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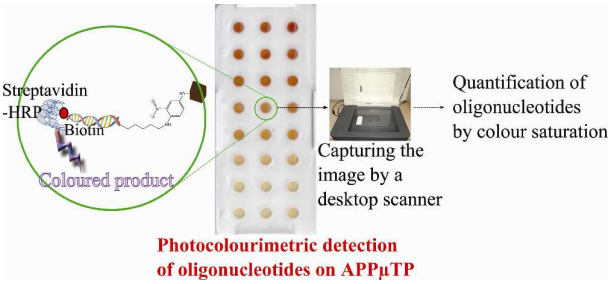
Graphical Abstract

Graphical representation of image-based (photocolorimetric) detection of oligonucleotides on APPμTP



Novel aspects

A simple and low cost image-based quantitative detection of oligonucleotides without using spectrophotometer or fluorometer.

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Image-based detection of oligonucleotides- a low cost alternative to spectrophotometric or fluorometric method

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Abstract

Herein, we report a sensitive and low cost image-based (photocolourimetric) method for detection of oligonucleotides on an activated polypropylene microtest plate (APPµTP). The assay was developed on APPµTP by covalently immobilizing 20-mer amino modified oligonucleotides. Biotin-tagged complementary target sequences were then hybridised with the immobilised oligonucleotides. Colour was developed by streptavidin-HRP conjugate and the image of the colour assay solution was taken by a desktop scanner and analysed using colour saturation. Developed method was analysed for its detection limit, accuracy, sensitivity and interference. The linearity range was found to be 1.7 - 170 ng mL^{-1} while the lower limit of detection and limit of quantification were 1.7 and 5.6 ng mL^{-1} respectively. The method showed comparable sensitivity to fluorometric method, and found to be correlated to fluorescence (R^2 = 0.8081, p-value < 0.0001) and absorbance (R^2 = 0.9394, p-value < 0.0001) based quantification. It discriminates mismatched base sequences from perfectly matched sequences efficiently. Validation of the method was carried out by detecting *por A* DNA of *Neisseria meningitidis* in bacterial meningitis samples. The *por A* specific probe having 6-carbon spacer at its 5´-NH_2 terminus was immobilised covalently to APPµTP and hybridised with different samples of biotinylated single stranded *por A* DNA.

Keywords

Photocolourimetric detection; activated polypropylene microtest plate; *Neisseria meningitidis*; *por A* DNA; hybridisation assay.

Introduction

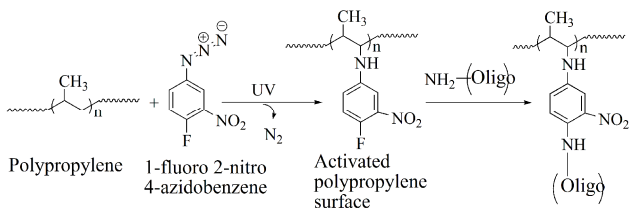
Detection of specific oligonucleotide sequences is crucial in medical research, disease diagnosis and bacterial detection in food and drinks[1]. Most of the assays developed for identification of specific sequence involve the hybridisation of an immobilised probe to the labelled target analytes[2-4]. Miniaturized solid surfaces for oligonucleotide immobilisation and hybridisation detection and analysis have become increasingly more attractive in many molecular applications including oligonucleotide microarrays[5], biosensors[6-10] and drug discovery methods[11]. These platforms utilise solid support bound oligonucleotide probes to capture single-strand targets through hybridisation. And the most convenient way to produce an oligonucleotide immobilised surface is to synthesize individual modified oligonucleotides and subsequently immobilise them to a solid surface[12]. For immobilisation to take place, solid surface must undergo surface treatment to introduce active functional group. Commonly available surface treatment chemistry for two dimensional solid surfaces employs epoxy silane, isothiocyanate, aminophenyl, aldehydes and epoxides. Choice of an appropriate support and attachment chemistry depends upon the functional group on oligonucleotides,

chemical stability of construct, background scattering inherent to support and modification, loading capacity and the degree of non-specific binding of the analytes[13]. Optical, electrochemical and surface plasmon resonance (SPR) are mostly used methods reported for detection of hybridisation to surface bound probes[14-20]. Commercial preparations available for oligonucleotide immobilisation and further hybridisation based detections are costly and cumbersome along with involvement of number of parameters to be optimized for efficient and effective experimental design. Even the colourimetric and fluorometric detection require expensive experimental setup and measurement instrumentation along with large volume of analytes.

Here, we develop a photocolourimetric method for detection of oligonucleotides. The unique photocolourimetric detection strategy consists of covalent immobilisation of oligonucleotide onto 1-fluoro-2-nitro 4-azidobenzene (FNAB) derivatized polypropylene microtest plate (APPµTP). The biotin-labelled target sequence hybridises to immobilised probes, and assayed by streptavidin-HRP conjugate. The developed colour is then scanned by a desktop scanner and quantified by a colour space. The method is validated by detecting *Neisseria meningitidis* DNA causing bacterial meningitis in samples from meningitis patients. Bacterial meningitis, caused by the presence of *N. meningitidis* in cerebrospinal fluid, is usually detected through biochemical tests[21], microscopy[22], PCR based assays[23], microarrays[24] and DNA biosensors[6-10]. The present photocolourimetric approach for DNA based detection of bacterial meningitis on APPµTP does not require costly instrument.

Results and discussion

We have activated polypropylene microtest plate (PPµTP) and polystyrene microtiter plates through a photolinker, FNAB. FNAB coated wells are made by adding methanolic solution of FNAB followed by evaporation of methanol under dark. FNAB coated wells are then subjected to UV irradiation at 365 nm. UV exposure of FNAB results in generation of highly reactive nitrene intermediate that inserts into a C-H bond of the polymer surface. Surface activation through FNAB is the quickest step in whole method, taking 5-10 min for surface coating and 12 min for UV mediated insertion of linker onto the plate (Scheme 1).



Scheme 1. Photochemical activation of polypropylene polymer surface using FNAB.

Resulted surface is slightly hydrophobic and is used for immobilisation of aminated oligonucleotides. In fact, any biomolecule bearing nucleophilic group can be immobilised on such surface.

Presence of $-NH_2$ moieties on nitrogenous bases of oligonucleotides make them susceptible to compete with 5'-end labelled amino group for binding to activated surface. Thus, to check if any nucleophilic groups on nucleotide bases, is involved in covalent linkage, we have immobilised 5'- amino modified oligonucleotide (probe A) as well as oligonucleotide without any modification (probe B) to APPµTP. Efficient immobilisation is observed with probe A whereas probe B which lacked terminal amine group shows negligible immobilisation (Fig. S1). This confirms that APPµTP specifically immobilise to 5'-amino modified oligonucleotides which is desirable. Further, we have optimized time and temperature for oligonucleotide immobilisation on APPµTP (Fig. S2). Maximum immobilisation of oligonucleotides was observed at 60^0C in 60 minutes. The reaction occurs through thermochemical nucleophilic substitution of fluoro group on the activated surface by the end labelled amine moiety of the probe. Optimum time for hybridisation of *por A* gene to immobilised probe at room temperature is also studied (results not shown); Prior to hybridisation, double stranded PCR product of *por A* gene is denatured at 95°C to get single stranded target. At RT the maximum hybridisation is achieved in 30 min when denatured targets are allowed to hybridise. This implies that the time frame of 30 min is enough for unfolded single stranded target sequences present in denatured sample to interact and form stable hybridisation with their complementary probes present on APPµTP.

Lower limit of detection (LOD) of 254 pM (1.7 ng mL^{-1}) and limit of quantification (LOQ) of 852 pM (5.6 ng mL^{-1}) for photocolourimetric method on APPµTP is determined by hybridising different concentrations (10 fM to 1000 nM) of 20-mer target to immobilised probe and plotting the oligonucleotide concentrations against colour saturation percentage. LOD and LOQ are calculated using following equation:

$$LOD = 3\,(SD/S)$$

$$LOQ = 10\,(SD/S)$$

Where SD is the standard deviation of the response and S is the slope of the calibration curve. The standard deviation of the response is determined based on the standard deviation of y-intercept of regression line. The photocolourimetric detection shows a linear rise in colour saturation with increase in oligonucleotide concentrations in dynamic range of 254 pM to 25 nM (1.7 ng mL^{-1} to 170 ng mL^{-1}), with limit of linearity (LOL) of 25 nM and correlation coefficient of 0.9690 (Fig. 1). The method is further checked with equimolar concentrations of three distinct probes (probe 1, 2 and 3) and is found to produce similar hybridisation for all perfectly matched targets (target 1a,2, 3) when quantified photocolourimetrically (Fig. 2).

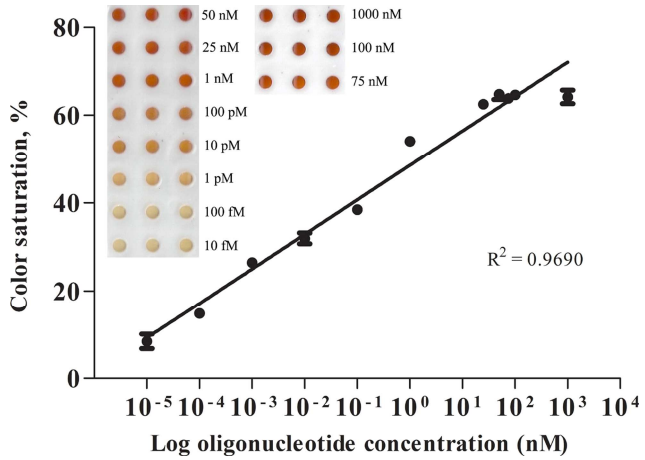


Fig.1. Concentration dependent hybridisation assay on APPµTP. Calibration curve is drawn by assaying different concentrations of target 1a by hybridising with immobilised-probe. Quantification is done by colour saturation values. The readings are plotted after subtracting blank. Inset shows respective scanned images.

Detection of single base mismatch mutations is quite important in disease diagnostics. Clear base-line separations of randomized sequences from target sequence in all four sets of probe-targets compel us to seek for mismatch discrimination potential of the photocolourimetric method. Mismatch detection is studied by hybridizing four distinct sequences (Target 1a, M1, M2 and R) varying in their underlying base sequences from each other by one or more bases, to immobilised-probe 1 on APPµTP. Salt concentration is an important aspect for hybridisation. We have performed hybridisation experiment using different salt concentrations. Lower salt (0.2M NaCl) concentration is found to improve mismatch sequence discrimination by photocolourimetric method (Fig. S3).

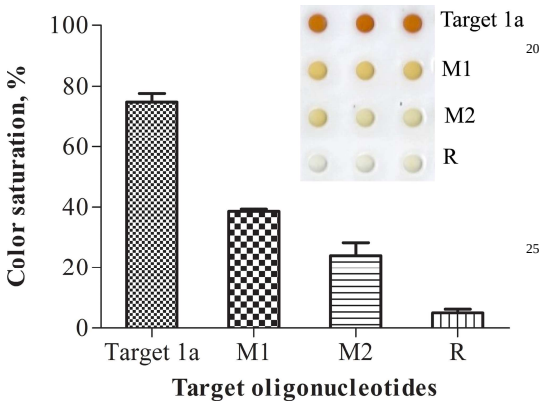


Fig. 3. Mismatch hybridisation assay. Probe 1 was hybridised to four distinct target oligonucleotides differing in their complementarities. Perfect complementary sequence (Target 1a) showed maximum hybridisation which decreases in single base mismatch (M1) and three base mismatch sequences (M2). Random sequence (R; used as a control) showed negligible hybridisation. Inset shows respective scanned images.

Target 1a shows maximum hybridisation whereas decrease in hybridisation is observed upon introduction of single base compared to perfectly matched sequence.

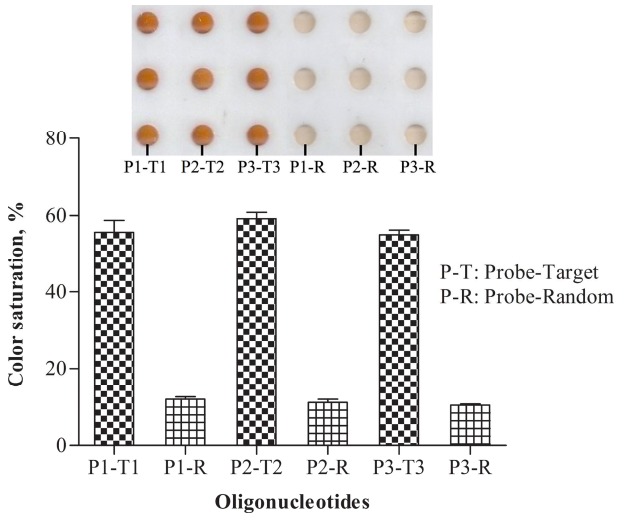


Fig.2. Hybridisation of three different target oligonucleotides to their respective probes immobilised on APPµTP. P1, P2 and P3 are Probe 1, Probe 2 and Probe 3, whereas T1, T2 and T3 are Target 1a, Target 2 and Target 3, respectively. Random sequence (R) was taken as control in each set of hybridisation assay. Hybridisation was quantified photocolourimetrically. Inset shows respective scanned images.

Therefore, the method excellently discriminates perfectly matched sequences from base mismatched and random sequences showing its mismatch (M1). In contrast, triple base mismatch (M2) and random mismatch (R) sequences show negligible hybridisation specificity (Fig. 3; S3).

Further, to illustrate the validity of photocolourimetric method for oligonucleotide detection, we compare the photocolourimetric method with spectrophotometric and fluorometric methods. For this we have hybridised same concentrations (0.5 nM to 100 nM) of biotin and FAM labelled targets to immobilised-probe. Hybridisation is detected spectrophotometrically, fluorometrically and photocolourimetrically on transparent microtiter plates, black microtiter plates and APPµTPs, respectively. Colorimetric measurements are performed by measuring the absorbance at 490 nm on spectrophotometer whereas the fluorometric measurements are carried out by measuring the fluorescence at 495/516 excitation/ emission wavelength on fluorimeter. The photocolourimetric detection of hybridised sequences is carried out on a desktop scanner captured images by measuring the colour saturation of the image. Concentration dependent detection of oligonucleotides through photocolourimetric (colour saturation) and spectrophotometric (absorbance) methods show strong correlation of 0.9394 (Pearson's product-moment correlation, $p < 0.0001$) (Fig. 4. A), while photocolourimetric and fluorometric methods show a positive correlation of 0.8081 (Pearson's product-moment correlation, $p < 0.0001$) (Fig. 4.B).

Sensitivity of the method is assessed by calculating the slope of calibration curves obtained by plotting normalized values of fluorometric, colorimetric and photocolourimetric quantifications against oligonucleotide concentration. The slope of calibration curves is calculated to be 41.35, 39.96 and 37.59

for fluorometric, colorimetric and photocolourimetric quantifications, respectively (Fig. 5).

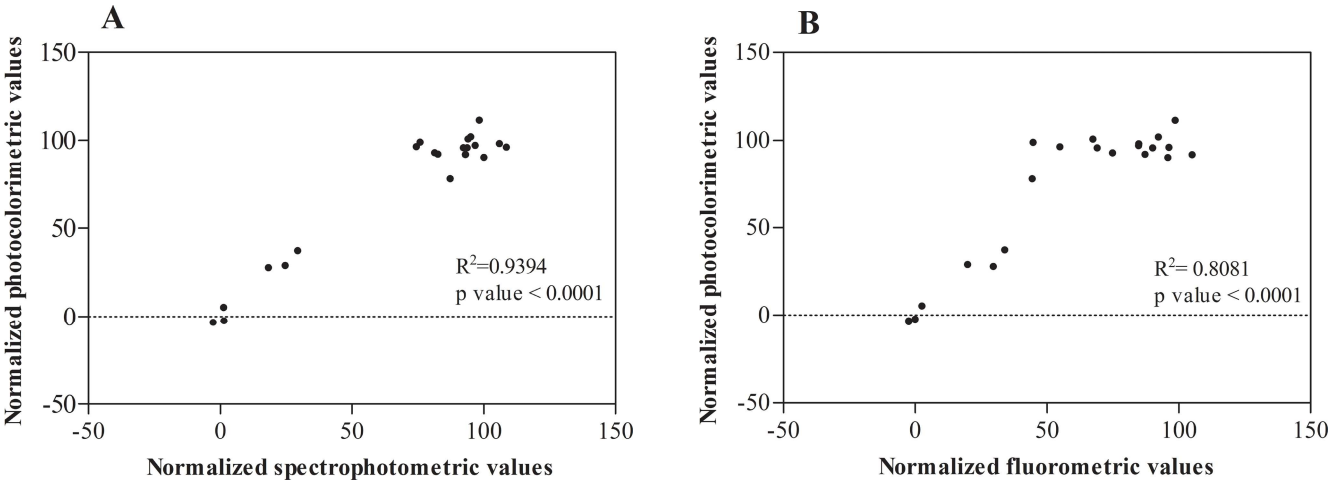


Fig. 4. Comparative study of image-based detection with spectrophotometric (A) and fluorometric (B) quantifications. Different concentrations (0.5, 1, 2.5, 5, 10, 50 and 100 nM) of a target oligonucleotide were hybridised and detected by different methods. All values were normalized. R values indicate correlation.

This signifies that the present method is comparable to colourimetric and fluorometric methods. Preciseness of the method is expressed as coefficient of variation (CV). The photocolourimetric method is found to be precise, with CV < 5-15% when analysed for 1 nM, 10 nM, 25 nM and 100 nM oligonucleotide concentrations. Within-run precision is validated by analysing five samples per concentration for mentioned concentrations in a single run. The within-run CV values are 10.95%, 12.56%, 7.53% and 4.71% for 1 nM, 10 nM, 25 nM and 100 nM respectively.

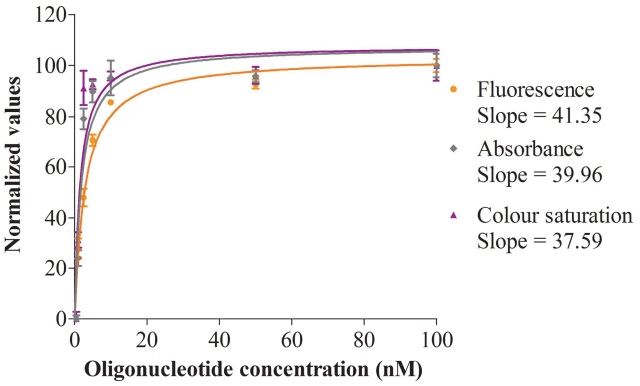


Fig. 5. Various concentrations (0.5-100 nM) of target 1a detected through fluorometric, colourimetric and photocolourimetric methods. Normalized values of colour saturation, absorbance and relative fluorescence units were plotted against various concentrations of oligonucleotide detected.

The method is also validated by performing detection of bacterial meningitis. A *N. meningitidis* specific probe (5´-TTCCTATCGCTTCGGTAATG-3´) based on *por A* gene is used to diagnose the disease in patient samples. Whole genomic DNA isolated from bacterial samples of meningitis is PCR (asymmetric) amplified to get labelled amplified product. The labelled 211 bp product is hybridised to immobilised *por A* probe and detected photocolourimetrically. When same amount of *por A* DNA from three different samples of meningitis is analysed by photocolourimetric method, equivalent colour saturation is achieved. *N. meningitidis* DNA is easily distinguished from random DNA, showing the high sensitivity and specificity of photocolourimetric method (Fig. 6). In all sets of experiments, no DNA sequence and random DNA sequence are used as blank and control, respectively to rule out the false colour development in absence of target sequence and specify the specificity of the photocolourimetric method.

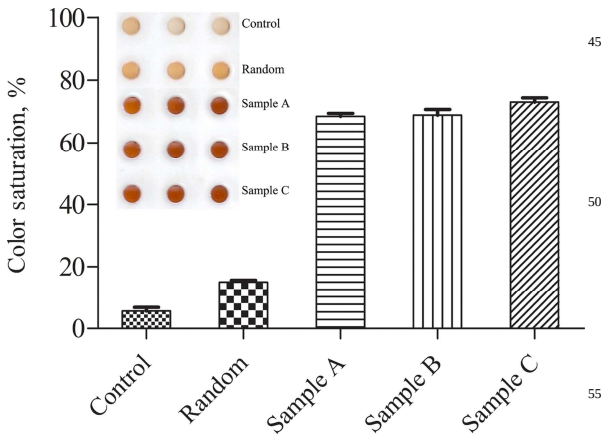


Fig. 6. Photocolourimetric detection of *N. meningitidis* samples on APPμTP. Immobilised *por A* probe was hybridised with A, B and C (100 ng each) *por A* DNA from three different samples of meningitis.

Thus, the photocolourimetric method of oligonucleotide detection is sensitive and accurate with minimum interference. For the first time, we have used FNAB to immobilise aminated oligonucleotides on a polypropylene surface. The complete process of surface modification and probe immobilisation is time saving, requiring only 60-90 minutes. The method does

not require spectrophotometer or fluorimeter for quantitation of coloured assay solution; instead the colour is detected and quantified photocolourimetrically. For this, a common desktop scanner is used to get the image of the assay solution and the image is quantified digitally by a colour space (colour saturation). The concept of utilising saturation colour space for colour quantification provides an economic alternative to conventional colorimetric quantifications. Present photocolourimetric method can be applied to quantify oligonucleotides in range of 1.7-170 ng mL^{-1} with least detection at 1.7 ng mL^{-1}. Validation of method, performed by detecting bacterial meningitis proves the potential of present method to be used in point of care diagnostics.

Experimental

Materials

Aminated *por A* probe (5´-NH2-TTCCTATCGCTTCGGTAATG-3´), forward primer (5´-TTCCTATCGCTTCGGTAATG-3´) and reverse primer (5´-Biotin/FAM-GGCGGCATTAATTTGAGTGT-3´) were purchased from Integrated DNA Technologies, USA. Other oligonucleotides (Table 1) were purchased from Midland Certified Reagent Company, USA. Bovine serum albumin (BSA), streptavidin- horse radish peroxidase (HRP) conjugate, sodium dodecyl sulfate (SDS), o-phenylenediamine dihydrochloride (OPD), DNA taq polymerase, PCR buffer, dNTPs and DNA ladder were purchased from Sigma-Aldrich, USA. 96-Well polystyrene microtiter plates were purchased from Greiner Bio-One, Germany. Phosphate buffer saline (0.1M, pH 7.2± 0.2, 0.7 M NaCl) and PBS-SDS (0.1M, pH 7.2 ± 0.2, 0.7 M NaCl, 0.1% SDS) were prepared in Millipore MQ water. HRP substrate dye (2.5 µg OPD, 2.5 µl H_2O_2 in 6 mL 0.1 M citrate buffer, pH 5.0 ± 0.2) was used for colour development. The image of the coloured solution was scanned using HP Photosmart C6300 All-in-One Printer. Non-linear regression, Pearson's correlation and column statistics were performed using GraphPad Prism version 5.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. All experiments were performed in triplicates and the mean values were used for graphical presentation.

Methods

Photochemical activation of PPµTP and polystyrene plate

Activation of PPµTP was carried out by a photolinker, 1-fluoro-2-nitro-4-azidobenzene (FNAB) as reported earlier[25]. Briefly, FNAB solution (0.125 mg/3 µl of methanol/well) was poured to each well of PPµTP and allowed to air dry in the dark. After complete evaporation of solvent, the photolinker-coated plate was exposed to UV light at 365 nm for 12 min in an UV Stratalinker (Model-2400; Stratagene, USA). After irradiating for stipulated time, the wells of the APPµTP were washed three times with methanol and dried. Polystyrene plates were activated as described earlier[26].

Table 1. Oligonucleotides used in the study

Oligonucleotide	Length	Sequence
Probe A	20	5´ Biotin-GCAACTGTAAAACGTCTTGT-NH_2 -3´
Probe B	20	5´ Biotin- GCAACTGTAAAACGTCTTGT -3´
Probe 1	20	5´ NH_2- ACAAGACGTTTTACAGTTGC-3´
Probe 2	20	5´ NH_2- ATGTGGAAAATCTCTAGCAG-3´
Probe 3	20	5´ NH_2- TCGGGGTTTTGGGTCTGACG-3´
Target 1(a)	20	5´ Biotin-GCAACTGTAAAACGTCTTGT-3´
Target 1(b)	20	5´ (6-FAM)-GCAACTGTAAAACGTCTTGT -3´
Target 2	20	5´ Biotin-CTGCTAGAGATTTTCCACAT -3´
Target 3	20	5´ Biotin-CGTCAGACCCAAAACCCCGA-3´
Mismatch 1 (M1)	20	5´ Biotin-GCAACTGTACAACGTCTTGT-3´
Mismatch 2 (M2)	20	5´ Biotin-GCAGCTGTACAACGACTT GT-3´
Random (R)	20	5´ Biotin-AGTTCGATCATTCATCTAAG-3´

Immobilisation of oligonucleotide onto the activated plates

The covalent coupling of 5´-amine labelled (probe A) and unlabelled (probe B) oligonucleotides was carried out by adding 10 µl of 100 nM probes dissolved in PBS to the wells of APPµTP. Time and temperature dependent immobilisation of aminated probe onto APPµTP were optimized by incubating at 40°C, 50°C and 60°C for 15, 30, 60 and 90 minutes. After incubation for the stipulated time periods, any unbound probe not adhered to APPµTP were washed by four washes of washing buffer (PBS-SDS).

Hybridisation of target oligonucleotides

Probe 1 was immobilised onto APPµTP at 60°C for 60 min incubation as described. Different amount of analyte oligonucleotides (10 fM -1000 nM target 1a) in PBS buffer were added (10 µl each) to the wells of the probe-immobilised APPµTP and incubated at room temperature for 30 min for hybridisation. Similarly immobilisations were carried out with Probe 2 and Probe 3 followed by their hybridisation with Target 2 and Target 3, respectively.

Photocolourimetric detection of hybridisation

Wells of APPµTP having hybridised sequences were loaded with 10 µl of streptavidin-HRP conjugate (1:3000 dilution) in 0.01 M PBS (10 mM phosphate buffer, pH 7.2, 150 mM NaCl) and incubated at room temperature (~27°C) for 30 minutes. Unbound conjugate was removed by washing with washing buffer. Colour development was carried out by adding 8 µl/well of substrate- dye solution. After 10 min, the colour development reaction was stopped by addition of 2 µl of 5% H_2SO_4. The developed colour was captured as an image and quantified as reported earlier[26]. Briefly, The APPµTP was scanned on a desktop scanner (HP photo smart C6388) by placing the plate upside down to get the image. Alternatively, a mobile camera can also be used to get the image of the assay solution. Using Adobe Photoshop, the mean value for R, G and B colour of the scanned image was obtained. These RGB values were then converted to HSB (Hue, Saturation, and

Brightness) using freely available "Macbeth colour calculator" software. Image was then quantified as saturation percentage.

Mismatch detection

Covalent immobilisation of probe1 on APPµTP was carried out as mentioned in oligonucleotide immobilisation section. To optimize the salt concentration, 100 nM of each target sequences (M1, M2, R and Target 1a) was dissolved in phosphate buffer having different salt concentrations (0.2M, 0.4M, 0.8M 1.0M) and added to the wells of APPµTP coated with probe 1. The hybridised sequences were then detected using streptavidin-HRP conjugate and the developed colour was quantified photocolourimetrically.

Correlation of photocolourimetric method with absorbance and fluorescence based assays

For correlation study, 100 nM of probe 1 (100 µl/well) was immobilised to FNAB activated transparent microtiter plate and black microtiter plate by incubating at 60°C for 60 minutes. The immobilised probe was then hybridised with different amount of targets (0.5, 1, 2.5, 5, 10, 50 and 100 nM). In the wells of transparent microtiter plate, biotinylated oligonucleotide (target 1a) was added, whereas the wells of black microtiter plate were loaded with FAM labelled oligonucleotide (target 1b). Biotinylated target 1a on transparent microtiter plate was detected colorimetrically by taking absorbance at 490 nm in a spectrophotometer. On the other hand, the FAM labelled target 1b on black microtiter plate was detected through fluorescence measurements carried out at 495/516 excitation/emission on TECAN Infinite 200 PRO (Switzerland). The photocolourimetric detection was performed using probe 1 and target 1a on APPµTP.

Detection of *N. meningitidis* using photocolourimetric method

The *por A* probe (100 nM in 10 µl PBS/well) was added to the wells of APPµTP and incubated at 60^0C for 60 minutes. Unbound probe were removed by four washes of washing buffer. Whole genomic DNA of *N. meningitidis* was isolated from patient samples and amplified by asymmetric PCR using primers specific for *por A* gene. The amplification conditions were, initial denaturation- 95^0C for 5 min, denaturation- 95^0C for 4 s, annealing- 55^0C for 5 s, extension- 72^0C for 1 s and a final extension of 72^0C for 3 min for total cycles of 30. The PCR amplified biotinylated product was heat denatured followed by addition of 10 µl of product to the well of APPµTP immobilised with probe. The hybridised *por A* DNA was detected photocolourimetrically. Two more samples of bacterial meningitis were analysed in the similar way by photocolourimetric method. Experiments were done in the similar way using random sequence instead of *por A* DNA.

Conclusions

We have developed a novel and low cost photocolourimetric method for oligonucleotide detection on polypropylene microtest plates. Photoreactive FNAB provides excellent choice of linker for modification of vast range of polymer surfaces including polypropylene and polystyrene. Probe immobilisation is less cumbersome and achieved easily at room temperature to 60°C incubation in 30-60 min, compared to 2-4 h or overnight incubation required in most of the commercial preparations. The hybridisation is detected photocolourimetrically. The developed colour was captured as an image by a desktop scanner and quantified using saturation colour space. This eliminates the need of costly instruments for colour quantification. The method is sensitive, exhibiting low limit of detection and limit of quantification. In case of unavailability of scanner, the colour quantification can be performed on a camera or mobile captured images too. The method is highly specific and easily discriminates single mismatch, double mismatch and multiple mismatched sequences from target sequence. Also, the photocolourimetric method is comparable to spectrophotometric and fluorometric methods for quantification of oligonucleotides in terms of its sensitivity, reproducibility and precision. Therefore, the photocolourimetric method could be a useful alternative either to spectrophotometric or fluorometric method or point of care diagnostics for rapid detection of oligonucleotides.

Acknowledgements

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Notes and references

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