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A generic sandwich-type biosensor with nanomolar detection limits

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Abstract A quantitative and highly sensitive, yet simple and rapid, biosensor system was developed for the detection of nucleic acid sequences that can also be adapted to the detection of antigens. A dipstick-type biosensor with liposome amplification, based on a sandwich assay format with optical detection, was combined with a simple coupling reaction that allows the transformation of the generic biosensor components to target specific ones by a mere incubation step. This biosensor platform system was developed and optimized, and its principle was proven using DNA oligonucleotides that provided a nucleic acid biosensor for the specific detection of RNA and DNA sequences. However, the coupling reaction principle chosen can also be used for the immobilization of antibodies or receptor molecules, and therefore for the development of immunosensors and receptor-based biosensors. The generic biosensor consists of liposomes entrapping sulforhodamine B that are coated with streptavidin on the outside, and polyethersulfone membranes with anti-fluorescein antibodies immobilized in the detection zone. In order to transform the generic biosensor into a specific DNA/RNA biosensor, two oligonucleotides that are able to hybridize to the target sequence were labeled with a biotin and a fluorescein molecule, respectively. By simultaneously incubating the liposomes, both oligonucleotides, and the target sequence in a hybridization buffer for 20–30 min at 42 °C, a sandwich complex was formed. The mixture was applied to the polyethersulfone membrane. The complex was captured in the detection zone and quantified using a handheld reflectometer. The system was tested using RNA sequences from *B. anthracis*, *C. parvum* and *E. coli*. Quantitation of concentrations between 10 fmol and 1000 fmol (10–1000 nM) was possible without altering any biosensor assay conditions. In addition, no changes to hybridiza-

tion conditions were required when using authentic nucleic acid sequence-based amplified RNA sequences, and the generic biosensor compared favorably with those previously developed specifically for the RNA sequences. Therefore, the universal biosensor described is an excellent tool, for use in laboratories or at test sites, for rapidly investigating and quantifying any nucleic acid sequence of interest, as well as potentially any antigen of interest that can be bound by two antibodies simultaneously.

Keywords Biosensor · Nucleic acid · Antibody · Detection · Nanomolar

Introduction

In recent years, biosensors have matured into bioanalytical devices that find their application in clinical diagnostics, food safety and environmental monitoring [1]. Glucose sensors, pregnancy tests, rapid pathogen analysis, and DNA microarrays are just a few examples of biosensors available today on the market. There is still an obvious need for biosensors and bioanalytical microsystems with low detection limits that can perform for rapid analysis with high specificity in all of the above mentioned application areas, and today more research than ever is being carried out toward these goals.

Each biosensor consists, in general, of a biorecognition element that provides the assay with specificity toward an analyte of interest, and a transducer as well as other components, such as amplification elements and solid supports, all of which are ideally assembled within one housing. Combinations of the different elements provide assays with different sensitivities, dynamic ranges, specificities, as well as characteristics such as portability, ease-of-use, and analysis time. While an ideal biosensor is highly specific and sensitive, yet also simple, rapid and portable, few of those that are currently available fit this description.

Nucleic acid-based biosensors recently discussed include optical sensors with a variety of different approaches,

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such as those based on intercalating dyes [2], molecular beacons [3, 4], simple visual or reflectance detection [5, 6], or microfluidic biosensors using dye-entrapping liposomes [7, 8] reaching very low detection limits in the low-pM range. Electrochemical biosensors were suggested, utilizing nanotubes detecting 0.1 nM concentrations of DNA [9], using carbon electrodes with horseradish peroxidase as a signal amplification system [10] or methylene blue as intercalating dye [11], and enzyme amplification systems [12] achieving low-fM detection limits. Other transduction principles include microcantilevers [13], the quartz crystal microbalance [14], and surface plasmon resonance [15]. A good overview of most recent DNA-based biosensors is given by Vercoutere et al. [16].

Even though interesting biosensors have been developed and described in literature, they are rarely commercialized or even used in R&D laboratories that urgently need new bioanalytical tools, such as research focusing on the identification of nucleic acid sequences in genomics and proteomics, in fundamental and applied microbiological, molecular biological, biotechnological, and clinical diagnostic research, and even in the field of biosensor development (identifying ideal biorecognition elements). Research laboratories still use methods such as agarose and polyacrylamide gel electrophoresis, and Southern, Northern and Western Blotting as standard procedures [17]. These are methods that do not provide enough specific information, or are very laborious and time consuming: requiring up to 48 hours of meticulous lab work. Newer methods include Taqman and molecular beacons in combination with real time PCR, the surface plasmon resonance-based BIAcore instrument, and DNA microarrays, but high equipment costs prevent their use in many laboratories.

An obvious reason that biosensors are often not applied in research labs is one of the biosensor's advantages: its specificity toward one specific analyte or a group of analytes. Even if a switch to a new analyte is not too complicated, they require substantial additional optimization in order to provide desirable results. In order to ameliorate this situation, we present here the development of a generic biosensor as a platform technology that can be adapted very easily to the identification and quantification of a new analyte. We have proven its principle using nucleic acid detection; however, the generic components of the biosensor are designed to be adaptable into an immunosensor or a receptor-based sensor as well. It can be transformed into a specific biosensor by a straightforward 20–30 min incubation at 42 °C. In addition, no optimization of assay conditions is required in general. The generic biosensor is based on earlier, specific biosensors developed in our laboratory [5, 6, 18]. It utilizes two generic components: (1) dye-entrapping liposomes that bear streptavidin on their outer surface, and (2) polyethersulfone membranes with anti-fluorescein antibodies immobilized in the detection zone. The two specific biorecognition elements are the reporter and capture probes that can hybridize to two different segments in the target sequence; these are labeled with a biotin and fluorescein molecule, respectively. By mixing

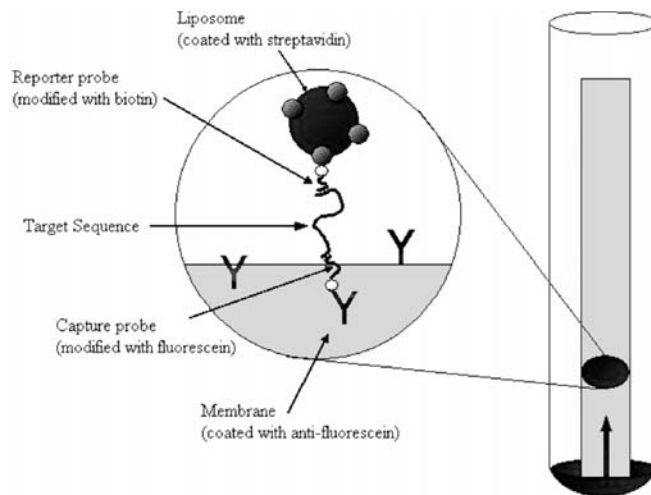


Fig. 1 The set up for a generic biosensor assay. The generic liposomes bear streptavidin covalently attached to their surface. The generic polyethersulfone membranes have anti-fluorescein antibodies immobilized in the detection zone. During a biosensor assay, the liposomes are mixed with a biotinylated reporter probe, the target DNA or RNA sequence, and a fluorescein-labeled capture probe. The two probes can specifically hybridize with two segments in the target sequence and form a sandwich. This hybridization mixture is incubated in a test tube for 20–30 min at 42 °C. Subsequently, the membrane is inserted into the tube so that the mixture migrates up the strip via capillary action. After about 8 min the amount of liposomes can be quantified in the detection zone, and correlated directly to the concentration of target sequence in the sample

and incubating the liposomes, reporter probes, target sequence and capture probes with a hybridization buffer for 20–30 min at 42 °C, a sandwich is formed. The mixture is pipetted onto the polyethersulfone membrane and allowed to migrate along the strip via capillary action for about 8 min. If the target sequence is present, the complex will be captured in the detection zone via the antibody-antigen binding. Therefore, the amount of liposomes present in the detection zone are directly proportional to the concentration of target sequence in the sample. Including signal quantification using a portable reflectometer (or visual detection), the assay can be completed within 30–40 min. The general principle of the biosensor is shown in Fig. 1.

Materials and methods

Reagents

All general chemicals and buffer reagents were obtained from Sigma Company, St. Louis, MO. Organic solvents were purchased from Aldrich Chemical Company, Milwaukee, WI. Predator membranes were obtained from Pall/Gelman Company, Port Washington, NY. Lipids were purchased from Avanti Polar Lipids, Alabaster, AL. Sulforhodamine B and streptavidin were acquired from Molecular Probes Company, Eugene, OR. Anti-fluorescein antibody was ordered from Cortex Biochem, San Leandro, CA. All nucleic acid sequences – probes, primers, and synthetic targets – were purchased from Qiagen, Valencia, CA.

Table 1 DNA sequences of all reporter, capture, and synthetic target sequences used to optimize the generic biosensor. Reporter probes for the generic assay were modified with biotin on the 3'

end of the sequence, and capture probes were modified with fluorescein on the 5' end of the sequence

Sequence name	Sequence 5' to 3'	Modification
<i>E. coli</i> - reporter probe	GTCTggTgAATTggTTCCg	3'-biotin
<i>E. coli</i> - capture probe	CCgTTggCACAgCAAATA	5'-fluorescein
<i>E. coli</i> - STS	GgC AAC CgT gTC gTT TAT CAg ACC ACT TAA CCA Agg C	none
<i>C. parvum</i> - reporter probe	GTgCAACTTTAgCTCCAgTT	3'-biotin
<i>C. parvum</i> - capture probe	AgATTGGAAGAAGACTCTGCGC	5'-fluorescein
<i>C. parvum</i> - STS	A CCA gCA TCC TTg AgC ATT TTC TCA ACT ggA gCT AAA gTT gCA Cgg AAg TAA TCA gCg Cag AgT TCT TCg AAT CTA gCT CTA CTg ATg gCA ACT gAA	none
<i>B. anthracis</i> - reporter probe	CAAgATgTCCGCGTATTTAT	3'-biotin
<i>B. anthracis</i> - capture probe	CTAgAAATATCgggAAGAGAA	5'-fluorescein
<i>B. anthracis</i> - STS	AT AAA TAC gCg gAC ATC TTg TC TTC TCT TCC CgA TAT TTC TAg	none

Sequences

DNA oligonucleotides (probes) for the detection of *E. coli*, *C. parvum*, and *B. anthracis* were previously designed [5, 18, 19]. The probes were stored at a concentration of 300 nmol/mL in TE at a pH of 7. Probe sequences and modifications are listed in Table 1. The melting temperatures of the capture and reporter probes calculated at 50 mM salt concentration are as follows: *E. coli* RP (55 °C), CP (52.1 °C), *B. anthracis* RP (53.1 °C), CP (50.4 °C) and *C. parvum* RP (49.2 °C), and CP (62 °C).

Preparation of membranes

Polyethersulfone membranes were cut into 7.5 cm by 4.5 mm strips. Anti-fluorescein antibody was diluted in 0.4 M Na₂CO₃/NaHCO₃ buffer, pH 9.0, containing 5% methanol so that its final concentration was as desired (typically 30 pmol/μL, variations were done between 0–36 pmol/μL). 1 μL of the prepared mixture was deposited onto the designated capture zone of each membrane strip, exactly 2.5 cm from the bottom of the strip. The membranes were then dried, first under a fume hood for 5 min, then in a vacuum oven at 53 °C and 15 psi, for 1.5 h. The membranes were then blocked by soaking them in a blocking reagent (0.5% polyvinylpyrrolidone (PVP) and 0.015% casein in Tris Buffered Saline (0.02 M Tris Base, 0.15 M NaCl, 0.01% NaN₃, pH 7.0)) for 30 min on a shaker at room temperature and blotted dry with tissue paper. They were allowed to fully dry in a vacuum oven at 25 °C, 15 psi, for 2–3 h, and finally stored until use in vacuum-sealed bags at 4 °C.

Preparation of liposomes

Liposomes were prepared using a slightly modified protocol of the reverse-phase evaporation method [6]. Initially, 7.2 μmol (5.0 mg) dipalmitoyl phosphatidylethanolamine (DPPE) was dissolved in 1 mL of 0.7% triethylamine in chloroform by 1 min sonication. Subsequently, 14.3 μmol (3.5 mg) *N*-succinimidyl-5-acetylthioacetate (SATA) was added. The mixture was sonicated again and incubated for 20 min forming a DPPE-ATA compound. To remove the triethylamine, 3 mL of chloroform was added to the mixture and evaporated under vacuum in a rotary evaporator at 45 °C. Finally, 1 mL of chloroform was added to this product. Subsequently, the DPPE-ATA, 40.3 μmol (0.0296 g) dipalmitoyl phosphatidylcholine (DPPC), 21.0 μmol (0.015 g) dipalmitoyl phosphatidylglycerol (DPPG), and 51.7 μmol (0.020 g) cholesterol were dissolved in a mixture of chloroform, methanol, and isopropyl ether in a 6:1:6 ratio by sonication in a round-bottomed flask in a water bath at 45 °C. To the lipid mixture a total of 4 mL of a 150 mM sulforhodamine B (SRB) in 0.02 M phosphate buffer (K₂HPO₄/KH₂PO₄), pH 7.5 (542 mmol/kg) was added and sonicated for 5 min. The organic solvents were evaporated in a rotary evaporator so that liposomes

formed spontaneously, entrapping SRB. To obtain a uniform particle size, the liposomes were subsequently extruded through 2 μm, then 0.4 μm filters (each 11 times) using the Avanti mini-extruder and polycarbonate filters from Avanti Polar Lipids, Alabaster, AL. Liposomes were purified from free dye by gel filtration using a Sephadex G50 column followed by dialysis against a 0.01 M PBS buffer, pH 7.0, containing sucrose to increase the osmolarity to 617 mmol/kg.

Coupling of streptavidin to the liposome surface

In order to couple streptavidin to the outside of the liposomes, streptavidin was dissolved in 0.05 M potassium phosphate buffer containing 1 mM EDTA, pH 7.8, to a concentration of 100 nmol/mL. For a final mol% tag of 0.4 for streptavidin, 30 nmol of streptavidin was allowed to react with 7500 nmol of total lipids on the liposomes (the appropriate volume of liposomes previously made was used for the coupling reaction). A solution of *N*-(κ-maleimido-dodecanoyloxy) sulfosuccinimide ester (sulfo-KMUS) dissolved in dimethyl sulfoxide (DMSO) was prepared at 10 mg/mL (20.8 μmol/mL), and this stock was added to the dissolved probe at a molar ratio of 3:1. Therefore, for 30 nmol of dissolved probe, 4.3 μL of a 10 mg/mL sulfo-KMUS in DMSO solution was added. This mixture was incubated for 2–3 h to derivatize the amino-modified nucleotide probes with maleimide groups.

The ATA groups on the liposomes were deprotected by deacetylating the acetylthioacetate groups on the surface of the liposomes, generating sulfhydryl groups. 0.5 M hydroxylamine hydrochloride in 0.4 M phosphate buffer pH 7.5 (K₂HPO₄/KH₂PO₄) with 25 mM EDTA was added to the volume of liposomes at a ratio of 0.1 mL hydroxylamine solution per 1 mL liposome solution, such that the final concentration of hydroxylamine was 0.05 M. The deacetylation reaction was allowed to proceed in the dark, at room temperature, and on a shaker for 2 h.

At the end of both incubation periods, the pH of both mixtures was adjusted to 7.0 using 0.5 M KH₂PO₄. Then, for actual conjugation of the streptavidin to the liposomes, the SH-tagged liposomes and the maleimide-derivatized streptavidin were mixed and allowed to react on a shaker at room temperature for 4 h, and then overnight at 4 °C. To quench the excess SH groups on the liposomes and the unreacted sulfo-succinimidyl groups on the sulfo-KMUS, ethylmaleimide and Tris were added equivalent to 10× the molar quantity of SH and 20× the molar quantity of sulfo-KMUS, respectively. A 200 mM ethylmaleimide solution in 0.05 M Tris-HCl, 0.15 M NaCl, 0.1 M sucrose, pH 7.0 was prepared. The appropriate volume (37.6 μL for a 30 nmol preparation) was added to the liposomes and incubated for 30 min on a shaker. The tagged liposomes were purified from free reporter probe by gel filtration using a Sepharose CL-4B column and subsequently by dialysis using a 0.01 M PBS buffer, pH 7.0, 617 mmol/kg (osmolarity adjusted with sucrose). Liposomes were stored in the dark at 4 °C.

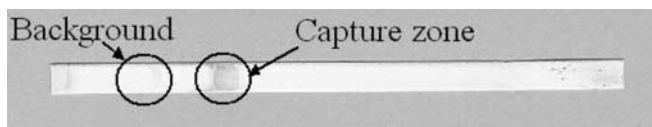


Fig. 2 Determination of the specific signal in the capture zone and the background signals of the strip. Reflectometer readings were taken at both the capture zone and about 5 mm before the capture zone. If false assay conditions were used, liposomes could non-specifically bind to the membrane and cause the background signal to increase significantly. It could therefore be considered an internal background standard, and was determined for each assay

Biosensor assay

The biosensor assay was a general dipstick-type assay. All optimization experiments were carried out with *E. coli* sequences. First, for the coupling of the specific reporter probes to the liposomes and the formation of the target-probe complex, 2 μ L liposomes (0.2 mol% surface tag), 0.5 μ L reporter probe (1 pmol), 1 μ L target sequence, 1 μ L capture probe (4 pmol), and 1 μ L master mix (45% formamide, 10 \times SSC (0.9 M NaCl, 0.09 M Na citrate, pH 7.0), 0.6% Ficoll, 0.6 M sucrose) were combined in a glass tube. This hybridization mixture was incubated at 42 $^{\circ}$ C for 20–30 min. After incubation, a membrane strip (with 30 pmol of antibody) was inserted into the glass tube and the hybridization mixture was allowed to migrate up the strip. Subsequently, 32 μ L of running buffer (30% formamide, 4 \times SSC, 0.4% Ficoll, 0.2 M sucrose) was added to the glass tube and allowed to traverse the entire length of the strip. After 8–10 min, all of the running buffer had run the length of the strip and the signal at the capture zone was analyzed using the BR-10 reflectometer (from ESECO Company, Cushing, OK). The reflectometer measures the reflectance of light at a wavelength of 560 nm, which is close to the maximum absorbance of the sulforhodamine B that is encapsulated within the liposomes. Each experimental strip had two measurements taken with the reflectometer. One measured the intensity of the signal at the capture zone. The other measured the level of background noise just below the capture zone, about 2 cm above the bottom of the strip (see Fig. 2). In addition, a negative control containing water instead of the target sequence was included in every experiment to account for any variation of the protocol during the optimization.

The concentrations of each component in both the master mix and the running buffer, the percent tag of streptavidin on the liposome, and the amounts of reporter and capture probe per assay were varied for optimization purposes. In addition, the incubation time and possible storage of the hybridization mixture was investigated.

Results

The most important design criterion of the development of the generic biosensor was the simplicity with which it could be transformed into an analyte-specific assay, so that the difficult coupling reactions required in most biosensor assays were avoided. The chosen reactions only required a single incubation step at 42 $^{\circ}$ C; no additional chemicals or treatment were needed. A number of different approaches were considered. For example, if only nucleic acid biosensors were targeted, generic oligonucleotides coupled to liposomes and the membranes could have been used, alternatively, for antibody-based biosensors, protein A or G, or anti-Fc antibodies would have been useful. However, we chose to utilize antibody-small antigen interactions as one binding reaction (in other words

anti-fluorescein antibody and fluorescein), and streptavidin-biotin as the second coupling mechanism to provide the possibility of adapting the generic biosensor to nucleic acid and also immunological target analytes. Therefore, the biorecognition elements of a desired specific assay simply have to be labeled with biotin and fluorescein. Easy-to-perform labeling procedures are available for proteins and DNA oligonucleotides, and are generally carried out for current standard detection techniques such as Northern, Southern, and Western Blotting. Alternatively, the oligonucleotides and some antibodies can be purchased pre-labeled. The liposome membrane-based biosensor format was chosen for its ease-of-use, proven sensitivity of detecting nucleic acid sequences in the low-nM range, and its speed (the assay takes a total of only 20 min, plus 10–20 min for coupling of the biorecognition elements to the biosensor components). Liposomes entrapping hundreds of thousands of dye molecules generate sufficient signal for low nucleic acid concentrations so that visual or quantitative reflectance measurements are feasible [18]. Alternatively, the membranes could be scanned using a computer scanner, and the grayscale intensity measured for quantification if no reflectometer is available [20].

Optimization of the generic biosensor assay

Initially, capture probe (0.5, 1, 2, 4, 7.5, and 15 pmol per assay) and reporter probe (0.5, 1, 2, 3, and 10 pmol per assay) concentrations were optimized. A clear optimum for the capture probe concentration was found at 4 pmol per assay. In the case of the reporter probes, it was found that the optimal concentration depended on the percent streptavidin surface tag of the liposomes (0.2, 0.4 and 0.6 mol%). The results are summarized in Table 2. Therefore, with increasing streptavidin tag, an increasing reporter probe concentration was optimal. The overall signal height obtained for each optimized condition varied only insignificantly (signals were between 55 and 57 arbitrary units (AU) with background signals between 9 and 11 AU). However, the optimal concentrations were significantly different from other concentrations investigated. In comparison, signals for the next highest reporter probe amount (0.5, 1 and 2 pmol, respectively) ranged between 40 and

Table 2 The optimal reporter probe amounts per assay were determined depending on the streptavidin concentration per liposome. Each assay condition was investigated in triplicate with 1 pmol of *E. coli* target sequence per assay. Reporter probe concentrations were varied between 0.5 and 10 pmol per assay. Maximum signals were around 55–57 arbitrary units (AU) with 9–11 AU as signal of the negative control

Streptavidin tag on the liposome surface (mol %)	Optimal reporter probe amount per assay (pmol)
0.2	1
0.4	2
0.6	3

47 AU. Therefore, two observations can be made from this experiment. If too little streptavidin is available on the liposome surface, more reporter probe added to the experiment results in the availability of free reporter probe in solution. These compete with the liposome-bound probes for the binding to the target sequence and so lower the signal of the assay. On the other hand, at lower reporter probe concentrations, if too much streptavidin is available on the liposome surface, not all liposomes may be coated with reporter probes sufficient for binding to the target sequence, again lowering the overall signal obtainable. Future experiments could include the investigation of varying target sequence concentrations. Preliminary experiments suggest that for lower target sequence concentrations, lower streptavidin and therefore lower reporter probe concentrations will result in higher signals, and so lower detection limits than higher reporter probe and streptavidin concentrations. However, these will also result in a shorter dynamic range, since these liposomes will be already saturated with target sequence at lower target concentrations than the liposomes with more streptavidin surface tag.

In subsequent assays, the hybridization conditions were optimized. In the hybridization buffer, formamide (30–55%), and SSC (7–11 \times) were varied, in order to determine the optimal stringency of the assay. Sucrose and Ficoll type 400 concentrations were used as previously determined to be optimal for liposome stability. The optimal hybridization buffer composition was determined to be 45% formamide, 10 \times SSC, 0.6 M sucrose, and 0.6% Ficoll type 400. The final concentration of each component in the final hybridization mixture was therefore 9% formamide, 2 \times SSC, 0.12 M sucrose, and 0.12% Ficoll type 400. With respect to the running buffer, again formamide and SSC concentrations were varied, between 10 and 50% and 0 and 12 \times , respectively. The optimal running buffer composition was determined to be 30% formamide, 4 \times SSC, 0.2 M sucrose, and 0.4% Ficoll type 400.

The polyethersulfone membranes were optimized with respect to the anti-fluorescein antibody concentration immobilized. The antibody purchased was available at a concentration of 36 pmol/ μ L. In two sets of experiments, the amount of antibody per membrane applied was varied between 10 and 50 pmol with the application volume at either 1 or 1.5 μ L. Even though, in general, it was found that the higher the antibody concentration, the higher the signal and also the signal to noise ratio obtained, mixing the antibody in the sodium carbonate immobilization buffer and applying only 1 μ L instead of 1.5 μ L per membrane was optimal. This correlates well with earlier findings in our lab; that a 0.4 M sodium carbonate buffer at pH 9.0 is optimal for protein immobilization on polyethersulfone membranes. Therefore, optimized membranes were prepared with 1 μ L containing 30 pmol of antibody. However, if a higher concentrated anti-fluorescein antibody could be obtained, this concentration could be further optimized.

The incubation time required was optimized with respect to the time needed for coupling of the reporter and

capture probes to the generic biosensor components, and with respect to the necessary hybridization time with the target sequence. Liposomes, target sequence, reporter and capture probes, and the hybridization buffer were mixed and incubated at 42 $^{\circ}$ C between 5 and 120 min. Best results were obtained for incubation times of 20–30 min. While the signal at 5 min was 70% of the maximum signal obtained, the signal at 120 min was only 44%. Therefore, shorter incubation periods can be used, especially if higher target sequence concentrations are expected, incubation periods that are too long should be avoided.

Finally, since long incubation times had a significantly negative effect on the signal, longer-term storage of the hybridization mixture was investigated. It was found that when storing the entire mixture (with 500 fmol target sequence), either at –20 $^{\circ}$ C for 14 days or for 6 days at 4 $^{\circ}$ C, the signals remained at 65 and 63% of the original signal, respectively. Therefore, storing under either condition is considered safe, if qualitative results are desired. For quantitative results, however, the preparation of fresh hybridization mixtures is suggested.

Detecting synthetic DNA sequences from *B. anthracis*, *C. parvum*, and *E. coli* with the universal biosensor

Dose response curves were generated for three different sequences, *atxA* from *B. anthracis*, *hsp70* from *C. parvum* and *clpB* from *E. coli*. In order to be able to quantify the detection limit and the dynamic range of the assays, synthetic DNA sequences were designed that mimicked the reporter and capture probe regions of the authentic sequences. In the case of *E. coli* and *B. anthracis*, short syn-

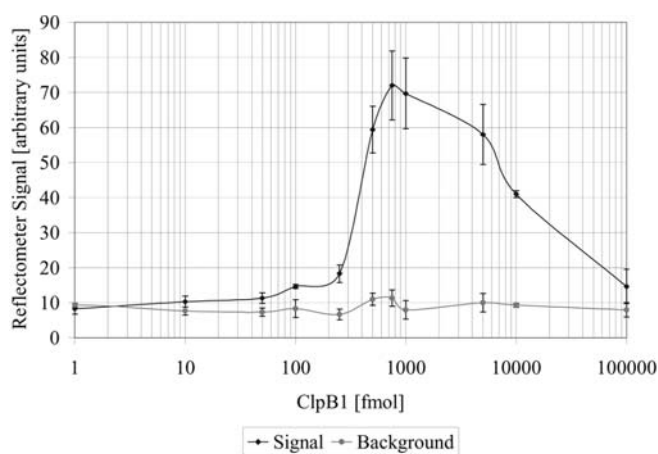


Fig. 3 Determination of the limit of detection for *E. coli* using the generic biosensor assay. Different concentrations of synthetic target sequence were analyzed under optimized conditions using 0.2 mol% tag liposomes. Data are presented in an x-axis log-scale plot, with reflectometer signals in arbitrary units plotted against the amount (fmol) of target sequence used per assay. While concentrations ranging from 0.1–100,000 fmol were analyzed, only those ranging from 1–100,000 are shown here. The negative control contained water instead of target sequence, and had a signal of 7 arbitrary units (AU)

Table 3 Summary of upper and lower limits of detection obtained for the universal biosensor assays detecting *E. coli*, *C. parvum* and *B. anthracis*. The data were compared to those published earlier, generated by biosensors specific for the respective organisms [5, 18, 19]

	Lower limit of detection (fmol)	Upper limit of detection (fmol)
<i>E. coli</i> specific	5	1000
universal	50	750
<i>C. parvum</i> specific	80	2900
universal	10	1000
<i>B. anthracis</i> specific	1.5	100
universal	10	750

thetic sequences were used; in the case of *C. parvum* a longer, 97 nt long segment consisted of most of the *hsp70* sequence of interest. These synthetic sequences had been designed previously for an organism-specific biosensor developed in our lab. Their sequences are given in Table 1. The *E. coli* target sequence was investigated, ranging from 0.1–100,000 fmol per assay (Fig. 3). Triplicate analyses of each concentration were performed. An excellent detection limit of at least 50 fmol (50 nM) was determined, calculated by adding three times the standard deviation of the negative control to its average value, and correlating that to the next highest concentration tested on the curve, (so that $x_0=7\pm 1.0$; $x_0+3\times sd=10<11\pm 1.5$ (signal of 50 fmol)). The upper limit of the dynamic range was 750 fmol. Above target concentrations of 750 fmol, a classical hook effect, typical for sandwich assays, was noted.

Similar studies were performed with *C. parvum* and *B. anthracis*. The data for the lower and upper limits of detection are summarized in Table 3. In addition, the data are compared to those of the specific biosensors published previously. It can be seen that all three assays using the generic biosensor were comparable to the specific biosensors. The discrepancy between the specific and the universal assay for *C. parvum* detection is due to the difference in assay format. Esch and colleagues used a competitive approach in comparison to the sandwich format of the universal biosensor, resulting in a higher value for both the lower and upper detection limit, as expected.

Detection of authentic RNA sequences from *B. anthracis*, *C. parvum* and *E. coli*

All prior experiments used synthetic target sequences in order to optimize the universal biosensor assay and to determine lower and upper limits of detection. In this set of experiments, mRNA sequences isolated from the target organisms were amplified using nucleic acid sequence-based amplification (NASBA) and analyzed with both the universal and the specific biosensors. Details about the amplification reactions, including amplification primer sequences, are given in the respective publications [5, 18, 19]. The specific biosensor for *C. parvum* has been transformed into a sandwich assay (data not published), which

is used here for comparative purposes. Analyses were performed in triplicate. Comparing the universal and specific biosensors, it was again observed that there were no difficulties in detecting amplified authentic RNA sequences with the universal biosensor. Therefore, in all cases the universal biosensor approach can be utilized; no specific biosensor would need to be developed for the specific sequences.

Conclusions

A generic sandwich assay for the rapid detection of analytes has been successfully developed, using the identification of pathogenic organisms via their nucleic acid sequences as examples. Avoiding the need for difficult coupling reactions, generally required in most bioanalytical assays, the generic biosensor can be transformed into a specific assay via a simple incubation step of approximately 20 min. Antigen-antibody and streptavidin-biotin coupling mechanisms were shown to function well under nucleic acid hybridization conditions. No special training and no specific equipment other than a water bath or heating block were required in order to perform the analysis. As a result, from the detailed studies with nucleic acid sequences we conclude that any nucleic acid sequence can be identified and quantified within 30–40 min (20–30 min of pre-incubation, plus 8–10 min for the membrane assay), as long as reporter and capture probe sequences are known. Signals can be evaluated qualitatively by visual detection, or can be quantified using a computer scanner or a portable reflectometer to obtain low limits of detection of 10 nM (10 fmol per assay). The reporter and capture probes tested here ranged from 18–21 nt in length and had T_m values 49.5–62 °C. It is expected that probes with similar characteristics will perform as well in the universal assay as the sequences tested here, and that small signal decreases can be expected with much shorter or much longer probes (those having much lower and higher melting temperatures). In that case, the formamide and SSC concentrations in the master mix and running buffer could be adjusted accordingly, if necessary. If double-stranded DNA molecules are the target analytes, an additional denaturing step is suggested, in which the probes are mixed with the target sequence and hybridization buffer, and brought to 95 °C prior to adding the liposomes and initiating the biosensor assay.

While no specificity studies were presented in this paper, it is known from previous studies that under the given stringency conditions, highly specific biosensors are obtained [5, 6] which should be directly transferable to the generic biosensor approach. Studies of effects from varying pH, and salt concentrations of the target analyte solution will be made in the future. However, due to the high buffering capacity of the hybridization buffer and the small volume of target analyte added, these effects should be limited. This universal biosensor is therefore ideal for research laboratories as a substitute for laborious methods such as Northern and Southern Blotting, for improving on

techniques in which only agarose gel electrophoresis is used for sequence identification, and will also be useful for the development of specific biosensors, since many target and detection sequences can easily be tested with the same biosensor assay during the testing stage. While a similarly-detailed study of antigen detection is needed to confirm that the generic biosensor format can also be applied to the use of any immunological sandwich assay, we can conclude from our data that this will likely be feasible, since antibody-antigen binding for analyte detection should be highly compatible with the antibody-antigen coupling mechanism employed in the generic assay.

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