A cellulose-based bioassay for the colorimetric detection of pathogen DNA

Deepika Saikrishnan, Madhu Goyal, Sharon Rossiter, Andreas Kukol*

School of Life and Medical Sciences, University of Hertfordshire, Hatfield AL10 9AB, United Kingdom

* Corresponding author, e-mail: a.kukol@herts.ac.uk

Running title: Cellulose-based bioassay for DNA detection

Abstract

Cellulose-paper based colorimetric biosensors may be used at the point of sampling without sophisticated equipment. This study reports the development of a colorimetric bioassay based on cellulose that can detect pathogen DNA. The detection was based on covalently attached single-stranded DNA probes and visual analysis. A cellulose surface functionalised with tosyl-groups was prepared by the dimethylacetamide/lithium chloride method. Tosylation of cellulose was confirmed by scanning electron microscopy, FTIR and elemental analysis. Sulfhydryl-group modified oligonucleotide probes complementary to a segment of the DNA sequence IS6110 of *Mycobacterium tuberculosis* were covelently immobilised on the tosylated cellulose. Upon hybridization of biotin labelled DNA oligonucleotides with these probes, a colorimetric signal was obtained with streptavidin conjugated horseradish peroxidase catalysing the oxidation of tetramethylbenzamidine (TMB) by H_2O_2. The colour intensity was significantly reduced when the biosensor was subjected to DNA oligonucleotide of randomised base composition. Initial experiments have shown a sensitivity of 0.1μM. A high probe immobilization efficiency (>90%) was observed with a detection limit of 0.1 μM corresponding to an absolute amount of 10 pmol. In addition detection of *M. tuberculosis* DNA was demonstrated. This work shows the potential use of tosylated cellulose as the basis for point-of-sampling bioassays.

Keywords: cellulose tosylation, oligonucleotide probe, visual detection, mycobacterium tuberculosis

Introduction

Biosensors and bioassays play an important role in clinical diagnosis, food processing safety applications, environmental monitoring, and forensic science [1,2]. Biosensor technology growth in the last couple of decades was primarily due to development of various advanced bio-recognition, transduction and signal processing elements. The biorecognition elements used in biosensors are enzymes [3,4], ion channels [5], antibodies [6-8], micro-organisms [9] and nucleic acids such as deoxyribonucleic acid (DNA) [10], peptide nucleic acid (PNA) [11], ribonucleic acid (RNA) [12], microRNA and locked nucleic acids (LNA) [13]. The use of single stranded DNA has increased due to its stability, sensitivity and high specificity arising from the ability of complementary strands to form a duplex [14] as well as due to the commercial availability of custom oligonucleotides with chemical modifications. Various

transduction methods have been applied, for example electrochemical [15,16] and optical methods [17], leading to numerous kinds of signal processing. In electrochemical transduction of DNA hybridization, detection is based on electrical current signal changes of redox molecule labels or on changes in parameters of the biomolecular layer, such as capacitance and conductivity [13]. The majority of electrochemical DNA biosensors make use of differential pulse voltammetry, cyclic voltammetry, impedance, amperometric, potentiometric, surface plasmon resonance and piezoelectricity techniques [18-20]. A differential pulse voltammetry based DNA biosensor to detect *Enterobacteriaceae* DNA including a target DNA recycling system was recently developed with sensitivity of 8.7 fM [21]. Impedimetric sensors constructed with gold nanoparticles and graphene oxide modifications to a carbon electrodes have been reported as high sensitivity DNA biosensors with detection limits around 11 fM [22]. An anodic aluminium oxide microfluidic 3D channel was utilized as an electrode for DNA probe immobilization and target detection using cyclic voltammetry to measure electrochemical response using redox indicators, $[Fe(CN)_6]^{4+}$ and/or $[Ru(NH_3)_6]^{3+}$ [23]. Although electrochemical biosensors are one of the important contributors to biosensing, a potential disadvantage is that they are very difficult to manufacture in large scale, may not be available as portable devices in many cases and require expensive materials for their manufacture [18].

DNA hybridization can also be optically detected using fluorescence, surface plasmon resonance (SPR), chemiluminescence, colorimetry, interferometry, or surface-enhanced Raman scattering (SERS) spectroscopy [reviewed in 24]. Fluorophore labelled DNA targets are easily detectable with an imaging fluorescence apparatus and this method has been exploited extensively for DNA biosensors as well as in DNA microarrays. Sensitivities down to 10 pM were achieved with fluorescein labelled targets [25]. In addition to fluorophores, molecular beacons (stem-loop structured oligonucleotides with fluorophore and quencher on each end) and quantum dots (QDs) have also been used as efficient labels that use fluorescence resonance energy transfer (FRET) to produce intense signals [reviewed in 24]. Some molecular beacons have been used to effectively detect single nucleotide polymorphisms [26] and mutations in rpoB gene of *Mycobacterium tuberculosis* [27]. QDs have many advantages over organic fluorophores, for example a brighter emission and enhanced stability [28]. Single QD nanosensors were designed for multiple detection of genes HIV-1 and HIV-2 [29] and in another case a silica-QD for pathogenic bacteria was able to detect up to 0.8 fM DNA and single base mismatches [30]. SERS does not require

fluorescent labels and has a higher spectral specificity owing to narrower bandwidths compared to dyes used in fluorescence [31]. Surface Plasmon resonance (SPR) explores changes in the refractive index at a surface due to the binding of biomolecules, which makes SPR a unique optical method because it does not require DNA labelling and has been exploited for DNA detection with sensitivities around pM with gold nanoparticles [32-34]. Although these methods yield very good results their usage is limited by the high cost of equipment, instability, manufacturing issues and non-portability [reviewed in 24].

Of all the optical methods colorimetric assays are preferred for initial diagnostic tests as they give visual readouts, have stability, and often reagents are less expensive thus suitable for low resourced areas [35,36]. Enzyme labels such as horseradish peroxidise [37,20] and metal nanoparticles, especially gold nanoparticles have been widely used for colorimetric nucleic acid assays in solution and on solid surfaces [38-40]. Various materials have been routinely employed as a basic surface to construct the biosensors that range from glass supports [41], polysterene [42], gold nanoparticles [43,44], to cellulose in its various forms including paper [45,46]. Paper as a biosensing surface has many advantages over other materials – it is a very good filter and barrier medium, cost effective, can be easily coated or impregnated, is biodegradable, has high porosity, is conducive to lateral flow and has a low non-specific absorption of biomolecules [47]. These properties of paper have led to its use in a number of microfluidic devices for many applications in diagnostics [10,35,36,48-50,44]. Another important property that contributes to the wide use of cellulose for immobilization of biomolecules is the presence of hydroxyl groups which can be subjected to many chemical reactions such as oxidation [51], esterification [52], acylation and tosylation [53] among others [54] that allow chemical groups of a biomolecule to covalently bind to the cellulose. Adsorption of biomolecules on cellulose has also developed in the last two decades and the adsorbed surface has been used as a very effective biosensing platform [10,55,38,56,9]. Although there is a surge in the number of colorimetric biosensors that use paper microfluidics, there are few simple assays that address the issue of pathogen diagnostics that could be used in high burden low resourced areas; most biosensors are primarily targeted at food borne bacteria [57-59]. In this work, we demonstrate the use of tosylated cellulose strips for the immobilization of sulphhydryl-modified oligonucleotide probes and visual detection of target DNA. The sequences correspond to the IS*6110* transposable element present in *Mycobacterium tuberculosis* and colorimetric detection is carried out with streptavidin-conjugated horseradish peroxidase (HRP) and chromogenic tetramethylbenzidine substrate.

The sensitivity and specificity of the system is evaluated with synthetic DNA and isolated DNA from the pathogen.

Materials and Methods

Tosylation of Cellulose

Tosylated cellulose was prepared by down-scaling and modifying a method described by Rahn et al. [60]. 2.0 g microcrystalline cellulose (Avicel® PH-101, Sigma Aldrich UK) was heated in 40 mL of *N,N*-dimethylacetamide at 160°C for one hour with stirring. 4.0 g of anhydrous lithium chloride was added after cooling down the solution to 100°C and then the reaction mixture was further cooled to room temperature. 5 mL of triethylamine was added to the highly viscous solution. This viscous solution was then cooled in an ice bath to 8-10°C. 4.0 g of p-toluene-sulphonyl chloride dissolved in 6 mL of *N, N*-dimethylacetamide was added to the solution. After 24 h the reaction mixture was poured into glass petridishes, immersed in ice cold water and allowed to precipitate for 2 h. As a modification of the original method [60] the precipitated sheets were washed in ice cold water and allowed to dry between sheets of strong tissue paper for 24 h. A control cellulose film was prepared in a similar procedure without addition of tosyl chloride. The tosylated and control cellulose strips were then subjected to Attenuated Total Reflection (ATR) Fourier-transform infrared (FT-IR) spectroscopy, scanning electron microscopy (SEM) and Elemental Analysis.

FTIR Analysis

A Nicolet 6700 FTIR instrument (Thermo Scientific, UK) with a diamond attenuated total reflection accessory was used to analyse the samples. The ATR-FTIR spectra of cellulose powder, control cellulose film and tosylated cellulose were recorded with 30 scans per spectrum and a resolution of 4 cm^{-1} using the DTGS detector. The spectrum was processed with level 2 zero filling and the Norton-Beer apodization.

SEM Analysis

Tosylated cellulose, control cellulose film, Whatman filter paper and cellulose powder were analysed with the SEM.A JEOL JCM-7500 scanning electron microscope in the SEI (secondary electron) mode. All the specimens were coated with a layer of gold approximately 30 nm thick. Low accelerating voltages of 5 or 10 kV were used for the

measurement. The measurements were carried out with spot sizes (diameter of electron beam) of 31 and 35 and at working distances (from the lens) of 15 mm and 28 mm

Elemental Analysis

Elemental analysis was performed by Medac Ltd. to find percentage composition of sulphur and chlorine in the samples and this was used for determining the degree of substitution. The degree of substitution (DS_S) is defined as the number of tosylated hydroxyl groups per glucose unit and it was calculated from the percentage of sulphur $S_{\%}$ obtained from elemental analysis using the formula $DSs = (S_{\%} \times M_G) / (M_S \times 100 - M_{Tos} \times S_{\%})$ [60]. Whereby the molecular mass of a glucose unit was $M_G = 180.16$ g/mol, the molecular mass of sulphur was $M_S = 32.06$ g/mol and the molecular mass of a tosyl group was $M_{Tos} = 155.19$ g/mol .

Immobilization of oligonucleotide probes

All synthetic oligonucleotides were obtained from Eurogentec Ltd, Belgium. 100 µL of a 2.5 µM solution of the 5'-end hexanethiol (SH) and hexaethyleneglycol (HEG) spacer modified oligonucleotide (29 nt), 5'-SH-HEG-GGCGAACCCTGCCCAGGTCGACACATAGG-3' (IS*6110* element of *Mycobacterium tuberculosis)* containing dithiothrietol (300 µM) and phosphate buffer solution (92 mM, pH 7.2) was pipetted onto the tosylated cellulose surface placed in a closed petri dish and allowed to react for 16 – 18 h in dark at room temperature.

Synthetic target Hybridization

5'-Biotinylated target (complementary to the probe) oligonucleotides, 5'-biotin-CCTATGTGTCGACCTGG GCAGGGTTCGCC-3' and a randomised (non-complementary), oligonucleotide of the same base composition, 5'-biotin-GTGTGCCCCATCGTACGCG AGTCGTGCGT -3' were prepared to a final concentration of 1 µM with hybridization buffer (270 mM NaCl, 4.5 mM $MgCl_2 \cdot 6H_2O$ and 22.5 mM Tris, pH 8.3) and 100 µL of each solution was pipetted on individual tosylated cellulose strips with immobilized probes and incubated for 1 hour at 60° C. After incubation the samples were washed three times in sterile distilled water for 15, 5 and 5 minutes respectively to remove any unbound probe and target/random oligonucleotides. 100 µl of hybridization buffer was pipetted onto probe immobilized tosylated cellulose strips and these were used as negative controls. The experiment was performed in triplicates.

Detection

After hybridization the cellulose strips were washed with freshly distilled water three times for 15, 5 and 5 minutes at room temperature. The strips were then blocked with 5 mL of 10% blocking solution containing non-fat milk powder (ECL blocking agent, GE Healthcare Ltd) and 0.1% PBS Tween-20 for 1 h at room temperature. After the blocking step the samples were washed in PBS Tween-20 three times for 15, 5 and 5 min. 50 µL of Streptavidin-Horseradish Peroxidase (HRP) conjugate in 0.1% PBS-tween (1:1000) was added to each sample and incubated for 1 h and washed in PBS-Tween-20 three times for 15, 5 and 5 minutes. The HRP substrate 3, 5, 3',5' –tetramethylbenzidine at a concentration of 0.55 mg/ml was prepared with 1 mL of dimethyl-sulphoxide (DMSO), 20 µM H_2O_2 (30% w/v) and buffer (4.5 mM $CaCl_2.2H_2O$, 22.5 mM citric acid, 45 mM $NaH_2PO_4.H_2O$). 75 µL of this solution was added onto each cellulose sample and observed for colour change. Once the colour developed the sample was scanned with a flatbed scanner and quantified with ImageJ software [9].

Image Analysis

ImageJ 1.47v [61] was used to measure the signal intensity from images of assay samples produced by a scanner. Each image was split into three channels, red, blue and green. The red channel image provided the highest contrast and was used for analysis. The image was inverted, so lighter grey areas (higher pixel intensities) corresponded to a detection signal. The mean pixel intensity of an area was measured by using the oval tool for keeping a constant area measurement.

Quantification of probe immobilization

The amount of a fluorescent probe immobilised on cellulose was estimated by subtracting the cummulative amount of probe removed in successive washing steps from the total amount of probe added onto cellulose. 5' hexanethiol - HEG-GGCGAACCCTGCCCAGGTCGACACATAGG-fluorescein-3' oligonucleotide probes (HPLC-RP purified) were purchased (Eurogentec Ltd, Belgium). 100 µL oligonucleotide probe solutions at concentrations between 0.5 and 5 µM were prepared with dithiothrietol (300 µM) and PBS (92 mM, pH 7.2). The samples were diluted to 1 mL with PBS and the initial fluorescence intensity was measured in a fluorimeter with an excitation wavelength of 490 nm and an emission wavelength of 520 nm. The solutions were pippetted on individual tosylated strips and allowed to react for 18 h and washed three times in 5 mL fresh PBS. Each set of washing was individually collected and measured in a fluorimeter. The third wash did

not yield any fluorescence signal and hence further washing of the samples was not required. The difference between the integrated peak area value of the solutions and cumulative peak area values of the three washings were calculated to measure the amount of probe immobilized.

Sensitivity

A series of solutions of target and random oligonucleotides of various concentrations (1, 0.5, 0.1, 0.05 and 0.01 μM) were prepared with hybridization buffer (270 mM NaCl, 4.5 mM $MgCl_2 \cdot 6H_2O$ and 22.5 mM Tris, pH 8.3) and 100 μL of each solution was pipetted onto the tosylated cellulose strips and hybridized for 1 h at 60°C. Detection was performed using the method described above.

Specificity

For analysing the specificity of the method 1 μM solutions of 5'- biotin labelled target, single base mismatch, double base mismatch, triple base mismatch and randomised oligonucleotide sequences with respect to the probe sequence were prepared with dithiothreitol (300 μM) and and PBS (92 mM, pH 7.2). 100 μL of each solution was pipetted onto probe immobilized tosylated cellulose strips. The strips were hybridized for 1 h at 60°C and detected as mentioned above.

Hybridization time

1 μM solutions of target and random oligonucleotide sequences were prepared with hybridization buffer (270 mM NaCl, 4.5 mM $MgCl_2.6H_2O$ and 22.5 mM Tris, pH 8.3) and 100μL of each solution was pipetted on probe immobilized tosylated cellulose strips and hybridized for .5, 1, 1.5 and 2 h at 60°C and detected as before. This experiment was performed in duplicates.

PCR

PCR was performed on H37Rv *Mycobacterium tuberculosis* DNA (provided by Brian Robertson, Imperial College, London, UK) to amplify the specific regions and also to incorporate biotin labels in the sample. Primers were chosen from IS6110 transposable element. Two sets of primers with biotin labels were obtained from Invitrogen. One set was used to amplify a region containing the complementary region with respect to the probe. The primers for complementary sequence amplification was from *Mycobacterium tuberculosis*

transposable insertion element, IS*6110* at position 791(forward primer) 5'-TAA CCG GCT GTG GGT AGC A-3'; and at position 864 (reverse), 5'-CGG TGA CAA AGG CCA CGTA-3' and the other set was used to amplify the region non-specific to the probe, also from IS*6110* at position 1062 (forward primer), 5'-CCGAGGCAGGCATCCA-3' and at position 1132 (reverse primer), 5'-GATCGTCTCGGCTAGTGCATT-3'. The PCR was performed in 50 µL volume containing taq polymerase (1 U/µL), forward and reverse primers (25.6 nmol/L), 1x reaction buffer, $MgCl_2$ (3 mM) dNTPs (1µM), template DNA (7.2 ng/µL) and sterile distilled water. The amplification parameters were as follows: 94°C for 5 minutes followed by 40 cycles at 94°C for 1 minute, 58°C (complementary region) and 60°C (non-complementary region) for 1 minute, at 72°C for 1 minute. After the 40 cycles the samples were heated at 72°C for 10 minutes. The samples were then subject to gel electrophoresis in 2% agarose gel.

Assay with PCR product

The PCR products were used without further purification. The obtained products were heated to 95°C to denature the helical duplex and then rapidly cooled in ice. 10 µL of the PCR product was added to 90 µL of hybridization buffer and added on to probe immobilized tosylated strips and hybridized at 59°C for 1 h. The samples were washed 3 times with fresh distilled water each time for 15, 5 and 5 minutes respectively. PCR controls without template DNA were used as controls for the assay. The experiment was carried out in triplicates. The detection was carried out as mentioned above.

Results and Discussion

Tosylation of Cellulose

Cellulose was tosylated successfully with modifications to the method described by Rahn et al. [60]. The infrared (IR) absorption spectrum of tosylated cellulose in figure 1a shows characteristic peaks at 814 cm^{-1} (aromatic C-H bend vibration), 1177 cm^{-1} (symmetric SO_2 stretch vibration), 1364 cm^{-1} and 1598 cm^{-1} (aromatic C-C bend vibrations), which are not present in the spectra of control cellulose films and cellulose powder (figure 1b & c). These results show that cellulose was successfully tosylated and the modifications that were made to the reported tosylation method did not impact the formation of the tosylated cellulose product. The scanning electron microscope images show the difference in the structural appearance of the tosylated cellulose, control cellulose film, Whatman filter paper and cellulose powder (Figure 2a-d). The Scanning Eelectron Microscopy (SEM) image of

tosylated cellulose in figure 2a had a very porous and uneven surface. On the contrary in figure 2b the control cellulose film had smooth surface with bigger sized lumps of material appearing at random locations possibly caused by undissolved lithium chloride. Tosyl chloride disrupts the formation of dimethyl acetamide Li^+ and forms the tosyl derivative of cellulose which does not occur in the control cellulose film and this may attribute to the difference in the surface appearance. SEM images in figures 2c and 2d show that Whatman filter paper consists of a network of fibres, while cellulose powder has an uneven surface with thicker lumps of material..

Elemental analysis of tosylated cellulose and control cellulose films was carried out. The elemental analysis showed the expected high sulphur content relative to the chlorine content. However the chlorine content is higher than reported previously [60]. This may be a consequence of not subjecting the sample to washing in ethanol and re-precipitation in acetone. This step was avoided to retain the samples as rigid materials to enable a paper like surface for biomolecule immobilization. The degrees of substitution of hydroxyl groups with sulphur (DS_s) in tosylated cellulose are shown with elemental analysis results in table 1. An absolute maximum value for DS_s obtainable for tosylated cellulose is 3.0, since there are three hydroxyl groups per glucose unit. The DS_s of 0.28 (9.6%) and 0.3 (10%) are much smaller than the value of 1.36 (45.6%) obtained previously for a similar cellulose starting material [64]. The degree of substitution has a direct impact on the sensitivity of the system because it determines the number of sites available for biomolecule immobilization. However, as demonstrated in this work the use of this material for biosensing applications was possible.

Quantification of Probe immobilization

In order to estimate the amount of probe oligonucleotides that immobilise covalently on the tosylated cellulose surface fluorescein labelled oligonucleotides were used for immobilization at various concentrations. The amount of probe covalently bound to tosylated cellulose was calculated indirectly by determining the amount of probe that did not bind to cellulose, but could be washed off. The results show that there was an increase in covalent attachment with increasing amount of probes added (Figure 3). The linear increase is observed only until 0.25 nmol after which there was a fluctuating amount of covalent attachment of the probe. It is possible >0.25 nmol probe added leads to non-specific adsorption of probe including entrapment in the cellulose fibre network. The fluctuations in the amount of probe

immobilisation is then caused by variation in the cellulose sample. Producing tosylated cellulose paper in an automated paper manufacturing process would eliminate these fluctuations. For the present study, in order to ensure a reproducible quantity of probe for immobilization, a final quantity of 0.25 nmol was used corresponding to a concentration of 2.5 μM in 100 μL solution. Higher probe concentrations would also incur a higher cost in manufacturing of the biosensor. The fraction of covalent probe immobilization was higher than 90% of the total amount of probe added (Table 2). This immobilization efficiency is much higher than previously reported covalent oligonucleotide immobilization methods [62,63]. Despite achieving these high immobilization efficiencies, the total amount of probe molecules that can bind to tosylated cellulose remains low because of the observed low degree of substitution to a maximum of 10% of the available hydroxyl groups.

DNA detection

The bioassay used for DNA detection exploiting the specific hybridisation of a biotin-labelled oligonucleotide with a cellulose attached oligonucleotide probe is shown schematically in figure 4a. The streptavidin-HRP conjugate functions as a transducer that provides the visual readout of hybridisation as the biorecognition event. As the signal processing element, the human eye or a scanner, is not an integral part of the system, it must be termed a 'bioassay' instead of a 'biosensor'. The results in figure 4b show the successful detection of target DNA complementary to the probe. Intense blue coloured spots characteristic of TMB oxidation via HRP/H_2O_2 were obtained with target DNA, while a DNA oligonucleotide with randomized sequence showed blue coloured spots of lower intensity and no blue coloured spots were obtained in a negative control experiment without DNA olgonucleotide. The results show clearly that specific hybridization of target-probe sequences was achieved and provide an indirect confirmation of successful tosylation and probe immobilization. Covalent immobilization of oligonucleotides has been successfully used for electrochemical, optical and colorimetric DNA biosensor assays. Some of these include polypyrrole-polyvinyl sulphonate coated platinum electrodes covalently linked with 25 bp polydeoxycytidine (dc) using avidin-biotin binding and carbodiimide coupling [64], amide formation using amine functionalised probes and oxidised cellulose [9], thiol or amine modified oligonucleotides attached to a photoactive polystyrene surface [37] and thiol functionalised oligonucleotides covalently linked to gold nanoparticles [65]. In the present work the specific detection of DNA using tosylated cellulose-linked oligonucleotide probes was demonstrated for the first time. The paper strip has the potential to be developed into an array type sensor by

immobilization of oligonucleotides of different sequences at various spots, thus allowing for multiplexed detection of specific various characteristics of pathogens. Within the current set-up the spots are of irregular size, which could be circumvented by micropatterning the paper using wax printing [66].

Limit of detection

The detection limit of the cellulose-strip biosensor was determined by using a range of oligonucleotide concentrations from 0.01 μM to 1.0 μM (see figure 5a for examples). The quantitative analysis shown in figure 5b reveals a clear difference between target and random oligonucleotide samples up to a concentration of 0.05 μM, which corresponds to a total amount of 5 pmol in 100 μL volume. This detection limit is higher, but within a similar range of other similar DNA hybridization based biosensors developed using oligonucleotides immobilized onto polystyrene plates or streptavidin coated microtitre plates. These two systems also exploit H_2O_2 oxidation with streptavidin-hrp conjugate systems and have a limit of detection of 4 nM and within the μM ranges respectively [37,67]. The assay sensitivity is far less than gold nanoparticle based DNA biosensors which have detection limits of 200 pM and 1.1 fM [38,17]. Although such high sensitivities are achieved these methods involve either processes such as vacuum filtering for allowing adsorption of gold nanoparticle labelled oligonucleotides on cellulose acetate membranes or chemiluminescent detection of the signal which make them unsuitable for achieving results at the point of sampling. Electrochemical methods based on voltammetry and impedimetry are able to reach high sensitivity in the pM and fM ranges [64,68-70], however these methods require expensive electrodes, coating of glass electrodes carbon nanotubes for immobilization and hybridization which is not desirable [71]. Optical methods using FRET based on quantum dots and SERS for DNA detection have also demonstrated sensitivities of < 10nM and 2.5 pM respectively but require high cost fluorimeters and spectrometers for analysis which are not portable and cumbersome to use [72,73]. The use of cost effective modified cellulose strips with visual detection up to 0.1 μM (0.1 pmol/μL) could be advantageous over other methods once further optimised. It is possibly that the sensitivity can be improved, for example, by enhancing the degree of tosylation of cellulose.

Specificity

The specificity test was carried out to ascertain what level of base-pair mismatches could be tolerated. The biosensor was treated with oligonucleotide solution at 1 μM, while the oligonucleotide had zero, one, two and three base pair mismatches to the surface-attached probe (figure 6a). The averages of the signal intensity over various experiments are shown in figure 6b and are compared with the negative control randomised oligonucleotide sequence. The results show an increase in signal fluctuation with base pair mismatches, while there is an overal tendency to lower signal intensities with an increasing number of base mismatches. However, even at three base mismatches, the signal is above the level obtained by the negative control. This shows that the biosensor on the one hand is able to detect oligonucleotides of similar but variable sequences, but on the other hand the specificity is not very high. In a similar method based on DNA-oligonucleotides attached to a polystyrene surface and HRP detection, up to two base-pair mismatches between a biotinylated probe and target lead to a detectable signal, while three base-pair mismatches were undistinguishable from a random target [37]. Most likely the specificity depends on the combination of probe and target sequences (in particular the GC-content) and could be increased by choosing different probes, raising the hybridisation temperature and/or by adding denaturants such as formaldehyde or dimethylsulphoxide. The melting temperature of the probe-target pair used in the current study was 68°C.

Hybridization time

From a practical point of view, the time required for the assay should be as short as possible. Towards that goal the dependence of signal intensity on the hybridisation time was investigated between 30 minutes to 120 minutes (figure 7). Interestingly, shorter hybridisation times between 30 and 60 minutes produce the highest signal. It is possible that longer hybridisation times lead to irreversible non-specific attachment that cannot be removed be subsequent washing steps. A 30 minutes hybridisation time would be advantages in a clinical or point-of-care setting, as results could be provided to the end user in a short time frame. These hybridization times are much more preferable to those reported earlier which range from 5 h in a colorimetric detection method [74] to 18 h in electrochemical methods [75]. Some gold nanoparticle based assays can yield results in minutes, however the colour change in such cases are likely to be highly unstable making them less reliable [17].

PCR Assay

In order to test the cellulose-based biosensor with pathogen DNA, DNA from *Mycobacterium tuberculosis* was amplified by the polymerase chain reaction (PCR).
A short region from the transposable element IS*6110* was chosen, because it is a multiple copy element which is spread over the entire genome, and the location of IS*6110* can be used for the identification of a particular strain. IS*6110* based PCR is viable for routine use in clinical laboratories for *M. tuberculosis* in sputum samples [76]. The PCR was designed to amplify a small 74bp-sequence including the sequence complementary to the immobilized probe. Another 71bp-sequence non complementary to probe was amplified as well and used as negative control. The PCR products had the expected size as shown by agarose gel electrophoresis (figure 8a). Bands were obtained for both complementary and non-complementary products at a migration distance corresponding to 74bp and 71bp. The PCR products were used directly for detection without purification after denaturing at 95°C for 10 minutes and rapidly cooling in ice.

The results of the bioassay in figure 8b showed that the PCR products corresponding to the complementary region of the probe yielded an expected blue colour, and the non-complementary PCR products and controls did not yield any colour indicating that the assay was successful for the samples obtained from bacterial DNA isolates. While the PCR reaction is required to introduce biotin lables, even in combination with PCR the reported method has two advantages. Firstly, the method has the potential for array-type assays or biosensors on cellulose surface with multiple probes that may be used distinguish between various pathogens in one step or determine the specific genomic type of the pathogen and secondly that it has potential to provide same day results in the field using portable PCR systems [reviewed in 77]

In summary, we have demonstrated the successful use of tosylated cellulose strips for the immobilization of oligonucleotides, and the development of a colorimetric assay for pathogenic DNA. The method has a number of advantages – it is highly cost effective as the tosylated cellulose strips themselves could be produced easily in large scale possibly during the process of paper manufacturing. Multiplexed detection systems could be produced by attaching oligonucleotide probes to different spots on the surface of micropatterned paper [66]. Once the biosensor has been produced the detection method eliminates the requirement of sophisticated instruments, except a portable PCR, and the strips can be disposed after use. The reagents and probes are used in low concentrations and can be used for many assays hence avoiding recurring expenditure.

Acknowledgements

This project was partially funded by the School of Life and Medical Sciences, University of Hertfordshire, United Kingdom. We thank the Vetinary Laboratories Agency, Surrey, for providing a sample of *Mycobacterium tuberculosis* DNA.

References

1. Lei Y, Chen W, Mulchandani A (2006) Microbial biosensors. Analytica chimica acta 568 (1-2):200-210. doi:10.1016/j.aca.2005.11.065

2. Song S, Xu H, Fan C (2006) Potential diagnostic applications of biosensors: current and future directions. International journal of nanomedicine 1 (4):433-440

3. Albayrak N, Yang S-t (2002) Immobilization of Aspergillus oryzae β-galactosidase on tosylated cotton cloth. Enzyme and Microbial technology 31:371-383

4. Yin L-T, Lin Y-T, Leu Y-C, Hu C-Y (2010) Enzyme immobilization on nitrocellulose film for pH-EGFET type biosensors. Sensors and Actuators B: Chemical 148 (1):207-213. doi:10.1016/j.snb.2010.04.042

5. Schmidt EK, Liebermann T, Kreiter M, Jonczyk A, Naumann R, Offenhäusser A, Neumann E, Kukol A, Maelicke A, Knoll W (1998) Incorporation of the acetylcholine receptor dimer from Torpedo californica in a peptide supported lipid membrane investigated by surface plasmon and fluorescence spectroscopy. Biosensors & bioelectronics 13 (6):585-591

6. Arya SK, Chornokur G, Venugopal M, Bhansali S (2010) Antibody functionalized interdigitated micro-electrode (IDmicroE) based impedimetric cortisol biosensor. The Analyst 135 (8):1941-1946. doi:10.1039/c0an00242a

7. Corso CD, Dickherber A, Hunt WD (2008) An investigation of antibody immobilization methods employing organosilanes on planar ZnO surfaces for biosensor applications. Biosensors & bioelectronics 24 (4):811-817. doi:10.1016/j.bios.2008.07.011

8. Wen X, He H, Lee LJ (2009) Specific antibody immobilization with biotin-poly(L-lysine)-g-poly(ethylene glycol) and protein A on microfluidic chips. Journal of immunological methods 350 (1-2):97-105. doi:10.1016/j.jim.2009.07.011

9. Su S, Nutiu R, Filipe CDM, Li Y, Pelton R (2007) Adsorption and covalent coupling of ATP-binding DNA aptamers onto cellulose. Langmuir : the ACS journal of surfaces and colloids 23 (3):1300-1302. doi:10.1021/la060961c

10. Ali MM, Aguirre SD, Xu Y, Filipe CDM, Pelton R, Li Y (2009) Detection of DNA using bioactive paper strips. Chemical Communications 43:6640-6642

11. Stender H, Broomer A, Oliveira K, Perry-O'Keefe H, Hyldig-Nielsen JJ, Sage A, Young B, Coull J (2000) Rapid detection, identification, and enumeration of Pseudomonas aeruginosa in bottled water using peptide nucleic acid probes. Journal of microbiological methods 42 (3):245-253

12. Liang R-Q, Li W, Li Y, Tan C-y, Li J-X, Jin Y-X, Ruan K-C (2005) An oligonucleotide microarray for microRNA expression analysis based on labeling RNA with quantum dot and nanogold probe. Nucleic acids research 33 (2):e17-e17. doi:10.1093/nar/gni019

13. Liu A, Wang K, Weng S, Lei Y, Lin L, Chen W, Lin X, Chen Y (2012) Development of electrochemical DNA biosensors. TrAC Trends in Analytical Chemistry 37:101-111. doi:10.1016/j.trac.2012.03.008

14. Berney H, West J, Haefele E, Alderman J, Lane W, Collins JK (2000) A DNA diagnostic biosensor: development, characterisation and performance. Sensors and Actuators B: Chemical 68 (1-3):100-108. doi:10.1016/s0925-4005(00)00468-8

15. Grant S, Davis F, Pritchard Ja, Law Ka, Higson SPJ, Gibson TD (2003) Labeless and reversible immunosensor assay based upon an electrochemical current-transient protocol. Analytica Chimica Acta 495 (1-2):21-32. doi:10.1016/j.aca.2003.08.026

16. Kukol A, Li P, Estrela P, Ko-Ferrigno P, Migliorato P (2008) Label-free electrical detection of DNA hybridization for the example of influenza virus gene sequences. Analytical biochemistry 374 (1):143-153. doi:10.1016/j.ab.2007.10.035

17. Qi Y, Li B, Zhang Z (2009) Label-free and homogeneous DNA hybridization detection using gold nanoparticles-based chemiluminescence system. Biosensors & bioelectronics 24 (12):3581-3586. doi:10.1016/j.bios.2009.05.021

18. Dolatabadi JEN, Mashinchian O, Ayoubi B, Jamali AA, Mobed A, Losic D, Omidi Y, de la Guardia M (2011) Optical and electrochemical DNA nanobiosensors. TrAC Trends in Analytical Chemistry 30 (3):459-472. doi:10.1016/j.trac.2010.11.010

19. Lazerges M, Bedioui F (2013) Analysis of the evolution of the detection limits of electrochemical DNA biosensors. Analytical and bioanalytical chemistry 405 (11):3705-3714. doi:10.1007/s00216-012-6672-5

20. Mir M, Homs A, Samitier J (2009) Integrated electrochemical DNA biosensors for lab-on-a-chip devices. Electrophoresis 30 (19):3386-3397. doi:10.1002/elps.200900319

21. Luo C, Tang H, Cheng W, Yan L, Zhang D, Ju H, Ding S (2013) A sensitive electrochemical DNA biosensor for specific detection of Enterobacteriaceae bacteria by

Exonuclease III-assisted signal amplification. Biosensors & bioelectronics 48:132-137. doi:10.1016/j.bios.2013.03.084

22. Sahoo P, Suresh S, Dhara S, Saini G, Rangarajan S, Tyagi aK (2013) Direct label free ultrasensitive impedimetric DNA biosensor using dendrimer functionalized GaN nanowires. Biosensors & bioelectronics 44:164-170. doi:10.1016/j.bios.2013.01.023

23. Kim YJ, Jones JE, Li H, Yampara-Iquise H, Zheng G, Carson Ca, Cooperstock M, Sherman M, Yu Q (2013) Three-dimensional (3-D) microfluidic-channel-based DNA biosensor for ultra-sensitive electrochemical detection. Journal of Electroanalytical Chemistry 702:72-78. doi:10.1016/j.jelechem.2013.04.021

24. Sassolas A, Leca-Bouvier BD, Blum LJ (2008) DNA biosensors and microarrays. Chemical reviews 108 (1):109-139. doi:10.1021/cr0684467

25. Abel AP, Weller MG, Duveneck GL, Ehrat M, Widmer HM (1996) Fiber-optic evanescent wave biosensor for the detection of oligonucleotides. Analytical chemistry 68 (17):2905-2912

26. Liu C-W, Lin Y-W, Huang C-C, Chang H-T (2009) Fluorescence detection of single-nucleotide polymorphisms using a thymidine-based molecular beacon. Biosensors & bioelectronics 24 (8):2541-2546. doi:10.1016/j.bios.2009.01.003

27. Gomez DI, Fisher-Hoch SP, Bordt AS, Quitugua TN, Robledo J, Alvarez N, Correa N, McCormick JB, Restrepo BI (2010) Systematic interpretation of molecular beacon polymerase chain reaction for identifying rpoB mutations in Mycobacterium tuberculosis isolates with mixed resistant and susceptible bacteria. Diagnostic microbiology and infectious disease 67 (1):37-46. doi:10.1016/j.diagmicrobio.2009.12.007

28. Murphy CJ (2002) Optical sensing with quantum nanodots. Analytical chemistry:520A-526A

29. Zhang C-y, Hu J (2010) Single quantum dot-based nanosensor for multiple DNA detection. Analytical chemistry 82 (5):1921-1927. doi:10.1021/ac9026675

30. Zhao X, Tapec-Dytioco R, Tan W (2003) Ultrasensitive DNA detection using highly fluorescent bioconjugated nanoparticles. Journal of the American Chemical Society 125 (38):11474-11475. doi:10.1021/ja0358854

31. Isola NR, Stokes DL, Vo-Dinh T (1998) Surface-enhanced Raman gene probe for HIV detection. Analytical chemistry 70 (7):1352-1356

32. Green RJ, Frazier Ra, Shakesheff KM, Davies MC, Roberts CJ, Tendler SJ (2000) Surface plasmon resonance analysis of dynamic biological interactions with biomaterials. Biomaterials 21 (18):1823-1835

33. Homola J, Vaisocherová H, Dostálek J, Piliarik M (2005) Multi-analyte surface plasmon resonance biosensing. Methods (San Diego, Calif) 37 (1):26-36. doi:10.1016/j.ymeth.2005.05.003

34. Homola J, Yee SS, Gauglitz G (1999) Surface plasmon resonance sensors: review. Sensors and Actuators B: Chemical 54 (1-2):3-15. doi:10.1016/s0925-4005(98)00321-9

35. Cheng C-m, Mazzeo AD, Gong J, Martinez AW, Phillips ST, Jain N, Whitesides GM (2010) Millimeter-scale contact printing of aqueous solutions using a stamp made out of paper and tape †. Lab on a chip 10:3201-3205. doi:10.1039/c004903d

36. Martinez AW, Phillips ST, Carrilho E, Iii SWT, Sindi H, Whitesides GM (2008) Simple Telemedicine for Developing Regions : Camera Phones and Paper-Based Microfluidic Devices for Real-Time , Off-Site Diagnosis. Analytical biochemistry 80 (10):3699-3707

37. Kannoujia DK, Nahar P (2010) Single-step covalent immobilization of oligonucleotides onto solid surface. Analytical Methods:212-216. doi:10.1039/c001661f

38. Iii RAR, Mirkin CA, Letsinger RL (2000) A gold nanoparticle / latex microsphere-based colorimetric oligonucleotide detection method *. Pure and applied chemistry 72 (July 1999):229-235

39. Vaseghi A, Safaie N, Bakhshinejad B, Mohsenifar A, Sadeghizadeh M (2013) Detection of Pseudomonas syringae pathovars by thiol-linked DNA–Gold nanoparticle probes. Sensors and Actuators B: Chemical 181:644-651. doi:10.1016/j.snb.2013.02.018

40. Wu Z, Wu Z-K, Tang H, Tang L-J, Jiang J-H (2013) Activity-based DNA-gold nanoparticle probe as colorimetric biosensor for DNA methyltransferase/glycosylase assay. Analytical chemistry 85 (9):4376-4383. doi:10.1021/ac303575f

41. Joos B, Kuster H, Cone R (1997) Covalent attachment of hybridizable oligonucleotides to glass supports. Analytical biochemistry 247 (1):96-101. doi:10.1006/abio.1997.2017

42. Bora U, Chugh L, Nahar P (2002) Covalent immobilization of proteins onto photoactivated polystyrene microtiter plates for enzyme-linked immunosorbent assay procedures. Journal of immunological methods 268 (2):171-177

43. Song F, Zhou F, Wang J, Tao N, Lin J, Vellanoweth RL, Morquecho Y, Wheeler-laidman J (2002) Detection of oligonucleotide hybridization at femtomolar level and sequence-specific gene analysis of the Arabidopsis thaliana leaf extract with an ultrasensitive surface plasmon resonance spectrometer. Nucleic acids research 30 (14):e72-e72

44. Zhao W, Ali MM, Aguirre SD, Brook MA, Li Y (2008) Paper-based bioassays using gold nanoparticle colorimetric probes. Analytical Chemistry 80:8431-8437

45. Bora U, Kannan K, Nahar P (2005) A simple method for functionalization of cellulose membrane for covalent immobilization of biomolecules. Journal of Membrane Science 250 (1-2):215-222. doi:10.1016/j.memsci.2004.10.028

46. Martinez AW, Phillips ST, Butte MJ, Whitesides GM (2007) Patterned Paper as a Platform for Inexpensive , Low-Volume , Portable Bioassays. Angewandte Chemie International Edition 46:1318-1320. doi:10.1002/anie.200603817

47. Pelton R (2009) Bioactive paper provides a low-cost platform for diagnostics. TrAC Trends in Analytical Chemistry 28:925-942. doi:10.1016/j.trac.2009.05.005

48. Martinez AW, Phillips ST, Nie Z, Cheng C-M, Carrilho E, Wiley BJ, Whitesides GM (2010) Programmable diagnostic devices made from paper and tape. Lab on a Chip 10:2499-2504

49. Ornatska M, Sharpe E, Andreescu D, Andreescu S (2011) Paper bioassay based on ceria nanoparticles as colorimetric probes. Analytical Chemistry 83:4273-4280

50. Tian J, Shen W (2011) Printing enzymatic reactions. Chemical Communications 47:1583-1585

51. Kim UJ, Kuga S, Wada M, Okano T, Kondo T (2000) Periodate oxidation of crystalline cellulose. Biomacromolecules 1:488-492

52. Jain RK, Lal K, Bhatnagar HL (1989) Thermal Degradation of Cellulose Esters and their Tosylated Products in Air. Polymer Degradation and Stability 26 (2):101-112

53. Heinze T, Rahnc K, Jaspersa M, Berghmansa H, Jena D (1996) p-Toluenesulfonyl esters in cellulose modifications : acylation of remaining hydroxyl groups. Macromolecular chemistry and physics 197:4207-4224

54. Kong F, Hu YF (2012) Biomolecule immobilization techniques for bioactive paper fabrication. Analytical and Bioanalytical Chemistry 403:7-13. doi:10.1007/s00216-012-5821-1

55. Di Risio S, Yan N (2010) Adsorption and inactivation behavior of horseradish peroxidase on various substrates. Colloids and surfaces B Biointerfaces 79:397-402

56. Penfold J, Tucker I, Petkov J, Thomas RK (2007) Surfactant adsorption onto cellulose surfaces. Langmuir The Acs Journal Of Surfaces And Colloids 23:8357-8364

57. Ivnitski D, Abdel-Hamid I, Atanasov P, Wilkins E (1999) Biosensors for detection of pathogenic bacteria. Biosensors and Bioelectronics 14 (7):599-624. doi:10.1016/s0956-5663(99)00039-1

58. Kapperud G, Vardund T, Skjerve E, Hornes E, Michaelsen TE (1993) Detection of pathogenic Yersinia enterocolitica in foods and water by immunomagnetic separation, nested polymerase chain reactions, and colorimetric detection of amplified DNA. Applied and environmental microbiology 59 (9):2938-2944

59. Pires ACDS, Soares NDFF, da Silva LHM, da Silva MDCH, De Almeida MV, Le Hyaric M, Andrade NJD, Soares RF, Mageste AB, Reis SG (2011) A colorimetric biosensor for the detection of foodborne bacteria. Sensors and Actuators B: Chemical 153 (1):17-23. doi:10.1016/j.snb.2010.09.069

60. Rahn K, Diamantoglou M, Klemm D, Berghmans H, Heinze T (1996) Homogeneous synthesis of cellulose p-toluenesulfonates in N , N-dimethylacetamidel LiCl solvent system. ANGEWANDTE MAKROMOLEKULARE CHEMIE 238:143-163

61. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9 (7):671-675. doi:Doi 10.1038/Nmeth.2089

62. Defrancq E, Hoang A, Vinet F, Dumy P (2003) Oxime bond formation for the covalent attachment of oligonucleotides on glass support. Bioorganic & Medicinal Chemistry Letters 13:2683-2686

63. Okutucu B, Telefoncu A (2004) Covalent attachment of oligonucleotides to cellulose acetate membranes. Artificial cells blood substitutes and immobilization biotechnology 32:599-608

64. Arora K, Prabhakar N, Chand S, Malhotra BD (2007) Immobilization of single stranded DNA probe onto polypyrrole-polyvinyl sulfonate for application to DNA hybridization biosensor. Sensors and Actuators B: Chemical 126 (2):655-663. doi:10.1016/j.snb.2007.04.029

65. Storhoff JJ, Elghanian R, Mucic RC, Mirkin CA, Letsinger RL (1998) One-Pot Colorimetric Differentiation of Polynucleotides with Single Base Imperfections Using Gold Nanoparticle Probes. journal of American Chemical Society 7863 (97):1959-1964

66. Carrilho E, Martinez AW, Whitesides GM (2009) Understanding Wax Printing: A Simple Micropatterning Process for Paper-Based Microfluidics. Analytical Chemistry 81 (16):7091-7095. doi:Doi 10.1021/Ac901071p

67. Mir M, Lozano-Sánchez P, Katakis I (2008) Towards a target label-free suboptimum oligonucleotide displacement-based detection system. Analytical and bioanalytical chemistry 391:2145-2152. doi:10.1007/s00216-008-2119-4

68. Chen C-P, Ganguly A, Lu C-Y, Chen T-Y, Kuo C-C, Chen R-S, Tu W-H, Fischer WB, Chen K-H, Chen L-C (2011) Ultrasensitive in situ label-free DNA detection using a GaN nanowire-based extended-gate field-effect-transistor sensor. Analytical chemistry 83 (6):1938-1943. doi:10.1021/ac102489y

69. dos Santos Riccardi C, Kranz C, Kowalik J, Yamanaka H, Mizaikoff B, Josowicz M (2008) Label-free DNA detection of hepatitis C virus based on modified conducting polypyrrole films at microelectrodes and atomic force microscopy tip-integrated electrodes. Analytical chemistry 80 (1):237-245. doi:10.1021/ac701613t

70. Wang J, Li S, Zhang Y (2010) A sensitive DNA biosensor fabricated from gold nanoparticles, carbon nanotubes, and zinc oxide nanowires on a glassy carbon electrode. Electrochimica Acta 55 (15):4436-4440. doi:10.1016/j.electacta.2010.02.078

71. Teles F, Fonseca L (2008) Trends in DNA biosensors. Talanta 77 (2):606-623. doi:10.1016/j.talanta.2008.07.024

72. Kim Y, Sohn D, Tan W (2008) Molecular beacons in biomedical detection and clinical diagnosis. International journal of clinical and experimental pathology 1 (2):105-116

73. Qian X, Zhou X, Nie S (2008) Surface-enhanced Raman nanoparticle beacons based on bioconjugated gold nanocrystals and long range plasmonic coupling. Journal of the American Chemical Society 130 (45):14934-14935. doi:10.1021/ja8062502

74. Bai X, Shao C, Han X, Li Y, Guan Y, Deng Z (2010) Visual detection of sub-femtomole DNA by a gold nanoparticle seeded homogeneous reduction assay: toward a generalized sensitivity-enhancing strategy. Biosensors & bioelectronics 25:1984-1988. doi:10.1016/j.bios.2010.01.012

75. Tam PD, Van Hieu N, Chien ND, Le A-T, Anh Tuan M (2009) DNA sensor development based on multi-wall carbon nanotubes for label-free influenza virus (type A) detection. Journal of immunological methods 350:118-124. doi:10.1016/j.jim.2009.08.002

76. Menéndez C, Samper S, Otal I, García MJ, Universitario H, Servet M (2012) IS 6110 the Double-Edged Passenger. In: Cardona P-J (ed). InTech, pp 59-89. doi:10.5772/2479

77. Almassian DR, Cockrell LM, Nelson WM (2013) Portable nucleic acid thermocyclers. Chem Soc Rev 42 (22):8769-8798. doi:Doi 10.1039/C3cs60144g

Table 1: Elemental analysis of control films and tosylated cellulose and degree of substitution in these samples calculated based on sulphur content

Substance	Elemental Analysis		Degree of substitution (Sulphur)
	Sulphur (%)	Chlorine (%)	
cellulose (control)	<0.10	2.70	0.00565
Replicate	<0.10	2.68	0.00565
Tosylated cellulose	4.05	2.72	0.283
Replicate	4.33	2.84	0.308

Table 2: Efficiency of oligonucleotide probe immobilization on tosylated cellulose

Amount of Probe added (nmoles)	**Amount of probe immobilized covalently(nmoles)**	**Percentage immobilization(%)**
0.05	0.045	90.08
0.1	0.091	91.088
0.15	0.124	82.59
0.2	0.195	97.44
0.25	0.232	93.16
0.3	0.192	63.95
0.35	0.165	47.21
0.4	0.370	92.70
0.45	0.340	75.73
0.5	0.478	95.76

Figure legends

Figure 1: FTIR spectra of (a) tosylated cellulose, (b) cellulose film, (c) cellulose powder

Figure 2: Scanning electron microscope images of (a) tosylated cellulose, (b) cellulose film, (c) whatman grade 40 filter paper, (d) cellulose powder

Figure 3: Immobilization quantities of fluorescein labelled oligonucleotide probes on tosylated cellulose derived from area under the curve of fluorescence intensity measurements.

Figure 4: (i) Schematic representation of biosensor method. (ii) Colour development on thiol probe immobilized tosylated cellulose strips after hybridization with (a) complementary (target), (b) non-complementary (random) and (c) control

Figure 5: (i) Sensitivity analysis of the assay with target and random sequences at concentrations (a) 1µM (b) 0.5µM (c) 0.1 µM (d) 0.05 µM (e) 0.001µM (f) control. **(ii)** Mean gray area pixel intensity of scanned images analysed with ImageJ software.

Figure 6: (i)Specificity analysis of the assay with (a) 100% complementary target, (b) single base mismatch, (c) double base mismatch, (d) triple base mismatch, (e) non-complementary (randomised probe) and (f) negative control without addition of oligonucleotides. (ii) Assay specificity quantification with mean gray area analysis of pixel intensity of scanned samples.

Figure 7: Hybridization time analysis for assay with mean gray area analysis of scanned samples.

Figure 8: (i) ~74bp PCR products in 2% agarose gel. (1) 25bp ladder; (2 & 3) bands from a PCR product complementary to the probe; (5 & 6) bands from a non-complementary PCR product; (4 & 7) controls without template DNA; (8) 50bp ladder. **(ii)** Assay (colour development) performed with PCR products corresponding to (a) region complementary to probe (b) non complementary region (c) negative control (without DNA).