical modification. Fig. 2 includes a panel that highlights an idealized layering structure that might be expected from immobilization of the oligonucleotides, and the subsequent hybridization and denaturation of the probe molecules. Fig. 1a shows the spectra of a control sample after cleaning. The surface shows that there is no contamination by nitrogen-containing species. The small signal for C (1S) at 284.9 eV is probably caused by physical adsorption of hydrocarbons. The Si (2P) spectrum shows a peak with binding energy in the range of 104–108 eV that is characteristic of oxidized silicon. Fig. 1b shows data from a sample that was reacted with GOPS and then capped with TMSCl. In this case, no signal from nitrogen was

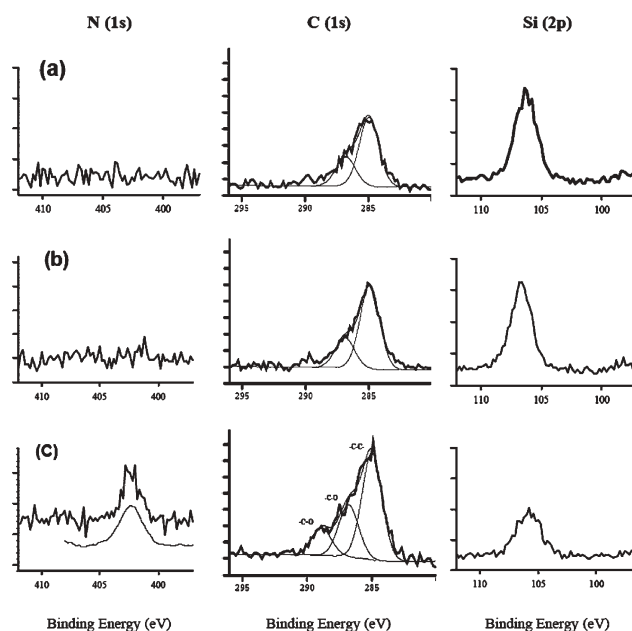


Fig. 1 XPS spectra at various stages of the chemical modification of fused silica wafers. The average thickness of immobilized oligonucleotides was 19.3 ± 2 Å. (a) Base and acid treated blank fused silica wafer; (b) fused silica wafer that was reacted with GOPS and then capped with TMSCl; (c) fused silica wafer that was covalently linked with **5**.

detected. The carbon (1S) spectrum suggests an increased signal for carbon relative to that in panel 1a, and more clearly shows a peak with binding energy of 286.5 eV, which is characteristic of C–O bond.³⁷ The Si (2P) spectrum of panel 1b shows one peak in the range of 104–108 eV, and a slightly reduced signal magnitude relative to the carbon signal when compared to Fig. 1a. This indicates the presence of a thin functionalized film on substrates without substantial silane polymerization. Fig. 1c shows spectra from a sample that was covalently linked with **5**. The N (1S) signal at 402 eV can be seen, and suggests that the procedure for immobilization of the oligonucleotides was effective. The binding energy represents a NH_3^+ -like chemical form, which is consistent with thymine (T).³⁸ The C (1S) signal is significant, with the shoulder at 289.5 eV suggesting the presence of a carbonyl group (C=O) from the nucleotides.³⁹ The Si (2P) spectrum shows a significant decrease of intensity, indicating further coverage of the surface. Scanning of various regions of the substrates showed the coverage to be quite uniform. The thickness of an overlayer composed of tethered TO oligonucleotides was estimated as 19 ± 2 Å.⁴⁰ This thickness was considerably smaller than the theoretical value of *ca.* 100 Å. The result is consistent with a monolayer film of medium density where collapse of the strands to the substrate surface under high vacuum is expected.

3.2 Hybridization at the surface of the DNA biosensor

Fig. 2 shows the fluorescence signal at different stages of a hybridization assay cycle using a typical fiber optic biosensor. The assay cycle started with a stable baseline, and binding of

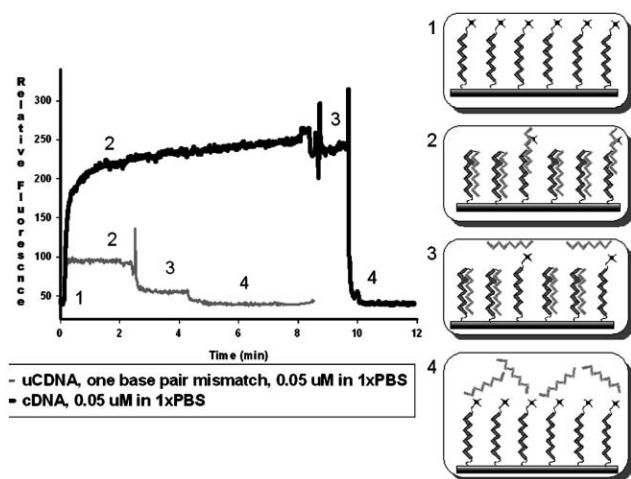


Fig. 2 Signal development during a hybridization assay cycle for biosensors prepared using probes of **3b**; (1) addition; (2) hybridization; (3) washing; (4) regeneration. Black line: for hybridization with the complementary sequence 5'-AAA AAA AAG AGA GAA-3'. Gray line: hybridization with one base pair mismatch 5'-AAA AAA AAA AGA GAA-3'. The sequence 5'- TTC TCT CTT TTT TTT-3', used as non-complementary sequence confirming signal, was same as that seen after regeneration in Step 4. Excitation at 488 nm, emission at 560 nm, 30 °C, for samples of 0.05 μM in 1 × PBS, delivery time 30 s, hybridization time 5 min, washing time 2 min at 1 mL min⁻¹ with 1 × PBS. Sensor was regenerated by treatment with 1 mL 90% formamide in TE buffer; 30 °C (derived from ref. 34).

the complementary strand to the immobilized TO tethered probe was monitored in real time by the increasing fluorescence intensity. A decrease of the fluorescence signal during the washing step indicated the removal of adsorbed material from the fiber optic surface. Compared to a blank sample when only hybridization buffer was introduced for the whole process, there was a 6-fold fluorescence intensity enhancement when the complementary sequence was introduced to the sensor surface. There was only an average 40% fluorescence intensity enhancement after washing the sensors to remove non-selectively adsorbed material when a sequence with a single base pair mismatch centered in the target was introduced to the surface (Fig. 2). The differential fluorescence enhancement upon hybridization with sequences of different complementarity suggests potential applications for distinguishing single nucleotide polymorphisms (SNPs) in nucleic acid analyses. Chemical regeneration of the sensor was done by pumping 90% formamide in TE buffer through the sample cell, and a transient signal peak was observed at the beginning of the regeneration. This was caused by the change in the refraction index of the hybridization buffer ($n = 1.31$) and the 90% formamide in TE buffer ($n = 1.40$).

3.3 Optimization of response

Immobilized tethered oligonucleotides at a solid surface experience a different environment from those in bulk solution.⁴¹ Several factors affect the hybridization event at the transducer/solution interface. These include salt concentration, temperature, the availability of the intercalating dye and the immobilization density.

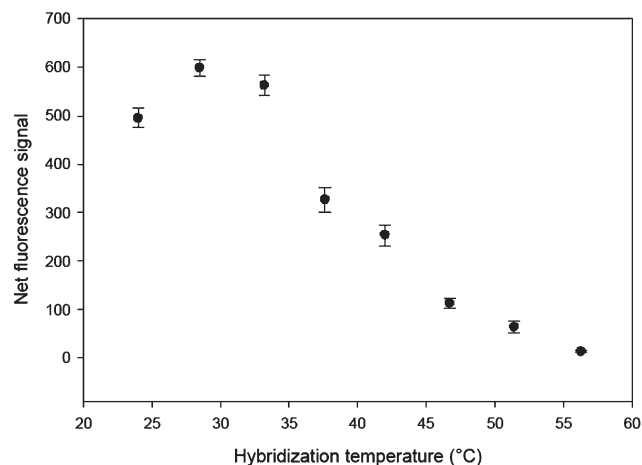


Fig. 3 Net fluorescence intensity (eqn. 1) from hybridization assays at different temperatures using TO-ssDNA probe **3b**. Complementary target was 5'-AAA AAA AAG AGA GAA-3', 0.5 μM in 1 × PBS buffer. The assay cycle was 10 min duration (2 min equilibration after regeneration, followed by 30 s delivery, 5 min hybridization, 2 min washing time at 1 mL min⁻¹ with 1 × PBS, and 1 min regeneration by the treatment with 1 mL 90% formamide in TE buffer. Excitation at 488 nm, emission at 560 nm, 30 °C.

3.3.1 Hybridization temperature. The net fluorescence intensity enhancement upon hybridization at different temperatures (between 24.0 and 56.2 °C) is shown in Fig. 3. The fluorescence signal at 9 min (equilibration of hybridization) of an assay cycle F was compared to the initial fluorescence signal (F_0) by using the eqn. 1 to determine the net fluorescence signal (dF):

$$dF = F - F_0 \quad (1)$$

The fluorescence signal resulted from two contributions, one of which was intercalation. However, a fluorescence intensity background was evident. The immobilization of the tethered oligonucleotides onto a surface could decrease the mobility of the TO dye. This can be largely attributed to an electrostatic phenomenon where the positively charged dye interacted with the relatively concentrated DNA at the solid interface. Increased temperature would reduce weak electrostatic interactions between the tethered dye and its surroundings, and could result in reduction of fluorescence intensity. A temperature increase would also lead to a larger proportion of denaturation, causing a reduction of intercalation. The dissociation of duplex DNA was apparent at hybridization temperatures up to 52 °C. A hybridization temperature of 30 °C was chosen for most work in order to increase fluorescence intensity while still assisting the hybridization selectivity (compared to rt).⁴²

3.3.2 Effects of tethered linker length on sensor performance.

In previous work⁴³ we completed molecular simulations using INSIGHT II, which is based on a self-consistent force field calculation (Accelrys, Inc., San Diego, CA). Energy calculations were done using a system based on a 20-mer ssDNA that was linked to TO using various lengths of poly(ether). The results indicated that the use of hexaethyleneglycol (HEG) as

the tether provided the most thermodynamically stable hybridization, while still minimizing the length of the tether to avoid possible interference with the neighboring sequences.

A series of related TO–ssDNA probes (**1b**, **2b**, **3b**) on fiber surfaces were used to experimentally evaluate the influence of the length of the tethers on the stability of the intercalation between the dye and dsDNA, and the results are shown in Fig. 4. A systematic evaluation of hybridization profiles indicated that an increase of the length of the tether induced an increase in thermal stability of the TO–dsDNA complexes. The trends of these results were consistent with predictions from the molecular simulation of energy minimization, and confirmed the results that had been predicted by modelling.

3.3.3 Non-selective adsorption at different densities of probe molecules. In order to quantify how much of the fluorescence signal change could be attributed to non-selective adsorption, fluorescein-labelled complementary DNA target was used for hybridization to immobilized TO–ssDNA probes (**4b**), at different packing densities and at different stringencies, based on use of buffers with different ionic strength. Fig. 5 provides some comparisons of the percentage of the signal that was lost by washing after hybridization. It was observed that films at low and medium oligonucleotide packing density had reduced loss of adsorbed material than those of high packing density, under conditions of identical ionic strength. The results suggest that low and medium packing densities provide the best rejection of adsorbed material at moderate ionic strength (presuming adsorbed materials can be efficiently removed by the washing protocol). At higher ionic strength, biosensors prepared at medium packing density provided for the best rejection of signal from adsorbed material on washing.

These results are significant in that it has been determined that the positively charged tethered intercalating dye could

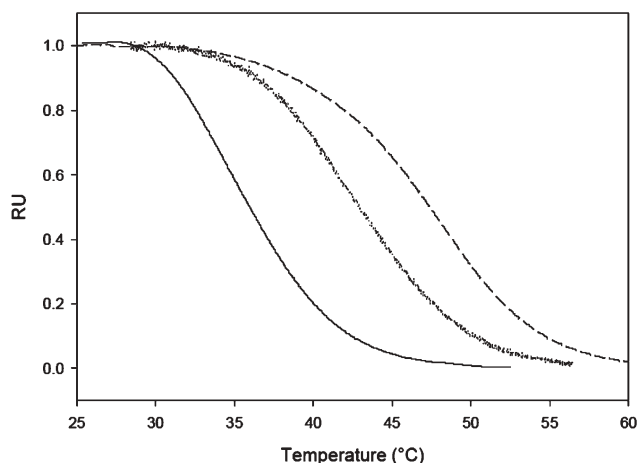


Fig. 4 Averaged melt curves for different tether lengths using TO–ssDNA probes; bold line: **1b**, dotted line: **2b**, dashed line: **3b**. Complementary DNA was 5′-AAA AAA AAG AGA GAA-3′, 0.05 μM in 1 \times PBS, with excitation at 488 nm, emission at 560 nm, hybridization temperature 30 $^{\circ}\text{C}$, hybridization time 5 min, followed by washing with 2 mL 1 \times PBS buffer; and then followed by a temperature ramp of 0.8 $^{\circ}\text{C min}^{-1}$ until 60 $^{\circ}\text{C}$ (examples of raw melt curves can be seen in ref. 34).

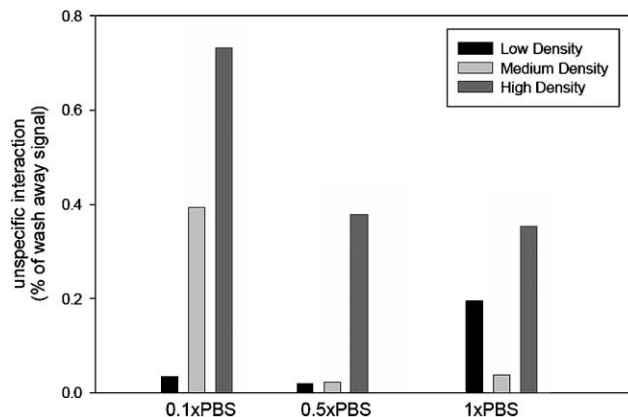


Fig. 5 Non-selective adsorption wash-off for low, medium and high immobilization packing densities of TO–ssDNA probe **4b**, in hybridization buffers of various ionic strengths at 30 $^{\circ}\text{C}$. The precision of each result is approximately 25%, and is based on a minimum of 5 replicates at each density for each condition.

electrostatically interact with immobilized probe DNA to increase background fluorescence intensity in the absence of target strands. It is known that this electrostatic influence can be suppressed by use of a high ionic strength buffer.⁴⁴ Medium density was chosen for an optimized biosensing system as a compromise between the need to reject adsorption, and the need to provide for a relatively large quantity of TO dye to develop an analytical signal.

3.3.4 Quantitative performance. The optimized conditions used a medium packing density of immobilized TO–ssDNA probes and hexaethyleneglycol as the tether. Experiments were done at 30 $^{\circ}\text{C}$ in high ionic strength buffer (1 \times PBS), and were repeated 3 times (2 min equilibration, 5 min hybridization, 2 min washing and 1 min denaturation). Fluorescence signal intensities after equilibration of hybridization and washing were averaged. The linear range of fluorescence response upon hybridization was between 10^{-9} to 10^{-7} M. The detection limit for hybridization assays was 3.1×10^{-9} M, which was similar to a sensor developed using free ethidium bromide in solution as a fluorescent intercalator to detect the formation of DNA.²¹ The hybridization assay cycle was much shorter (5–10 min) compared to the cycle time of 45 min used in previous hybridization assay. Fibers could be reused for at least 10 cycles of use, and photobleaching was not apparent.

3.4 Detection of PCR products from *Erwinia herbicola*

An *E. herbicola* phosphoglucose isomerase (PGI) gene fragment was cloned from an *E. coli* plasmid. In plasmid pPGI[pA1], the pA1 linker component contained 5′-AAA AAA AAG AGA GAA-3′. Plasmid pCA14PGI was the negative control. The specific regions of the plasmid that contained the *E. herbicola* insert was amplified by PCR and then gel purified to get PGI[pA1] (286 bp) and CA14PGI (344 bp). The PCR products were dissolved in 1 \times PBS buffer, preheated at 95 $^{\circ}\text{C}$ for 10 min, and then sat in an ice bath before each measurement. Experiments were done at 30 $^{\circ}\text{C}$ using a hybridization time of 8 min, and a washing time of

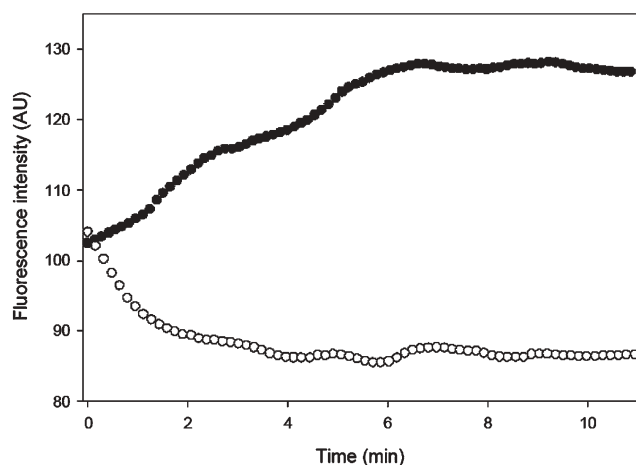


Fig. 6 Fluorescence response from fiber optic biosensor to two PCR products from *Erwina herbicola*; (●) PGI[pA1] ($3 \mu\text{g } 500 \mu\text{L}^{-1}$); (○) CA14PGI ($3 \mu\text{g } 500 \mu\text{L}^{-1}$). Excitation at 488 nm, emission at 560 nm, hybridization temperature 30°C , hybridization time 8 min, followed by washing with $2 \text{ mL } 1 \times \text{PBS}$ buffer.

2 min using hybridization buffer. Formamide in TE buffer was used to regenerate the sensor surfaces. Fig. 6 indicates an example of the response of the biosensor based on probe **3c** to PCR products PGI[pA1] and CA14PGI (all experiments were repeated 3 times). A significant increase in fluorescence intensity was observed when PGI[pA1] was introduced to the sample cell, and required about 6–7 min for equilibration. The equilibration was slow, relative to that seen when using 20-mer targets. Interestingly, a reduction in fluorescence signal was induced by introduction of the CA14PGI (the negative control where there was no complementary sequence present). A similar result was seen for addition of poly(T), which was selected to be large in molecular weight and not able to significantly hybridize with the probe molecules. The biosensors initially have a fluorescence intensity based on weak interaction of TO with materials at the surface of the optical fiber. Clearly the surface environment is changed by the presence of adsorbed long sequences of DNA, suggesting that there is disruption of the initial weak electrostatic interactions of the TO dye with the surface. This result suggests that further optimization for reduction of background might be achieved by addition of non-selective DNA.

4. Conclusions

An intercalating dye has been immobilized onto probe DNA for development of a fiber optic biosensor system based on detection using evanescent field excitation. The fused silica fiber optic biosensors were able to detect target oligonucleotides and PCR products in DNA hybridization assays. Silane chemistry was used to prepare a monolayer of HEG-DMT, and this was followed by solid-phase oligonucleotide synthesis to place oligonucleotide probes at different densities onto the fused silica surfaces. The intercalating dye thiazole orange was tethered at the 5' terminal phosphate group of the oligonucleotide probes using poly(ethyleneglycol) linkers by solid-phase oligonucleotide synthesis. XPS data demonstrated a

relatively homogenous monolayer of probes on the surface after immobilization. The fluorescence hybridization signal with target sequences was monitored in real time. This self-contained DNA biosensor system avoided the addition of free dye to stain dsDNA, and did not require fluorescently labelled target strands for development of an analytical signal. Adsorption could be largely eliminated by choice of probe density and washing conditions. Chemical denaturation of the hybridized double strands quantitatively regenerated the TO–ssDNA probes, allowing for multiple assay cycles using the same fiber. The detection system could distinguish a single base mismatch, and was able to reversibly and reproducibly generate signals for 280–350 bp PCR products from *E. herbicola*.

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References

- S. S. Deshpande and B. P. Sharma, in *Diagnostics in the year 2000*, ed. P. Singh, B. P. Sharma and P. Tyle, Van Nostrand Reinhold, New York, 1993, p. 459.
- A. Polizzi, R. Francavilla, G. Castaldo, T. Santostasi, R. Tomaiuolo, A. Manca, F. De Robertis, L. Mappa, F. P. Oliverio, F. Salvatore and N. Rigillo, *Am. J. Med.*, 2005, **132A**, 434.
- S. Lincoln, J. Wiley, T. Lynch, J. W. Langston, R. Chen, A. Lang, E. Rogaeva, R. P. Munhoz, J. Harris, K. Marder, C. Klein, G. Bisceglia, J. Hussey, A. West, M. Hulihan, J. Hardy and M. Farrer, *Neurology*, 2003, **60**, 1605.
- P. Fortina, S. Surrey and L. J. Kricka, *Trends Mol. Med.*, 2002, **8**, 264.
- J. C. Venter, M. D. Adams and E. W. Mayers, *Science*, 2001, **291**, 1304.
- M. J. Wolcott, *Clin. Microbiol. Rev.*, 1992, **5**, 370.
- J. Walker and G. J. Dougan, *J. Appl. Bacteriol.*, 1989, **67**, 229.
- M. Scarselli, M. M. Giuliani, J. Adu-Bobie, M. Pizza and R. Rappuoli, *Trends Biotechnol.*, 2005, **23**, 84.
- N. J. Armstrong and M. A. van de Wiel, *Cell. Oncol.*, 2004, **26**, 279.
- M. Thompson and U. J. Krull, *Trends Anal. Chem.*, 1984, **3**, 173.
- T. H. M. Lee, H. Cai and I. M. Hsing, *Analyst*, 2005, **130**, 364.
- J. M. Gibbs, S. J. Park, D. R. Anderson, K. J. Watson, C. A. Mirkin and S. T. Nguyen, *J. Am. Chem. Soc.*, 2005, **127**, 1170.
- M. Minunni, I. Mannelli, M. M. Spiriti, S. Tombelli and M. Mascini, *Anal. Chim. Acta*, 2004, **526**, 19.
- P. Skladal, C. D. Riccardi, H. Yamanaka and P. I. da Costa, *J. Virol. Methods*, 2004, **117**, 145.
- (a) H.-A. Ho and M. Leclerc, *J. Am. Chem. Soc.*, 2004, **126**, 1384; (b) K. Dore, S. Dubus, H.-A. Ho, I. Levesque and M. Leclerc, *J. Am. Chem. Soc.*, 2004, **126**, 4240.

- 16 (a) E. Ortiz, G. Estrada and P. Lizardi, *Mol. Cell. Probes*, 1998, **12**, 219–226; (b) X. Fang, X. Liu, S. Schuster and W. Tan, *J. Am. Chem. Soc.*, 1999, **121**, 2921; (c) F. J. Steemers, J. A. Ferguson and D. R. Walt, *Nat. Biotechnol.*, 2000, **18**, 91.
- 17 (a) B. Liu and G. C. Bazan, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 589; (b) F. J. Steemers, J. A. Ferguson and D. R. Walt, *Nat. Biotechnol.*, 2000, **18**, 91.
- 18 R. M. Sutherland, C. Dähne, J. F. Place and A. R. Ringrose, *J. Immunol. Methods*, 1984, **74**, 253.
- 19 Z. Guo, R. A. Guilfoyle, A. J. Wang and L. M. Smith, *Nucleic Acids Res.*, 1994, **22**, 5456.
- 20 S. F. Wolf, L. Haines, J. Fisch, J. N. Kremsky, J. P. Dougherty and K. Jacobs, *Nucleic Acids Res.*, 1987, **15**, 2911.
- 21 P. A. E. Piunno, U. J. Krull, R. H. E. Hudson, M. J. Damha and H. Cohen, *Anal. Chem.*, 1995, **67**, 2635.
- 22 J. H. Watterson, S. Raha, C. C. Kotoris, C. C. Wust, F. Gharabaghi, S. C. Jantzi, N. K. Haynes, N. H. Gendron, U. J. Krull, A. E. Mackenzie and P. A. E. Piunno, *Nucleic Acids Res.*, 2004, **32**, 18.
- 23 T. L. Mann and U. J. Krull, *Biosens. Bioelectron.*, 2004, **20**, 945.
- 24 T. Kempe, W. I. Sundquist, F. Chow and S.-L. Ho, *Nucleic Acids Res.*, 1985, **13**, 45.
- 25 J. Goodchild, *Bioconj. Chem.*, 1990, **1**, 165.
- 26 X. Wang and U. J. Krull, *Anal. Chim. Acta.*, 2002, **470**, 57.
- 27 H. S. Rye, S. Yue, D. E. Wemmer, M. A. Quesada, R. P. Haugland, R. A. Mathies and A. N. Glazer, *Nucleic Acids Res.*, 1992, **20**, 2803.
- 28 T. L. Netzel, K. Nafisi and M. Kubista, *J. Phys. Chem.*, 1995, **99**, 17936.
- 29 J. Nygren, N. Svanvik and M. Kubista, *Biopolymers*, 1998, **46**, 39.
- 30 S. F. Wolf, L. Haines, J. Fisch, J. N. Kremsky, J. P. Dougherty and K. Jacobs, *Nucleic Acids Res.*, 1987, **15**, 2911.
- 31 M. Durand, K. Chevrier, M. Chassignol, N. T. Thuong and J. C. Mauizot, *Nucleic Acids Res.*, 1990, **18**, 6353.
- 32 P. A. E. Piunno, J. H. Watterson, C. C. Wust and U. J. Krull, *Anal. Chim. Acta*, 1999, **400**, 73.
- 33 W. Kern and D. A. Puotinen, *RCA Rev.*, 1970, **6**, 187.
- 34 X. Wang and U. J. Krull, *Bioorg. Med. Chem. Lett.*, 2005, **15**, 1725.
- 35 M. Kubista, R. Sjoback, S. Eriksson and B. Albinsson, *Analyst*, 1994, **119**, 417.
- 36 K. M. R. Kallury, P. M. Macdonald and M. Thompson, *Langmuir*, 1994, **10**, 492.
- 37 *Handbook of X-ray photoelectron spectroscopy, 1st edition*, ed. J. F. Moulder, Elmer Corporation, Eden Prairie, Minnesota, 1992.
- 38 L. Zhang, T. Strother, W. Cai, X. Cao, L. M. Smith and R. J. Hamers, *Langmuir*, 2002, **18**, 788.
- 39 T. Strother, W. Cai, X. Zhao, R. J. Hamers and L. M. Smith, *J. Am. Chem. Soc.*, 2000, **403**, 1205.
- 40 K. M. Kallury, J. D. Brennan and U. J. Krull, *Anal. Chem.*, 1995, **67**, 2625.
- 41 T. Bunemann, *Nucleic Acids Res.*, 1982, **10**, 7181.
- 42 R. B. Wallace, J. Shaffer, R. F. Murphy, J. Bonner and K. Itakura, *Nucleic Acids Res.*, 1979, **6**, 3543.
- 43 J. Zeng, X. Wang and U. J. Krull, *Proc. SPIE-Int. Soc. Opt. Eng.*, 2001, **4414**, 1.
- 44 M. Singh-Zocchi, S. Dixit, V. Ivanov and G. Zocchi, *Proc. Nat. Acad. Sci. U. S. A.*, 2003, **100**, 7605.