AWARD LECTURE / CONFÉRÉNCE D'HONNEUR

A fibre-optic biosensor for detection of microbial contamination[1]

Amer Almadidy, James Watterson, Paul A.E. Piunno, Inge V. Foulds, Paul A. Horgen, and Ulrich Krull

Abstract: A fibre-optic biosensor is described for detection of genomic target sequences from *Escherichia coli*. A small portion of the LacZ DNA sequence is the basis for selection of DNA probe molecules that are produced by automated nucleic acid synthesis on the surface of optical fibres. Fluorescent intercalating agents are used to report the presence of hybridization events with target strands. This work reviews the fundamental design criteria for development of nucleic acid biosensors and reports a preliminary exploration of the use of the biosensor for detection of sequences that mark the presence of *E. coli*. The research work includes consideration of the length of the strands and non-selective binding interactions that can potentially block the selective chemistry or create background signals. The biosensors were able to detect genomic targets from *E. coli* at a picomole level in a time of a few minutes, and dozens of cycles of use have been demonstrated. In a step towards the preparation of a completely self-contained sensor technology, a new intercalating dye known as SYBR 101 (Molecular Probes, Inc.) has been end-labelled to the LacZ nucleic acid probe, to examine whether dye tethered onto an oligonucleotide terminus could fluorimetrically transduce the formation of hybrids. The results obtained from experiments in solution indicate that the use of tethered dye provides fluorescence signals that are due to hybridization, and that this process is functional even in the presence of a high concentration of non-selective background DNA obtained from sonicated salmon sperm.

Key words: biosensor, DNA, fibre optic, hybridization, fluorescence, pathogen, *E. coli*.

Résumé : On décrit une fibre optique servant de biosenseur pour la détection du génome cible dans la séquence du *Escherichia coli*. Une petite portion de la séquence du LacZ-ADN sert de base pour la sélection des molécules sondes d'ADN obtenues par la synthèse automatique d'acides nucléiques à la surface des fibres optiques. On a utilisé des agents intercalants fluorescents pour mettre en évidence la présence de phénomènes d'hybridation avec la fibre cible. Ce travail passe en revue le critère de conception fondamental de développement de biosenseurs d'acides nucléiques, et rapporte également une exploration préliminaire de l'utilisation des biosenseurs pour détecter les séquences qui témoignent de la présence du *E.coli*. Les travaux de recherche tiennent compte de la longueur de la fibre et des interactions liantes non sélectives qui peuvent potentiellement bloquer la sélectivité chimique ou créer un bruit de fond. Les biosenseurs ont été capables de détecter le génome cible à partir du *E. coli* au niveau de la picomole et en quelques minutes, on a ainsi pu effectuer des douzaines de cycles d'études. Dans une étape en vue de la préparation de la technologie du senseur auto-contenu, on a marqué un nouveau colorant intercalaire connue sous le nom de SYBR 101 (Molecular Probes, Inc.) à l'extrémité de la sonde d'acide nucléique LacZ, pour voir si le colorant attaché dans l'oligonucléotide terminal peut fluorométriquement induire la formation d'hybrides. Les résultats obtenus à partir des expériences en solution indiquent que l'utilisation de colorant attaché fournit des signaux fluorescents qui sont dus à l'hybridation, et que ce processus est fonctionnel même en présence d'une forte concentration d'ADN d'arrière plan non sélectif obtenue à partir du sperme de saumon traité à l'ultrason.

Mots clés : biosenseur, ADN, fibre optique, hybridation, fluorescence, pathogène, *E. coli*.

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Introduction

Design of a nucleic acid biosensor

Methods that are suitable for the routine determination of the presence of bacterial species are of obvious importance in the quality control of foodstuff and water resources everywhere. Classical methods of nucleic acid hybridization assay are often time consuming and so may be inappropriate for laboratories that require rapid turnover of results and high sample throughput (1–6). As a result, significant research has been devoted to the development of biosensors in an attempt to generate assays that are reversible, reusable, sensitive, selective, and relatively simple to use. Methods of signal transduction to detect nucleic acid hybridization on biosensor surfaces include electrochemical, piezoelectric, and optical approaches (7–11) and compete favourably with the recent developments in wet-chemical methods such as those based on the polymerase chain reaction (12, 13).

The development of biosensors must take into consideration the effects of the local environment on the binding capacity of the immobilized selective molecular recognition elements. This involves careful consideration of the nature of the solid substrate used, the method of immobilization, solution conditions under which experiments will generally be done, and probe density and length. Each of these parameters has direct consequences on the reproducibility and sensitivity of signal generation (14–17).

To examine the effects of immobilization on nucleic acid hybridization, thermal denaturation experiments were done at the surface of fibre-optic nucleic acid biosensors to determine whether trends in hybridization observed in bulk solution could be extrapolated to describe nucleic acid hybridization in an interfacial environment (17).

The immobilization of oligonucleotide probes onto the surface of fused-silica optical fibre substrates was achieved by means of a modification using a silane reagent (15, 17). The modified optical fibre substrates were then subjected to standard β-cyanoethyl-phosphoramidite oligonucleotide synthesis protocols in order to undergo the stepwise synthesis of oligonucleotides at controlled densities onto the surface of the substrates.

To examine the energetics of interfacial hybridization, the van't Hoff enthalpy changes and temperature-corrected standard enthalpy changes were computed for a series of denaturation experiments, conducted based on the method developed by Piunno and co-workers (17). This model applies to denaturation occurring within a film of immobilized nucleic acids, with the complementary DNA freely able to float in and out of the membrane. The model assumes no interaction between neighbouring strands and that the denaturation is a two-state transition. The enthalpic change accompanying denaturation in an interfacial environment was significantly lower than that observed in experiments conducted in bulk solution. The sensitivities of ΔH_{VH} (T_m) to changes in the characteristic melt temperature (T_m) were a factor of 2–4 smaller for the transitions occurring at the interface of the optical biosensors relative to those observed for the experiments done in bulk solution and were usually opposite in sign. This suggested that the changes in heat capacity that accompanied the denaturation were not the same in an interfacial environment as they were in bulk solution. This may be owing to local density changes in the nucleic acid films as a result of the denaturation. The results suggested that there may be significant differences in the nature of the base pairing in an interfacial environment compared with that which occurred in bulk solution. There did not appear to be a relationship between the packing density of immobilized oligonucleotides and the reduction in the endothermicity of the denaturation. The observed T_m values were still of comparable magnitude to those that were observed in experiments done in bulk solution, so it is likely that there is a significant difference in entropy changes accompanying hybridization and denaturation in an interfacial environment relative to those observed in experiments done in bulk solution.

The data further suggest that the selectivity of hybridization in an interfacial environment may be substantially different and advantageous in comparison with that observed in a bulk solution environment. Furthermore, the selectivity of hybridization does not necessarily follow the trend of T_m, which is seen as a function of ionic strength and oligonucleotide immobilization density. These results corroborate the notion that there is an ensemble of interactions that will occur, along with the hybridization–denaturation transition, in an interfacial environment. These interactions contribute to the overall stability of the binding of target DNA and therefore play an important role in defining the T_m values of a particular probe–target complex, as well as the shape of the thermal denaturation profile, and therefore ultimately affect the selectivity of hybridization.

The results of such work (15, 17) indicate that the choice of density of immobilized single-stranded DNA (ssDNA) does provide for control of selectivity. The optimization of the analytical function of a fibre-optic biosensor for any particular hybridization assay must consider both the issues of selectivity and the amount of fully complementary double-stranded DNA (dsDNA) that is formed, as this is the source of the analytical signal. A review of the data suggests that it is possible to select a combination of ssDNA density, solution ionic strength, and temperature that provides an improvement in the selectivity coefficient of about two orders of magnitude when comparing the formation of the fully complementary duplex to that which contains one central base-pair mismatch. Bulk solution experiments do not attain the same magnitude of selectivity coefficient. Importantly, only about 30% of the maximum amount of fully matched dsDNA is available under the conditions in bulk solution where the best case of selectivity is achieved. This does not compare favourably with the immobilized ssDNA system, where about 55% of the maximum amount of fully matched dsDNA is available under conditions where selectivity is maximized.

An important consideration in the evaluation of the sensitivity and selectivity of hybridization for a given sensor system is the nature of the sample that is being introduced. Samples may contain various levels of large, non-complementary genomic DNA and RNA molecules, which may interfere with analysis. Also, most nucleic acid sensor systems will be exposed to the target DNA of interest in double-stranded form. This imposes the requirement of denaturing these double-stranded targets so that selective hybridization may subsequently take place at the sensor

surface. In practice, this may result in a competition for hybridization of target strands in bulk solution between immobilized probe oligonucleotides and the complementary DNA in bulk solution. This competition for hybridization may impart some significant limitations on the sensitivity and selectivity of the assay. A balance can be struck between the desired sensitivity and selectivity of a given hybridization assay, and this balance is somewhat tunable by means of controlling the density of ssDNA immobilization. If non-selective adsorption occurs predominantly in regions between the immobilized oligonucleotide probes, then it may be that optimal assay sensitivity and selectivity would be achieved using a sensor with a higher density of probe molecules, where the number of exposed surface sites for non-selective adsorption is decreased.

To more accurately model the effects of interferences as experienced in a real sample, experiments were done to investigate the effects of the presence of large genomic DNA strands (20 kbp average size) on the response of the sensors to labeled oligonucleotides. The experiments were designed such that the relative concentrations of oligonucleotides and genomic DNA were adjusted. Experiments were done using concentration regimes where both fully complementary (cDNA) and non-complementary (ncDNA) oligonucleotide 20mers were introduced at a concentrations of about 10^{15} molecules L^{-1}, while genomic DNA from *E. coli* was introduced at a concentration of 10^{12} to 10^{14} molecules L^{-1} (15). The results suggest that the presence of genomic DNA as a background species does not substantially block hybridization of very short target oligonucleotides or the extent of non-selective adsorption of short non-complementary oligonucleotides. This trend was observed for all concentration regimes used. Additionally, the presence of genomic DNA did not affect the response times of the sensors in the first few minutes of an analysis. Interestingly, the pretreatment of the sensor surface with genomic DNA for 10 min reduced the response time of the sensors to cDNA. It may be that the larger genomic DNA acted to reduce the effective solution volume near the sensor surface and to increase the effective analyte concentration. This effect was more significant at the lower analyte concentration, where response times were more sensitive to changes in analyte concentration. The response time of sensors to the addition of 10^{15} molecules L^{-1} cDNA was 224 ± 5 s, while the response time after pretreatment with genomic DNA was 192 ± 5 s. The response time for addition of 10^{16} molecules L^{-1} cDNA was 28 ± 1 s, while the response time after pretreatment of the surface with genomic DNA was 21 ± 1 s.

These results have ramifications for analyses of real-world samples. The preliminary results suggest that the process of non-selective adsorption by interfering short and long nucleic acid sequences in solution may not occur in such a manner as to substantially inhibit the extent of hybridization of a target sequence when early in an analytical experiment.

A fibre-optic biosensor for *E. coli*

Coliforms are aerobic and facultatively anaerobic, gram-negative, non-spore forming bacilli, encompassing members of *Escherichia*, *Citrobacter*, *Klebsiella*, and *Enterobacter* (1–3). Although several of the coliform bacteria are not usually pathogenic themselves, they serve as an indicator of potential bacterial pathogen contamination. Using such indicators, researchers in the U.S.A. estimate that 40% of private water supplies and 70% of spring-fed supplies contain coliform bacteria (4). Coliform bacteria concentrations are determined using methods specified by the Environmental Protection Agency (EPA) and those found in ref. 5. These methods can be slow, and new biosensor technologies may offer substantial advantages in providing analyses within seconds to minutes.

Currently, several methods are used for the detection or enumeration of *E. coli* cells in water, including microbiological, serological, and immunological procedures. Polymerase chain reaction (PCR) methods have been developed, where LacZ, lamB, and uid genes have been used as targets for the design of primers for coliform detection (6). False positive and negative results can arise when using these techniques, and only a very limited number of strains have been used to test for specificity of the primers. Standard PCR generally only provides information about detection, and even when using quantitative real-time PCR, the analyses still often require hours.

Herein we report the development of a fibre-optic biosensor for the detection of short sequences of oligonucleotides that indicate the presence of *E. coli*. Single-stranded DNA (ssDNA) was immobilized by covalent binding to a fused silica optical fibre. Hybridization on the solid surface was detected by use of the fluorescent intercalating dye, ethidium bromide (EB). Testing to detect coliform contamination of water was demonstrated using selective hybridization of nucleic acid sequences. A 25mer sequence on the LacZ gene of the *E. coli* was targeted using a 25mer ssDNA probe. The investigation has shown that the biosensor was capable of detecting minute amounts of synthetic cDNA and also genomic DNA that was extracted from *E. coli*. The biosensor could provide analytical information in less than 1 min and was regenerable for many cycles of application.

Preliminary work that targets the development of a self-contained biosensor has involved attachment of the intercalating fluorescent reporter dye to the probe by means of a short molecular tether. The intercalating fluorescing dye (SYBR 101) was covalently attached through a short tether to the 25mer ssDNA (labelled DNA, L-DNA), and the fluorescence changes caused by hybridization have been investigated in bulk solution using free L-DNA. In the design of a self-contained biosensor, this approach may help reduce background fluorescence from free dye in solution, will allow internal standardization, and will substantially reduce the risk of exposure of the operator to toxic chemicals by confining the intercalating dye to the surface of the device.

Experimental

Chemicals

SYBR 101, succinimidyl ester, was donated by Molecular Probes, Eugene, Oregon. Biosynthesis-grade solvents were purchased (EM Science, Toronto, ON) and further purified or dried by standard laboratory protocols. Reagents for DNA synthesis were purchased from Dalton Chemical Laboratories Inc. (Toronto, ON) and were used as received or were prepared as below. Anhydrous acetonitrile (EM Science) was pre-dried by distillation from P_2O_5 and redistilled from

calcium hydride under dry argon. Tetrahydrofuran (EM Science) was pre-dried over CaH_2, filtered, and distilled immediately prior to use from sodium metal (Aldrich) / benzophenone (Aldrich). Water was double distilled in glass, treated with diethyl pyrocarbonate (Aldrich), and autoclaved. Molecular-biology-grade polyacrylamide gel electrophoresis reagents and apparatus were obtained through Bio-Rad (Hercules, California). Silica gel (Toronto Research Chemicals, Toronto, ON) had a particle size of 30–70 microns.

Instrumentation

Fluorescence studies of LacZ-target hybridization onto the probe at the surface of the *E. coli* biosensors were done using an optic-fibre spectrofluorimeter operated in an intrinsic-mode configuration (17). The spectrofluorimeter was equipped with a fluid-handling system for stop-flow fluorescence investigations of nucleic acid hybridization. Preliminary fluorescence studies of LacZ target to the dye labelled ssDNA in solution were done using a spectrofluorimeter instrument.

Attachment of SYBR 101 to ssDNA probe

The SYBR 101 – ssDNA probe consisted of three parts: the ssDNA, a C6 aminomodifier as a tether, and the fluorescent dye SYBR 101 (absorption 483 nm, emission 515 nm). The tether was attached to the ssDNA using an ABI 392 DNA/RNA synthesizer. C6 aminomodifier is a phosphoramidite synthon containing a six-carbon atom chain terminated by a protected amine moiety. The reagent was used in analogy to a phosphoroamidite nucleoside. The modifier was activated with tetrazole to form an active intermediate that coupled to the 5′-hydroxyl terminus of the oligonucleotide, which was bound to controlled pore glass (CPG) in the final coupling cycle. Oxidation and ammonium hydroxide cleavage – deprotection yielded the 5′-amine-modified oligonucleotide.

The ssDNA-linker at this stage bore a nucleophilic, unprotected primary amine group, which reacted with the electrophilic *N*-hydroxy succinamide group of the SYBR 101. SYBR 101 succinimidyl ester (reactive dye) was used to label the amine-modified oligonucleotide because it forms a very stable amide bond between the dye and the amine-modified oligonucleotide probe. The reactive dye is a hydrophobic molecule, so it was dissolved in high-purity dimethylsulfoxide before reaction with the amine-modified oligonucleotide probe. The reaction was done in a tetraborate buffer at pH 8.5, so that the reactive dye reacted with the non-protonated amine group on the modified oligonucleotide probe.

In this protocol, 250 μg of the reactive dye was dissolved in 14 μL dimethylsulfoxide. To this vial, 7 μL of deionized, distilled H_2O was added followed by 75 μL of the sodium tetraborate buffer and 4 μL of a 25 μg μL^{-1} of gel-purified 5′-amine-modified oligonucleotide. The vial was placed on an oscillating platform stirrer at low speed to insure that the reaction remained well mixed. The reaction was allowed to continue overnight. The labelled oligonucleotide (L-DNA) was purified from the reaction mixture by use of a Pharmacia NAP-10 column containing Sephadex G-25 medium of DNA Grade, in distilled water containing 0.15% Kathone CG/ICP Biocide as a preservative.

Preparation of optical fibres

The jacket material surrounding the fused silica optical fibres (400 μm core diameter, 3M Power Core™ Series Optical Fibre, FT-400-URT or FP-400-UHT, distributed by Thor Labs Inc., Newton, NJ, U.S.A.) was mechanically removed by use of a fibre-stripping tool (Thor Labs Inc.) to reveal the fused silica core material and cladding layer. Optical fibre pieces 48 mm in length were then made by use of a custom-built, diamond-edged fibre-scoring device. The termini of the fibre pieces were visually inspected at 40 × magnifications to ensure the fibre termini were flat, orthogonal to the length of the fibre, and free of chips and nicks.

The fused silica fibre segments were cleaned prior to surface modification according to the published methods (14). CPG was used to grow ssDNA in tandem with fused silica fibres and was subsequently used for recovery of ssDNA to determine the quality and quantity of synthesis. CPG was treated identically to fused silica fibres. The fibre substrates were first immersed and gently agitated in a 1:1:5 (v/v) solution of 30% ammonium hydroxide – 30% hydrogen peroxide – water at 80°C for 5 min. The substrates were then recovered, washed with copious amounts of water, and then treated with 1:1:5 (v/v) concd. HCl – 30% hydrogen peroxide – water for 5 min at 80°C with gentle agitation. The substrates were recovered and washed with 100 mL portions of water, methanol, chloroform, and diethyl ether, respectively, dried under reduced pressure, and stored in vacuo and over P_2O_5 until required.

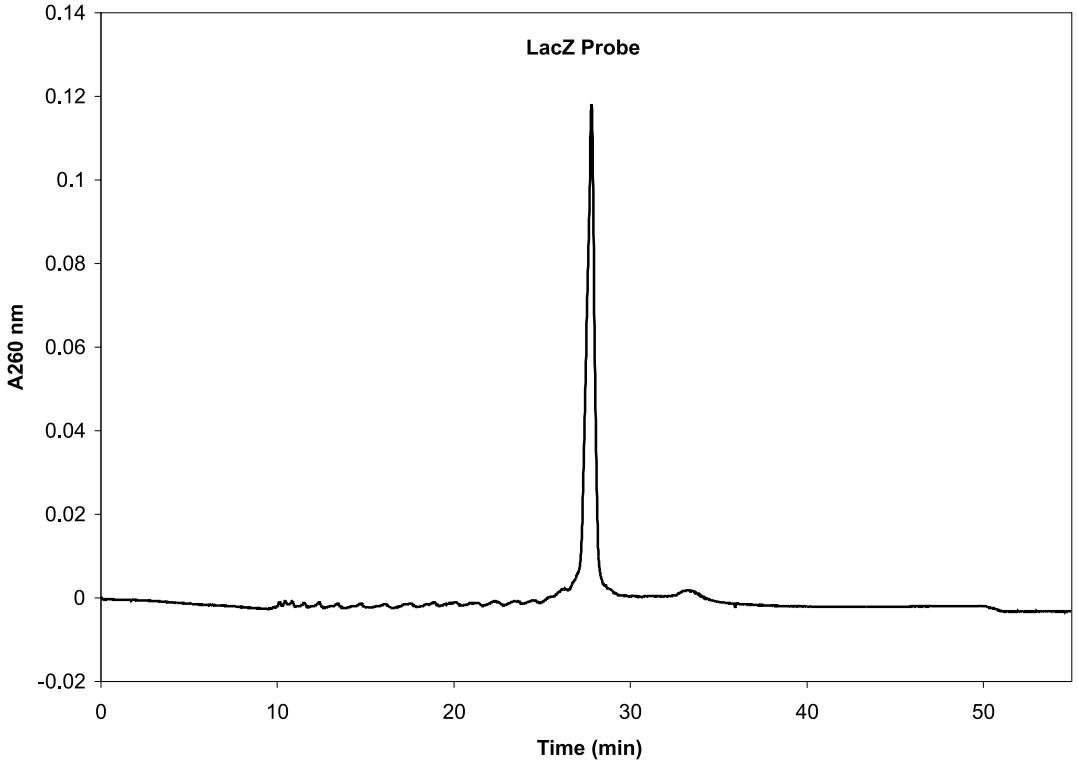
Functionalization of fused silica substrates with 3-glycidoxypropyltrimethoxysilane (GOPS)

The cleaned fused silica substrates were suspended in an anhydrous solution of xylene – 3-glycidoxypropyltrimethoxysilane – diisopropylethylamine (100:30:1 v/v/v). The reaction was stirred under argon at 80°C for 24 h. The fibres were then collected and twice washed with 50 mL portions of methanol, chloroform, and diethyl ether, respectively, and then dried and stored in vacuo and over P_2O_5 at room temperature until required.

Synthesis of dimethoxytrityl hexaethylene glycol (DMT-HEG)

A solution of dimethoxytrityl chloride (7.1 g, 21 mmol) in dry pyridine (10 mL) was added dropwise to a stirred solution of hexaethylene glycol (5.6 mL, 21 mmol in 5 mL pyridine) under an argon atmosphere. Stirring was continued overnight, after which time the reaction mixture was combined with dichloromethane (50 mL). The mixture was shaken against 5% aqueous bicarbonate (2 × 900 mL) and then with water (2 × 900 mL) to remove unreacted HEG, pyridine, and salts. The organic layer was dried under reduced pressure to yield the crude product. The product was purified by liquid chromatography using a silica gel column and an eluent of 1:1 dichloromethane – diethyl ether containing 0.1% triethylamine (2.9 g, 24% yield). ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.47–7.19 (m, 9H), 6.81 (d, 4H, J = 8.8 Hz), 3.78 (s, 6H), 3.74–3.51 (m, 22H), 3.22 (t, 2H, J = 5.8 Hz). Purity DMT-HEG = 96%.

Fig. 1. HPLC anion-exchange chromatogram of the 25mer mixed-base LacZ probe that was grown on CPG and then quantitatively removed by base cleavage. The chromatogram indicates the synthetic purity of the sample used in this investigation.



Linkage of DMT-HEG onto GOPS-functionalized substrates

DMT-HEG (10 equiv relative to the quantity of surface hydroxyl moieties, 700 mg DMT-HEG – 100 mg CPG) that had been dried by extended storage in vacuo and over P_2O_5 (>72 h) was dissolved in 20 mL of anhydrous pyridine and introduced to an excess of NaH (10 equiv) that had been thrice washed with dry hexane to remove the oil in which it had been suspended. The reaction was permitted to proceed with stirring for 1 h at room temperature under an argon atmosphere. The reaction mixture was filtered through a sintered glass frit under a positive pressure of argon and the filtrate immediately introduced to the reaction vessel containing the GOPS-functionalized substrates. One batch of GOPS-functionalized substrates containing both optical fibres and CPG was created, for which the DMT-HEG coupling reaction was permitted to proceed under a positive pressure of argon gas at room temperature with gentle agitation on an oscillating platform stirrer for durations of 4 h. Following the coupling reaction, the substrates were quickly recovered by filtration over a fritted glass funnel and washed with 150 mL portions of methanol, water, methanol, and diethyl ether, respectively, to quench the coupling reaction and remove non-specifically adsorbed reactants. The DMT-protected polyether-functionalized substrates were dried by placement in vacuo and over P_2O_5 and were maintained under these conditions until further required.

Capping of unreacted silanol and hydroxyl functionalities with chlorotrimethylsilane

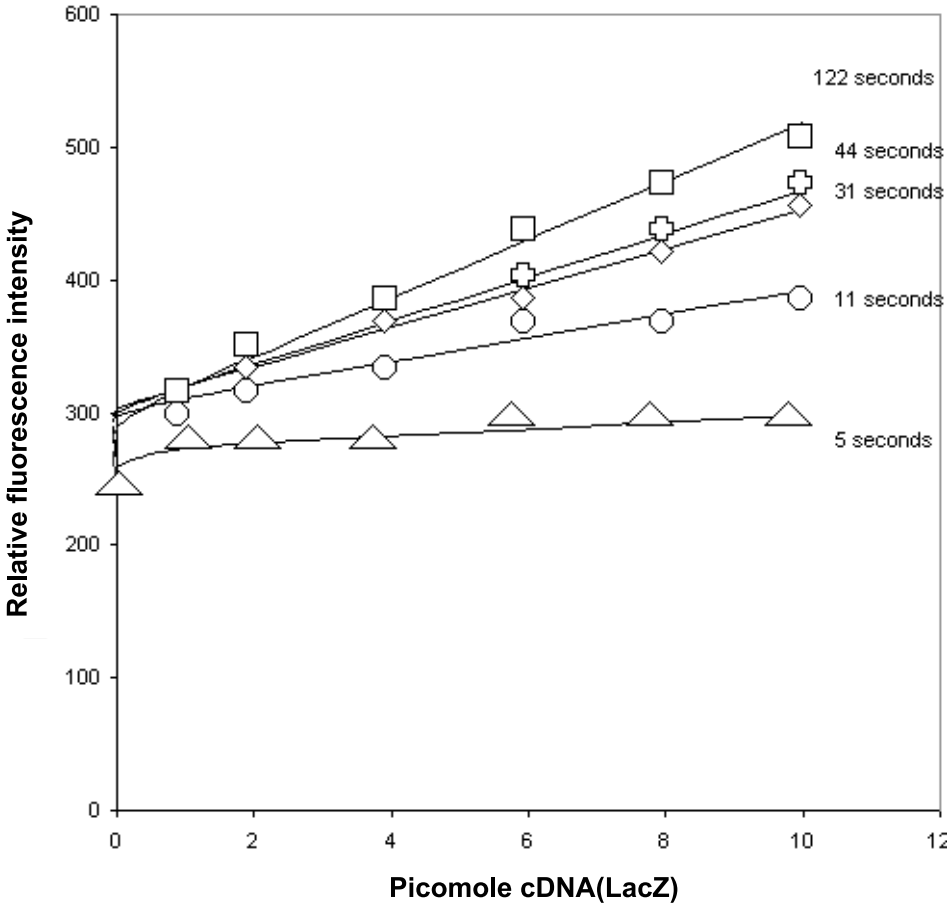
Sites on the surfaces of the fused silica fibres and CPG onto which undesired nucleotide synthon coupling could occur were capped prior to oligonucleotide assembly using chlorotrimethylsilane (TMS-Cl), as per the method of Watterson et al. (17). The substrates that had been dried by storage in vacuo and over P_2O_5 for a minimum duration of 16 h were suspended in a solution of 1:10 (v/v) chlorotrimethylsilane–pyridine for 16 h under an argon atmosphere at room temperature. The fused silica substrates were thrice washed with 20 mL portions of pyridine, methanol, and diethyl ether, respectively, and stored in vacuo and over P_2O_5 at 25°C until required.

Solid-phase phosphoramidite synthesis of oligonucleotides

All oligonucleotide synthesis was done using a PE-ABI 391-EP DNA synthesizer (PerkinElmer Applied Biosystems, Foster City, CA, U.S.A.). The manufacturer-supplied synthesis cycles were employed for oligonucleotide assembly with modifications to the delivery times of the reagents as required to completely fill the synthesis columns that were used. Oligonucleotide synthesis onto optical fibres (400 μm i.d. × 48 mm) was done in a custom-manufactured Teflon® synthesis column (6 mm i.d. × 50 mm) capable of holding 8 fibres in an evenly distributed and non-contacting fashion via cylindrical bores (400 μm i.d. × 2 mm deep) machined into one of the end caps (9).

A nucleic acid oligonucleotide having the sequence (5′)CAGGTAATGTGGCGGATGAGCGGCA(3′) was synthesized onto the sensor surface as the ssDNA probe. The target nucleic acid (cDNA) used to challenge the probe was an oligonucleotide having the sequence (5′)TGCCGCTCATCCGCCACATATCCTA(3′), which was derived from a portion of the LacZ gene sequence. The 25mer

Fig. 2. *E. coli* biosensor response to serial dilutions of cDNA as a function of cDNA concentration at various times. Maximum response was observed at about 2 min for all concentrations of cDNA.



oligonucleotides were prepared by use of a phosphoramidite synthon (Dalton) and standard protocols for oligonucleotide assembly, purification, and quantitation, as have previously been reported (15).

Determination of the extent of surface coverage of CPG substrates with covalently immobilized oligonucleotide–polyether conjugates was done by anion-exchange HPLC using methods that have been reported elsewhere (16).

E. coli and salmon sperm DNA preparation

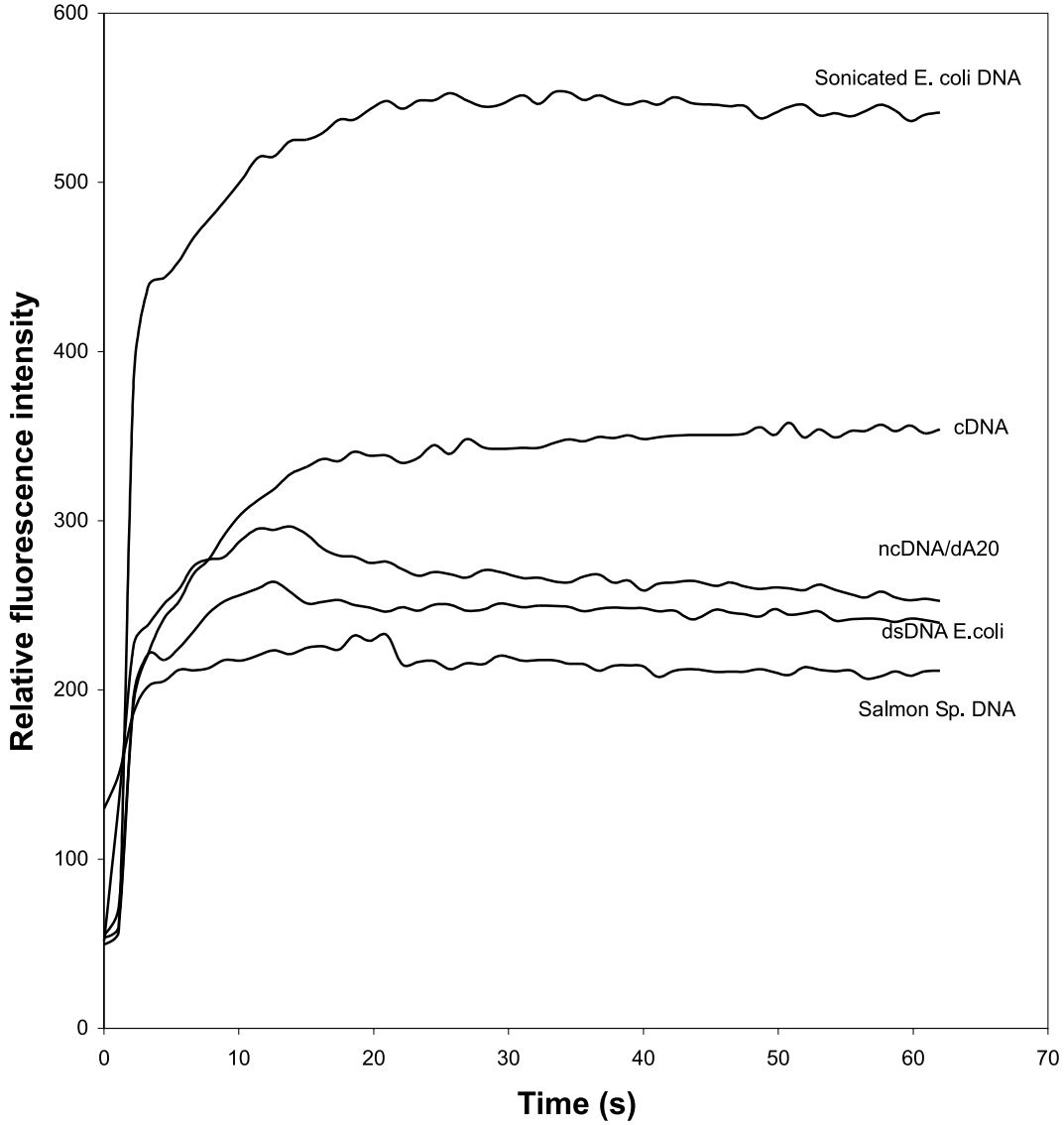
A 60 mL culture of *E. coli* was grown overnight at 37°C in LB media. Bacteria were harvested by centrifugation at 3000*g* for 10 min. Cells were lysed using TRIZOL reagent (Life Technologies, Canada) by repetitive pipetting, using 1 mL of the reagent per 1×10^7 cells of the *E. coli*. The homogenized sample was incubated for 5 min at room temperature, and chloroform was then added (0.2 mL chloroform per 1 mL of TRIZOL). Sample tubes were capped and shaken vigorously for 15 s and incubated at room temp for 2–3 min. The sample tubes were then centrifuged at 12 000*g* for 15 min at 2–8°C, to separate the mixture into a lower, red phenol–chloroform phase, an interphase, and a colourless upper aqueous phase. DNA was precipitated from the interphase and the organic phase by the addition of 0.3 mL of 100% ethanol per 1 mL of TRIZOL reagent originally used. Samples were mixed by inversion and permitted to equilibrate at room temperature for a few minutes, followed by precipitation of DNA by centrifugation at 2000*g* for 5 min at 2–8°C. Phenol–ethanol supernatant was removed and the DNA pellet was washed twice with ethanol and dried under vacuum. DNA was finally reconstituted by adding 1 mL of 1 × PBS and measurement of absorption at 260 nm showed that the DNA concentration of the resultant solution was about 350 μg mL^{-1}. The extracted *E. coli* DNA was sheared by syringe and then by sonication for 5 min with a Vibra cell sonicator (Sonics & Materials, Inc.) equipped with a 5-mm tip and set to 125 W maximum power at 20 kHz. Samples were kept on ice at all times until they were used for examination of hybridization.

Lyophilized salmon sperm genomic DNA was reconstituted in 1 mL of 1 × PBS to a concentration of 350 μg mL^{-1}, as indicated by measurement of absorption at 260 nm. This DNA was sheared by syringe and then by sonication for 5 min, as was done for the DNA from *E. coli.*

Hybridization assays of the *E. coli* biosensor

All sensors were cleaned by sonication in ethanol in a 40 W bath sonicator for 30 min to remove adsorbed contaminants from the sensor surface. In all cases, sensors were activated for hybridization by undergoing three consecutive thermal denaturation – re-annealing cycles, in which the sensors were exposed to a 1×10^{-7} M solution of the complementary 25mer oligonucleotide sequence (cDNA) in phosphate-buffered saline (PBS) hybridization buffer

Fig. 3. Chronofluorimetric response profile of a biosensor for *E. coli* using immobilized 25mer mixed-base probe on a fused silica optical fibre. Biosensor was exposed to 10 pmols of fully complementary LacZ 20mer (cDNA), 35 ng ssDNA from *E. coli (*prepared by treatment of whole genomic DNA by sonication and shearing), 35 ng of dsDNA from *E. coli*, and 35 ng ssDNA from salmon sperm (prepared by treatment of whole genomic DNA with sonication and shearing), in 1 × PBS containing 10^{-7} M ethidium bromide at 40°C with full washing and chemical regeneration with water at 90°C and formamide solution (90% in TE buffer) between samples.



(1.0 mol L^{-1} NaCl, 50 mmol L^{-1} total phosphate ion, pH 7.0) and subsequently subjected to a temperature ramp of 0.3°C per min, over a range from 20 to 80°C. Hybridization assays were done for optical sensors that were exposed to solution-phase cDNA mixed with the staining intercalator ethidium bromide at a final concentration of 0.1 μmol L^{-1}. Each sample solution had a total volume of 26 μL, once in the reaction chamber. The flow was stopped and the signal was recorded over a period of up to 10 min. After reaction, the sensor was washed at a flow rate of 3 mL min^{-1}. Removal of the bound DNA that had associated with the sensor surface from the previous analysis was done prior to each experiment by flushing 15 mL of 90°C water through the flow cell (3 mL min^{-1}, 5 min), followed by 1 mL of 95% ethanol, and final wash with 90% formamide in TE buffer (10 mm L^{-1} Tris HCl, 5 mmol L^{-1} EDTA, pH = 8.3). Salmon sperm DNA (0.1 μmole L^{-1}) served as a control for genomic non-complementary DNA. Assays were performed using a solution temperature of 40°C. All hybridization assays were done in triplicate.

The L-DNA probe hybridization assays were done in bulk solution, by titrating the tethered SYBR 101-probe with a fully complementary synthetic LacZ sequence. A concentration range, from 0.133 to 6.00 μg mL^{-1} of each sequence, was used in a total volume of 700 μL.

Results and discussion

The rapid detection of microbes in samples of water is becoming more critical as the population of the world increases. Our research group has focused on developing a biosensor that is rapid and sensitive for the detection of

Fig. 4. HPLC anion-exchange chromatogram of the mixed base 5′ amine-modified LacZ probe that was grown on CPG and then quantitatively removed by base cleavage. The chromatogram indicates the high purity of the synthetic samples used in this investigation.

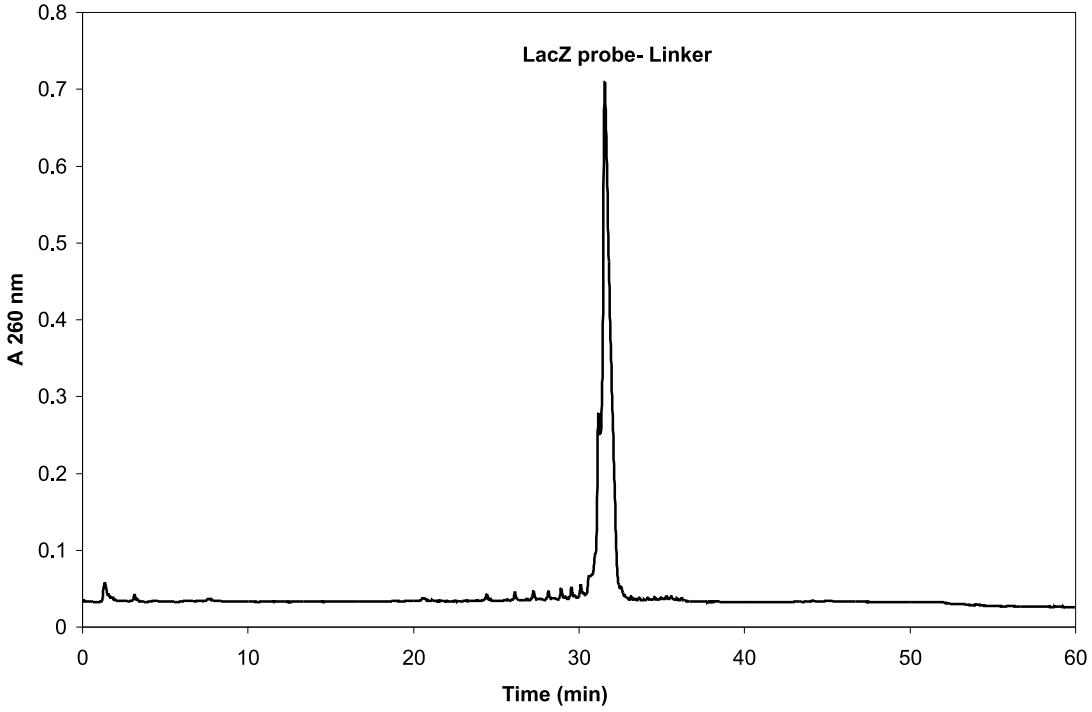
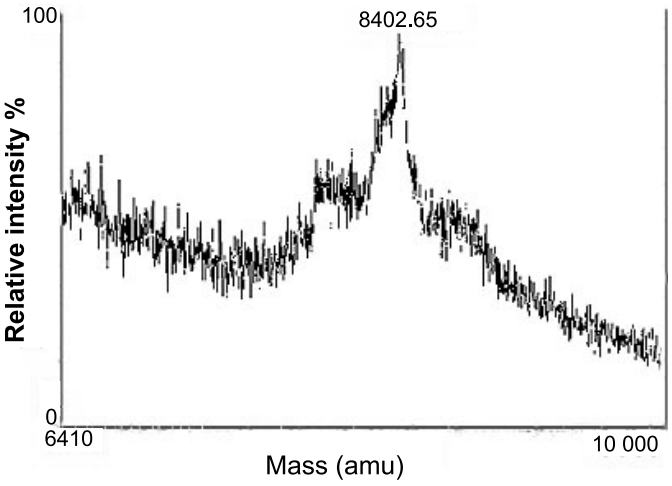


Fig. 5. MALDI-TOF mass spectrometric analysis of the mixed base SYBR 101-LacZ probe showing the molecular weight of the probe *m*/*z* (8402 amu). The results confirm the tethering of the dye to the probe.



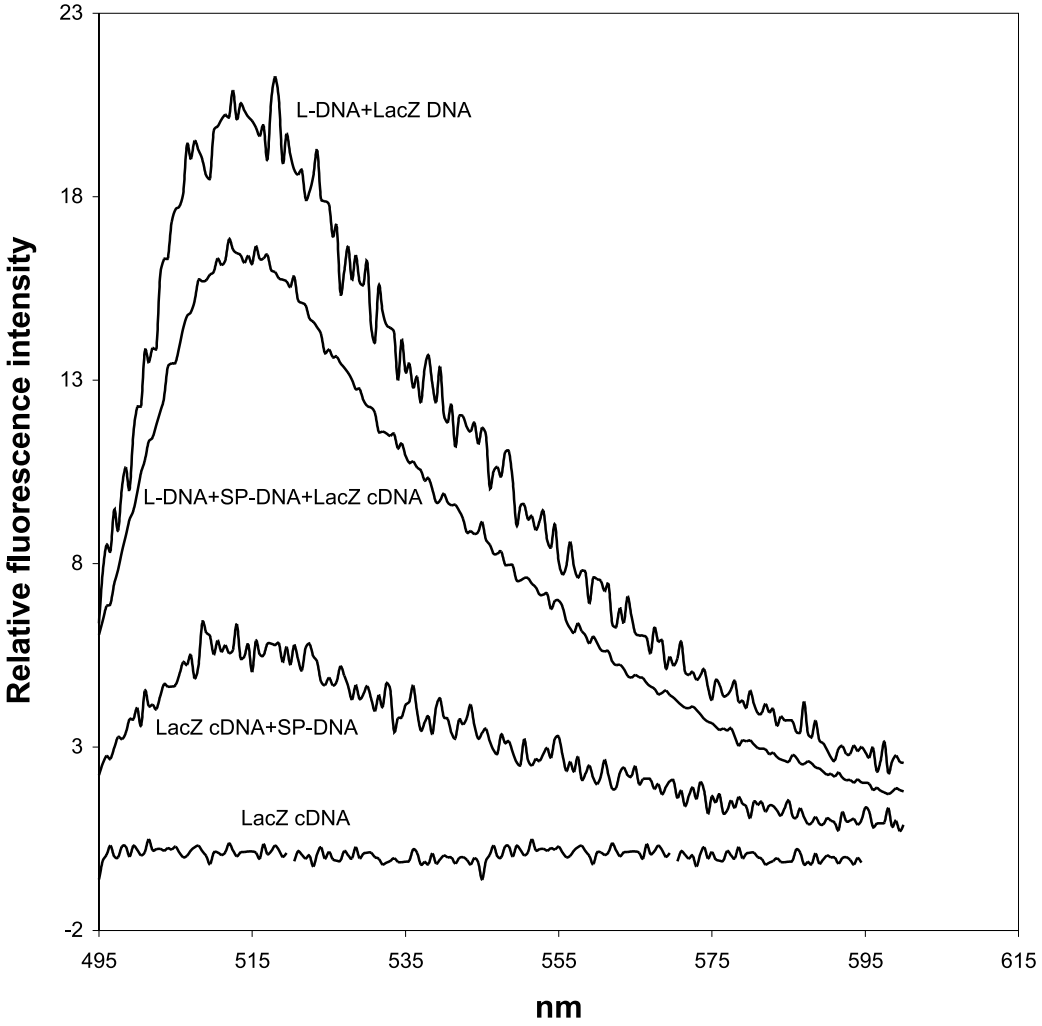
coliforms as an indicator of microbial contamination in surface and ground water. Immobilized oligonucleotides on solid supports for use as molecular recognition elements in bioassays and biosensors are short sequences conjugated at the strand terminus to a linker molecule, which in turn is covalently linked to the substrate. This strategy has proven advantageous in terms of the enhanced nucleotide coupling efficiency realized during solid-phase assembly of the oligonucleotide onto the linker and the rapid kinetics of hybrid formation of the immobilized strand with target sequences.

The single-stranded probe was immobilized via hexaethylene glycol linker (HEG) to functionalized fused silica substrates. This effectively provided an oligonucleotide surface where each molecule covered an approximate area of 400–1300 $Å^2$ when using linear co-polymer strands of ca. 100 Å lengths (18). This chemistry is highly stable toward water hydrolysis and physical cleavage, which can occur, owing to treatment of the surface by heating, cooling, and detergent washing. The use of HEG also provides advantages based on high solubility and hydrophilicity (19).

All sensors that were used were checked for quality of oligonucleotide immobilization by an indirect method based on concurrent immobilization of DNA on fibres and CPG. The material on the CPG was cleaved quantitatively from the surface with base, and anion exchange HPLC was then used for separation and analysis of recovered material (16). Figure 1 shows the HPLC analysis of the ssDNA LacZ probe that was synthesized on CPG. The chromatogram demonstrates the sequence integrity of the oligonucleotides that were used throughout this investigation.

Prior to hybridization, sensors were thermally activated by multiple cycles of heating (90°C) and cooling (30°C) in the presence of 1×10^{-7} M cDNA. The response of the biosensor to a series of samples introduced sequentially is shown in Fig. 2. The fluorescence signal was a function of the concentration of cDNA. In these experiments, the maximum signal was obtained at about 120 s after the cDNA was

Fig. 6. Spectrofluorimetric scan of SYBR 101-LacZ probe. The probe was treated with an equivalent amount of synthetic cDNA (LacZ) in the presence of an equivalent number of molecules of salmon sperm ssDNA, in 1 × PBS at 515 nm (room temperature). Salmon sperm ssDNA (SP-DNA*)* was prepared by treatment of whole genomic DNA with sonication.



introduced to the sensor. Figure 3 provides an indication of the shape of the curves and the speed of response. Quantities as low as about 100 fmole provided signals at the three-standard-deviation level. Fibres were washed and chemically regenerated using water at 90°C and 90% formamide solution in TE buffer between samples. The reproducibility was excellent, and dozens of cycles of use have been demonstrated.

Figure 3 shows that the sensor does respond selectively to the synthetic cDNA LacZ sequence in comparison to the non-complementary genomic sonicated DNA from salmon sperm. The fact that the signal for cDNA was present and reproducible after challenging the biosensor with sonicated genomic salmon sperm DNA provides an indication that, for real environmental samples, the possible co-existence of other non-complementary DNA would not block the biosensor from functioning. Upon challenging the biosensor with sonicated genomic *E. coli* DNA, the time dependence of the signal obtained demonstrates hybridization between the probe and the genomic LacZ.

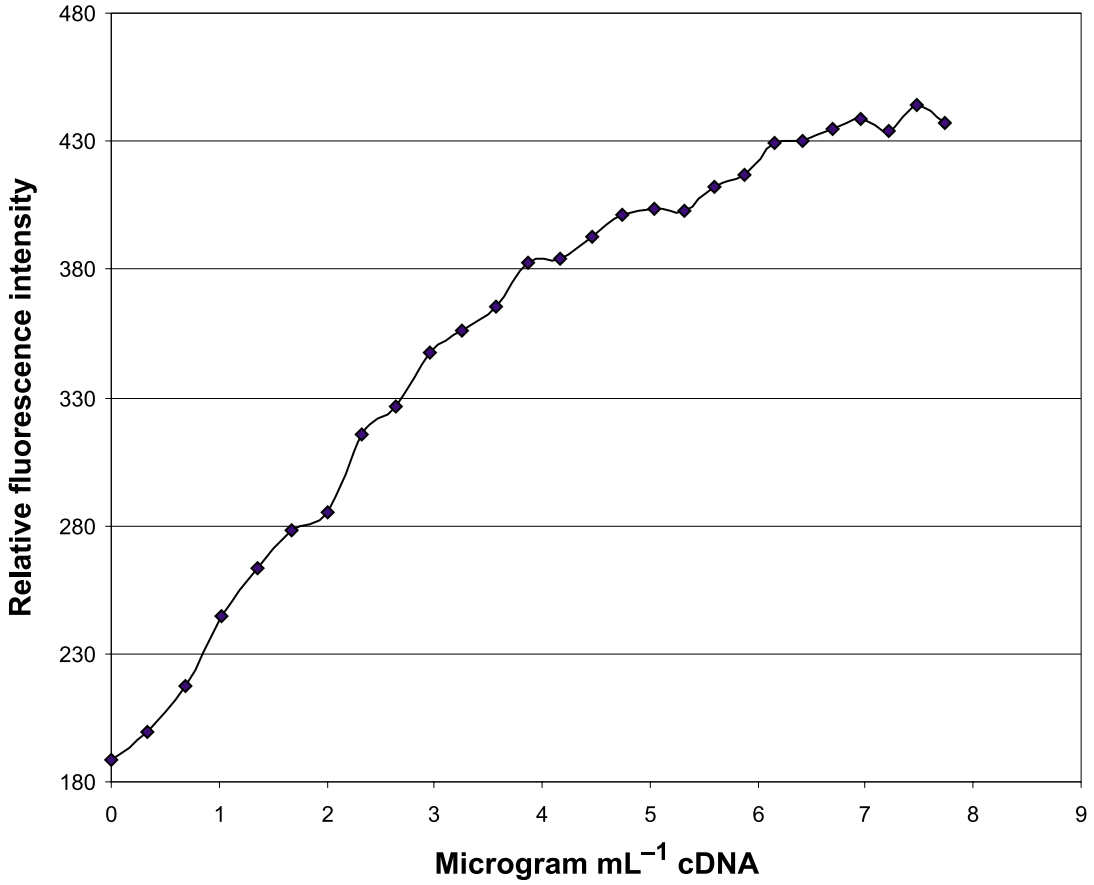
Further results investigating the use of PCR products with the target sequences located in various positions within a longer product have demonstrated that signal magnitude is somewhat dependent on the location of the target sequence (20), with the signals of greatest magnitude appearing when the target sequence is farthest removed from the biosensor surface.

Tethered dye

In a preliminary experiment that was designed as a first step towards preparation of tethered dye, a second probe was constructed in which the fluorescent intercalator SYBR 101 was chemically conjugated to the probe via a tether. The dye-conjugated probe was purified and examined by anion exchange HPLC for purity (Fig. 4). The mass spectral analysis confirmed the formation of SYBR 101 labelled LacZ probe (Fig. 5).

The ability of the L-DNA to hybridize with fully complementary DNA was shown by the change in fluorescence when complementary, in comparison to non-complementary, DNA was added. Importantly, the results confirmed that the tethered dye could still bind into double-stranded DNA and achieve a substantial change in quantum yield. A second important observation was that the fully complementary target

Fig. 7. Spectrofluorimetric titration curve of SYBR 101-LacZ probe (5.7 μg mL^{-1}) against concentrations of LacZ cDNA from 0.1 μg mL^{-1} to 7.7 μg mL^{-1} in 1 × PBS at 515 nm (room temperature).



could still be detected in the presence of a large background of salmon sperm DNA. Figure 6 provides spectral information about the tethered dye and a summary of the fluorescent signal due to binding events of the labelled probe at a concentration of 1×10^{14} molecules in 700 μL PBS solution. When the probe was mixed with an equivalent number of molecules of salmon sperm DNA, the signal remained relatively high.

To observe the response to hybridization at different concentrations of target cDNA of the SYBR 101 labelled probe, a titration curve was generated using 25mer fully complementary target LacZ. In this experiment, a PBS solution of cDNA containing 5.7 μg mL^{-1} (1×10^{14} molecules) was titrated against the SYBR101-LacZ probe. A maximum of hybridization was achieved when a stoichiometrically equivalent amount of the labelled probe was added to the cell. The intensity of fluorescence did not substantially change beyond the 1:1 stoichiometric equivalence point, indicating that hybridization was necessary to bring the tethered dye into close proximity to a duplex for stable intercalation to occur (Fig. 7).

Preliminary work using tethered thiazole orange labels on short mixed nucleotide probes that were immobilized to the biosensor surface confirmed that such tethered dyes can report selective hybridization (21). The results also indicated that adsorption of non-complementary DNA had a significant effect on the environment of the tethered TO. This appeared to be largely an electrostatic phenomenon where the positively charged dye interacted with the relatively concentrated DNA at the solid interface. The non-selective adsorption was largely eliminated by moving to high salt (3 × PBS), with the concurrent advantage being that the dsDNA stability was improved. The background intensity effect produced by non-complementary DNA could be reduced to less than 10%. Another limitation that was identified was sensitivity to photobleaching (21), which was easily ameliorated by use of gated detection. Further research has now demonstrated that a more complicated time-dependent chemical process, based on availability of intercalant after denaturation and biosensor regeneration, was the main cause of reduction in signal intensity as a series of experiments were done sequentially.

Conclusions

A fibre-optic biosensor for a portion of the LacZ gene was constructed as a diagnostic device to provide a surface for the hybridization with markers from *E. coli*.

The LacZ gene of *E. coli* was selected because conventional coliform monitoring is based on detection of the activity of the gene product (β galactosidase) produced by coliform bacteria. Also, the LacZ sequence was selected as a target because it is specific to total coliforms, while the lamB gene is within *E. coli*, *Salmonella*, and *Shigella spp*.,

and the uid gene is within *E. coli* and *Shigella s*pp. The 25mer length of the probe was shown to be sufficiently selective to hybridize genomic target from *E. coli* bacteria and was further investigated by searching within the GenBank nucleotide sequence data to insure that there were no other homologies with potential non-target sequences (22). The short length of the probe provides advantages in terms of reversibility and high speed of hybridization.

The LacZ probe was covalently attached through a C6 amino-modifier tether to SYBR 101 fluorescent intercalating dye. This labelled probe was used free in solution to investigate whether the dye could still participate in intercalation as hybridization with fully complementary target in the presence of a high concentration of non-complemetary DNA (salmon sperm DNA) proceeded. The results are encouraging, as there was no indication that the fluorescent signal from hybridization was dramatically affected by the presence of the non-complementary material.

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