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ORIGINAL PAPER

Biosensor-based on-site explosives detection using aptamers as recognition elements

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Abstract Reliable observation, detection and characterisation of polluted soil are of major concern in regions with military activities in order to prepare efficient decontamination. Flexible on-site analysis may be facilitated by biosensor devices. With use of fibre-optic evanescent field techniques, it has been shown that immunoaffinity reactions can be used to determine explosives sensitively. Besides antibodies as molecular recognition elements, high-affinity nucleic acids (aptamers) can be employed. Aptamers are synthetically generated and highly efficient binding molecules that can be derived for any ligand, including small organic molecules like drugs, explosives or derivatives thereof. In this paper we describe the development of specific aptamers detecting the explosives molecule TNT. The aptamers are used as a sensitive capture molecule in a fibre-optic biosensor. In addition, through the biosensor measurements the aptamers could be characterised. The advantages of the aptamer biosensor include its robustness, its ability to discriminate between different explosives molecules while being insensitive to other chemical entities in natural soil and its potential to be incorporated into a portable device. Results can be obtained within minutes. The measurement is equally useful for soil that has been contaminated for a long time and for urgent hazardous spills.

Keywords Aptamer · TNT · In-field measurement · Biosensor

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Introduction

The most widely used explosive worldwide is still trinitrotoluene (TNT). Besides the danger of forgotten explosive landmines or nondeactivated munitions or during a hazardous incident, TNT poses a considerable health risk for long periods to the population. TNT contaminates industrial wastewater or may leak from obsolete storage facilities, contaminating soil and water. To be able to protect the civilian and military population as well as to demilitarise and clean areas previously used for munitions storage and processing, there is an urgent need for a fast, reliable and inexpensive method to detect explosives. The method should even allow detection of trace amounts of TNT in soil, groundwater or as leftovers on materials or surfaces. Health problems after TNT uptake may occur in consequence of the metabolism of explosives in the human body, which produces cancerogenous derivatives [1, 2]. This illustrates an additional need for accurate analysis of these explosives and their metabolites in body fluids like blood or urine. To our knowledge, the production, storage and use of explosives have already resulted in a widespread environmental problem because mutagenic, toxic and persistent

compounds have leached from soils and have accumulated in the food chain [3–6].

So far a number of classic analytical methods, such as colorimetric methods, high-performance liquid chromatography (HPLC) and gas chromatography-mass spectroscopy, as well as different sensor techniques have been used to detect explosives. Sensor platforms are based on electrochemical methods [7], a micropourous membrane or microcapillary immunosensors [8]. In the field of explosives, Pinnaduwage et al. [9, 10] reported measurements of TNT in a small, localised explosion on an uncoated piezoresistive microcantilever.

Other systems including protein microarrays are used [11]. The biological detector components of those previous systems have been antibodies. A comprehensive test and characterisation of anti-TNT antibodies was described by Goldman et al. [12]. Fibre-optic methods were reported by Shriver et al. [13, 14] and in a review by Singh [15].

Notably, systems that are able to detect explosives with high sensitivity and that are able to discriminate effectively between different species are also systems that need a high level of expensive instrumentation, usually in a laboratory setting. This requires good handling skills (i.e. for chromatographic methods). Other systems that are easy to handle and at the same time cost-effective (i.e. colorimetric methods) usually do not exhibit very high sensitivity and cannot distinguish well between different chemicals. It should be noted, however, if the explosives are expected to occur in considerable amounts and if only a class of explosives needs to be detected, those methods will presumably continue to be the preferred methods of detection.

For the work presented here, high-affinity nucleic acids (aptamers) for TNT were developed and used for the first time as molecular recognition elements instead of antibodies.

Nucleic acid aptamers are single-stranded nucleic acids usually ranging between 30 and 70 nucleotides in length. As a result of the capacity of nucleic acids to form elaborate three-dimensional structures and shapes, aptamers can bind with high specificity and high affinity to a special target molecule [16, 17], e.g. in biosensors [18].

We designed and developed aptamers recognising the low molecular weight, hydrophobic target molecule TNT with high affinity and specificity. We found our aptamers to be significantly more sensitive and specific than antibodies against the same target molecule, while in addition rendering our sensor system considerably robust for different application systems, such as on-site detection of TNT contamination within minutes. This paper describes the development of a fibre-optic aptamer sensor for fast explosives detection.

Usually aptamers are selected by the SELEX procedure [19, 20], which is described as an iterative process of binding, partitioning and amplification steps to select single high-affinity aptamers out of a pool of unspecific, randomised oligonucleotide species. After a number of cycles of the iterative binding, selection by partitioning and an amplification process, the remaining library of molecules will bind tightly to the target of interest. Individual molecules in the mixture are then isolated, their nucleotide sequences determined and their properties, with respect to binding affinity and specificity, measured and compared.

Since Gold, Ellington and their groups developed their method, it has been used to isolate high-affinity aptamers against a multitude of targets and further methods for aptamer development are being explored.

The robustness of aptamers is also reflected in their temperature tolerance from ambient or room temperature up to higher temperatures. In contrast to antibodies, aptamers do not lose their shape and, therefore, their binding capacities when heated up to temperatures well above 60 °C and then cooled down again to room temperature or the working temperature needed. Unlike proteins, nucleic acid aptamers do not need to be constantly kept at 4 °C until use, but can be prepared and then used at room temperature or at a moderate ambient temperature for at least 2 days.

Materials and methods

Instrumentation

The principle of detection in the biosensor is based on fibre-optic measurement. As a measurement category we currently use fluorescence. The fibre-optic apparatus is presented schematically in Fig. 1 and has been described previously [21, 22].

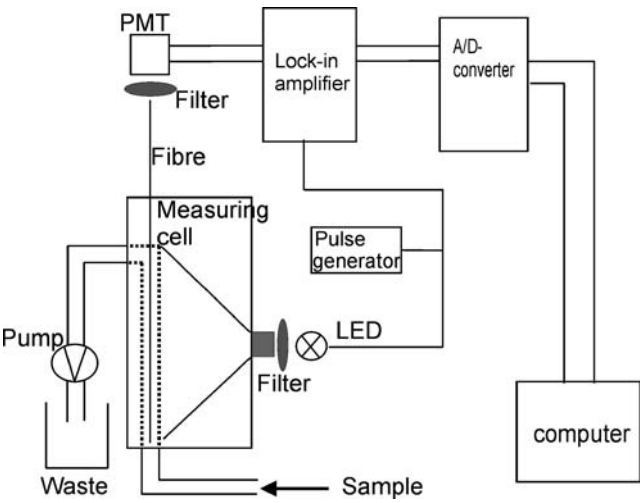

Fig. 1 Fibre-optic device based on the evanescent field technique. The sample is pumped through the measuring cell with an embedded sensing glass fibre. The excitation light is passed through an interference filter and is guided by a fibre bundle to the measuring cell. The sensing glass fibre guides the fluorescence through a filter to a photomultiplier tube (*PMT*). The PMT signal is collected by a lock-in amplifier, converted and passed to a computer for data sampling

The device consists of a blue LED (Nichia, Japan) for excitation. The light is passed through an interference filter (480 nm, bandwidth less than10 nm) and is then guided by a fibre to the measuring cell. The excitation light hits the sensing fibre in a glassy flow-through cell perpendicular to the fibre axis (500 nm cutoff) that leads to a photomultiplier tube (R1463, Hamamatsu, Japan) running at −1,000 V. The photomultiplier tube signal is collected by a lock-in amplifier (LA5101, EG&G), which is triggered by the same pulse generator also triggering the LED. The flow cell has an effective volume of 100 μl. Samples are drawn by a peristaltic pump behind the flow-through cell. Teflon tubing is used to minimise adsorption and dispersion. All parts are connected to a PC to coordinate the flow regime and the accumulated data. During the project, this classic instrument was developed into a portable in-field measurement system.

Fibres

The surface of unmodified fibres had to be modified for the binding of ligands such as TNT. The fibres were standard hard plastic clad silica fibres with a pure fused-silica core, a hard polymer cladding and a Tefzel® jacket with an internal diameter of 400 μm (Laser Components, Germany).

The modification process started with the removal of the polymer cladding at one end of the fibres. These were treated with 5 M NaOH for 3–4 h, activating the surface for the following silanisation procedure. The cleaned fibre was silanised with 10% (vol/vol) aminopropyltriethoxysilane in dilute HCl (pH 3.45) at 80 °C for 2 h, washed and dried at 100 °C.

On the silanised surface, activated TNT derivatives were coupled for 2 h at room temperature to create the sensor surface on our fibres. For this, TNT had been treated via acidic group derivatisation and activation. After immobilisation of the TNT derivatives on the fibre surface, the fibres were introduced into the flow cell.

Development of TNT-specific aptamers

The specific TNT-detection molecule of our sensor is an aptamer (RiNA, Berlin), recognising TNT either coupled to the surface or free in solution. This aptamer has been especially designed and developed against TNT. For the aptamer development, an aptamer precursor pool of 90 almost randomised nucleotides was designed, synthesised and produced as RNA using adjacent primers for T7 transcription and reverse transcriptase reactions. Aptamer precursors were incubated at room temperature in a tris (hydroxymethyl)aminomethane (Tris)/HCl buffer (10 mM, pH 7.6, 150 mM NaCl, 5 mM $MgCl_2$, 5% methanol) with immobilised TNT. TNT immobilisation was performed either on Sepharose (immobilised to saturation) or on glass fibres. Non-TNT-binding oligonucleotides were removed or reduced in quantity, i.e. by extensive washing processes with the same buffer. TNT-binding species were released from the immobilised TNT alternately either by unspecific incubation steps using detergents such as 2% sodium dodecyl sulfate (SDS) or using heat, or by specific incubation steps using high TNT concentrations up to 1.5 mM in buffer. After 13 different steps, a library of high-affinity binders could be identified and single aptamer species could be isolated and characterised.

To easily label the final aptamers with a fluorescent dye, the aptamers included a known specific sequence. An oligonucleotide with the complementary sequence was biotinylated and annealed to the aptamers. A fluorescent nanobead was attached to the biotinylated oligonucleotide. The beads used were amine-modified fluoSpheres (Molecular Samples) with a diameter of 200 nm. Using this labelling technique with a biotinylated oligonucleotide-bead format we can easily adapt our system setup to aptamers with other specificity. Because the aptamers have to be active in organic solvents, they were tested with methanol, propanol and acetonitrile.

Analytes

As a model our aptamer ligand of interest was TNT. For measurements of cross-reactivity, chemicals with isomorphic properties such as Tetryl (2,4,6-trinitrophenyl-*N*-methylnitramine) and 4-nitrophenol were used. Furthermore 2,4-D (2,4-dichlorphenoxyacetic acid) was tested for cross-reactivity to the aptamer. The concentration of the reactants was adjusted within the range from 1×10^{-5} to 10^{-10} M. TNT is only partially soluble in water or aqueous solutions but is fully soluble in acetonitrile or methanol. Both solvents are usually used to extract TNT from soil samples. For the extraction of TNT from soil, different amounts of diverse soil samples (5–100 g) were incubated with equal amounts (in millilitres) of either methanol or acetonitrile. After vigorous shaking for 5–10 min, an aliquot of the supernatant solute was measured directly in a mixture with the aptamers without the need for further concentration steps.

Control experiments for the TNT detection and concentration measurements were achieved using HPLC.

Measuring procedure in the sensor

The measurement in the flow system was performed following an indirect competitive immunoassay format in a microtiter plate as illustrated in Fig. 2. A derivative of the analyte TNT was activated and covalently bound to the previously silanised sensor surface. The procedure of TNT

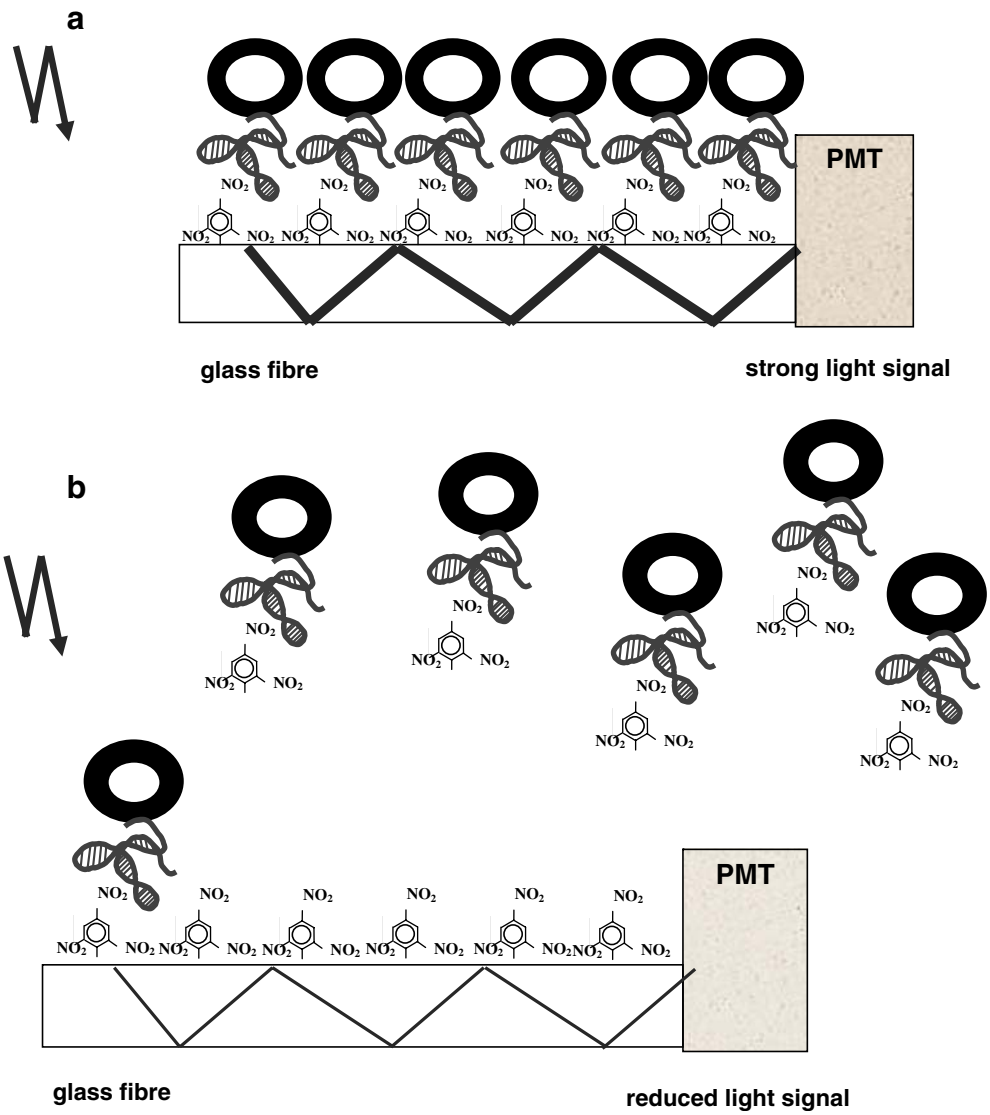

Fig. 2 The measuring principle of the TNT sensor. **a** Sensor fibre with immobilised TNT. Fluorescently labelled aptamer (shown as a *dark green structure* with a *yellow bead* attached) is added to the flow cell and binds to its target molecules on the surface. The fluorescence of the bead is induced by excitation with laser light (480 nm, shown as a *red arrow*). The PMT detects the light at the fibre. **b** Sensor fibre with immobilised TNT. Fluorescently labelled aptamer and sample are mixed, and then added to the flow cell. If TNT is in the sample, the aptamer will bind to the free TNT instead of the immobilised TNT on the surface, which results in a reduced light signal at the surface

coupling was identical for all experiments. For this kind of experiment it is necessary to ensure an overrun of binding sites on the fibre surface. This was checked before further procedures were carried out. After coupling and fibre insertion, the surface was rinsed with buffer containing 10 mM Tris/HCl pH 7.6, 150 mM NaCl, 5 mM $MgCl_2$, 5% methanol. For the measurement process 50 µl of fluorescently labelled aptamers (20 µM in buffer) was mixed with 50 µl of samples of TNT, soil extracts (extracted either in 100% acetonitrile or in 100% methanol) containing different concentrations of the analyte TNT or unknown soil extracts/samples to be tested for TNT contamination, respectively. The resulting 100 µl was then added to the flow cell by the pump mechanism of the system. The aptamers of the sample mixture bind to the immobilised TNT on the fibre immediately, unless they have already been captured and occupied by TNT in the sample mixture. After the samples had been loaded into the measuring cell, buffer was flown continuously through the cell. The aptamers that stay bound to the TNT on the fibre cause a fluorescence signal. The binding of free aptamer to TNT immobilised on the fibre was measured in real time and the signal was recorded as a curve of fluorescence intensity at the fibre versus time (Fig. 3). As a reference and a control, each measurement series contained one measurement of a mixture of aptamer and extraction solvent only. In the absence of TNT in the sample (or as in the reference), all the aptamers will bind unhindered to the surface and will give rise to a high signal (100%) (Fig. 2a). In the presence of the analyte TNT in the sample, the aptamer binding to the surface is inhibited accordingly (Fig. 2b), because the aptamers are already occupied by free TNT. The amount of signal inhibition corresponds to the concentration of free analyte in the sample.

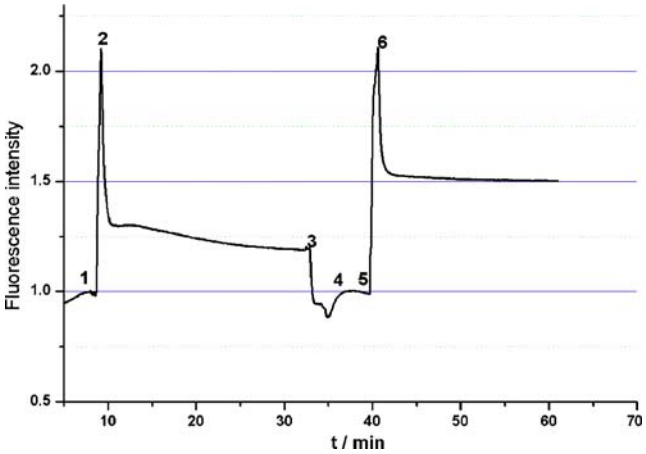

Fig. 3 Sensogram of TNT measurement. At point *1* aptamer sample loading into the flow cell is started; at point *2* the rinsing begins by changing to buffer flow. The signal of aptamers that stay bound to immobilised TNT is visualised as an elevated signal. At point *3* the experiment is stopped using a regeneration solution (i.e. sodium dodecyl sulfate or NaOH) that will release all aptamers from the fibre. At point *4* the flow cell is equilibrated with buffer and a new sample loading cycle can begin [point *5*, 50 μl of fluorescently labelled aptamers (20 μM) mixed with 50 μl of buffer], which will show aptamer binding after changing to buffer rinse (point *6*)

Results

A typical sensogram we achieved is shown in Fig. 3. TNT was covalently bound on a fibre surface as described in "Materials and methods" and the fibre was inserted in the system. As can be seen in Fig. 3, the measurement starts with a base line while buffer is flowing through the flow cell. At point 1 in Fig. 3 the aptamer sample loading into the flow cell is started; at point 2 the rinsing begins by changing to buffer flow. The signal of aptamers that stay bound to immobilised TNT is visualised directly. At the end of signal measurement the experiment is stopped using a regeneration solution (i.e. SDS or NaOH) (Fig. 3, point 3) that will release all aptamers from the fibre. The flow cell is equilibrated with buffer (Fig. 3, point 4) and a new sample loading cycle can begin (Fig. 3, point 5), showing aptamer binding after changing to buffer rinse (Fig. 3, point 6). The first loading cycle of Fig. 3 contained aptamers plus free TNT in the loaded sample, whereas the second loading cycle contained only aptamers in the sample. Figure 3 shows that loading of an aptamers-only sample (Fig. 3, point 5) that does not contain free TNT gives rise to a high fluorescence signal at the fibre because all aptamers are available to bind to the fibre. This second cycle in Fig. 3 is a measuring cycle that is performed in all of our measurement series as a control and reference to achieve the 100% signal value of the capacity of aptamer binding to the fibre when no free TNT is present. In contrast, loading of an aptamer sample mixed with free TNT (Fig. 3, point 2) results in a signal at the fibre that is much lower, because many of the aptamers are occupied by the TNT of the sample mixture and can therefore not bind to immobilised TNT on the fibre. Figure 3 nicely illustrates, in addition, the capacity of the fibre system to be regenerated. With use of various regeneration solutions, the basal signal of the fibre can be regenerated after aptamer binding (Fig. 3, point 3).

Moreover, repeated measurements with the same sample solution show exactly reproducible signal values. For ten measurements the standard deviation is around σ_n=0,062. A prepared fibre can be reused at least ten times not only with the same aptamer species, but also with different aptamers.

Even without regeneration, a fibre can be reliably used consecutively with at least five different sample–aptamer mixtures (as employed in the following experiments). Each mixture is applied only after reaching a stable signal plateau as the new base line. Until all immobilised TNT molecules on the fibre have been saturated with aptamers and thus are no longer available for measurement, new aptamer mixtures can be applied (including the reference sample).

Calibration and binding characteristics of the best aptamer species in the biosensor

Subsequent to the original selection strategy on Sepharose as described in "Materials and methods", the aptamer-development steps were refined using fibre-immobilised TNT until high-affinity recognition molecules could be isolated. The strong binders show high sequence similarity (94–98% identity, alignment using the ClustalW program). One of these best binders was used for all the following experiments. Figure 4 shows the calibration curve using an in-house TNT concentration standard and the curve starts with a blank.

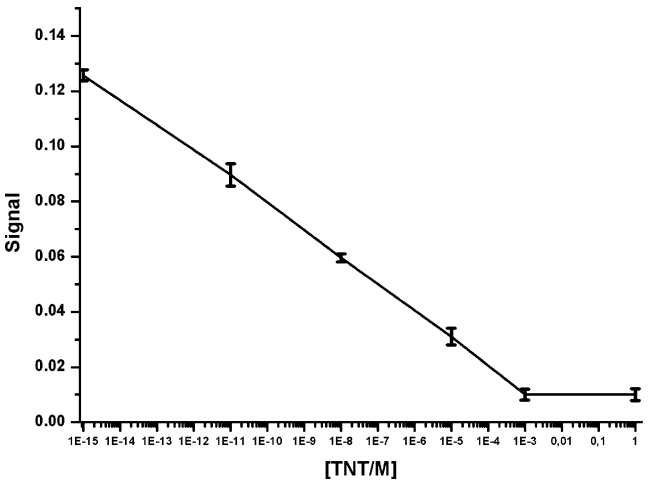

Fig. 4 Quantitative analysis of the best binding aptamer species in the biosensor. The measurements were performed as described for Fig. 4

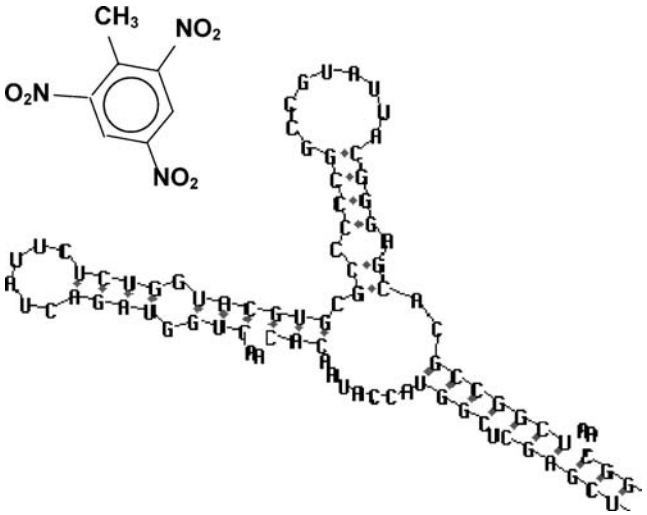

Fig. 5 Predicted secondary structure of a high-affinity aptamer candidate with possible vicinity to its target TNT

The calibration curve suggests a detection limit in the picomolar range. In comparison with former publications reporting use of classic antibody-based ELISAs, our aptamers show a very good detection limit. Zeck et al. [23] reported, e.g., a detection limit of 0.06 ng/ml and Sapsfort et al. [11] reported a detection limit of around 20 ng/ml in their indirect competitive immunoassays.

An example of the best binding sequences is shown in Fig. 5. It is a predicted two-dimensional estimation (M-fold by Zuker et al.) of the possible structure of one of our high-affinity TNT-aptamers and an estimation of the likely location of the TNT molecule with reference to the RNA structure. The TNT molecule is depicted in the vicinity of sequence parts that differ in single nucleotides between aptamer sequences and that result in minor binding affinity changes. A three-dimensional structure prediction and determination of the complex will give valuable insight into the binding characteristics of the two molecules.

Specificity and robustness of the TNT sensor system

Subsequent to our system evaluation experiments, we could show the applicability of our method for the reliable measurement of TNT contamination in water, in solvent and in soil samples. Soils of different origin were extracted with methanol or acetonitrile and the extracts tested directly with the aptamers in our sensor system.

First, the influence of solvent on the aptamer reactivity was tested. Neither methanol nor acetonitrile has any effect on the binding affinity behaviour of the aptamer.

Second, extractions with either methanol or acetonitrile showed the same TNT concentration results when extracting TNT from soil. During the course of the experiments described here all extractions were done using methanol. For detection control, soil samples known to be uncontaminated were measured as well as soil samples spiked with TNT.

In order to test the specificity of the TNT-aptamer sensor, TNT, Tetryl and 4-nitrophenol were also measured as laboratory standard solutions and their detection by TNT aptamers was compared. Figure 6 shows an overlay plot of examples of these measurements. The first measurements were done as an internal system control without any TNT concentration. It can be seen that the values are similar for a pure methanol-buffer solution (measurement 1), a methanol-water extraction from a soil sample (measurement 2) and a sample spiked with 0.1 mg/ml Tetryl (measurement 3). Measurement 4 is for a sample solution spiked with 1×10^{-11} M TNT and measurement 5 is for a TNT-spiked soil, spiked with a comparable amount of TNT. Measurements 6 and 7 are for a similar experiment with a sample solution containing 1×10^{-8} M TNT and with an extraction of TNT-spiked soil containing also Tetryl. Columns 8-10 represent measurements of extractions of real soil samples of military areas. In HPLC control measurements at the Wehrwissenschaftliches Institut für Werks-, Explosiv- und Betriebsstoffe (WIWEB) the concentration of TNT in the extraction sample of measurement 8 was determined to be 1×10^{-4} M and to be 1×10^{-5} M in the samples of measurements 9 and 10. The sample of measurement 10 included also 4-nitrophenol. Overall, the values obtained

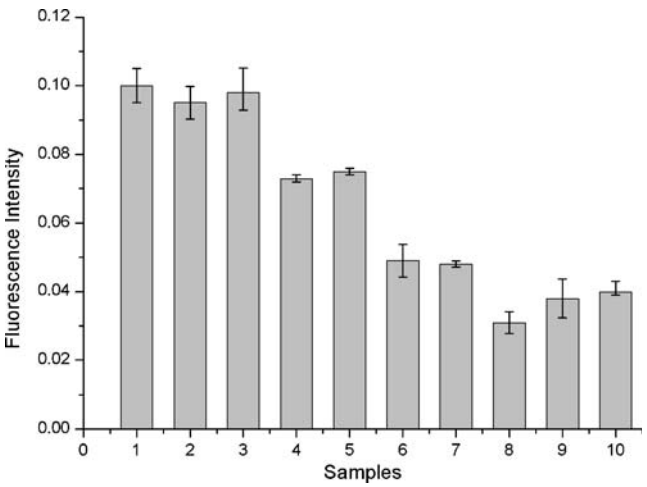

Fig. 6 Overlay plot of binding curves of different explosives compounds in the TNT sensor. *1* methanol-buffer solution, *2* methanol-water extractions from a soil sample, *3* the sample spiked with Tetryl, *4* solution spiked with 1×10^{-11} M TNT, *5* extractions from a spiked soil, *6* solution spiked with 1×10^{-8} M TNT, *7* extractions from a spiked soil including Tetryl, *8-10* real soil sample

in our TNT-aptamer biosensor are in good accordance with the results of the control HPLC analytes.

Discussion

This paper illustrates that biosensor systems based on aptamer technology seem to combine the properties of easy handling devices for on-site measurements and high specificity as well as sensitivity. Antibodies are successfully used in a variety of biosensor systems. Unfortunately, low molecular weight compounds such as TNT often seem to be recognised by antibodies only with considerably low affinity and specificity. The development of more sensitive antibodies needs a considerably more complex and extensive immunisation strategy, supposedly more laborious than the in vitro selection strategy of aptamer molecules. Like antibodies, nucleic acid aptamers recognise their targets through several contact points and folding pockets. So they seem to be advantageous especially for low molecular weight targets, since they tend to be more flexible and versatile in structure and seem to be able to unfold more three-dimensional contacts surrounding small target molecules.

In addition, aptamers can be developed (and have been shown by us to work) in concentrations of organic solvents suitable for solubilisation and detection of organic molecules such as TNT, a property that, amongst further advantages, gives aptamers a considerable additional advantage compared with proteinaceous antibody molecules when molecules such as TNT are targeted. We have shown our aptamers work well in acetonitrile and methanol concentrations of at least 50%.

The sensor system presented is sensitive enough to be able to directly measure TNT contamination in the field, because the facile extraction methods used in connection with the sensitive aptamer detector molecules do not need further concentration steps and the analysis results can be seen directly in real time and within minutes. Traditional HPLC analysis usually requires additional concentration steps and expensive instrumentation in a laboratory setting to detect environmentally relevant TNT concentrations.

In our present system, one fibre can be used for at least five consecutive measurements if needed, but the fibres can also be regenerated after each use, as shown in Fig. 3. Regeneration will, however, need a few extra minutes, extra handling and extra liquids per sample in the format we have developed so far. An automated process including these steps is conceivable and needs to be developed in the future.

The aptamers presented show high specificity to detect their target TNT versus other structurally distinct targets or by-products of TNT synthesis even if the targets show some similarity to TNT, such as Tetryl (Fig. 6). This is an essential property for a recognition molecule to unequivocally identify and qualify a chemical. In some applications, however, rather than a very specific explosives compound or chemical needing to be detected, a whole class of explosives needs to be detected. Our sensor system can be adapted easily to use a mixture of different aptamers or detector molecules, each recognising a certain chemical. In addition, aptamers could be developed to recognise only common structures of a class of chemicals.

An equally essential property of a field sensor system lies in its robustness to still reliably detect the target even in a crude mixture with other chemicals, as shown for our aptamer system (Fig. 6).

However, nucleic acid aptamers are highly susceptible to nucleases, in the case of RNA aptamers to RNases and in the case of DNA aptamers to DNases. So, caution needs to be applied when using unstabilised aptamers in different systems. In our case, the TNT-specific aptamers did not need to be stabilised and worked perfectly in the field measurements and with all the samples we tested. This could be due to the length and/or structure of our aptamers that positively influenced stability and also to a lack of considerable amounts of RNases in our samples. We tested a diverse number of laboratory and field water samples with our TNT-specific aptamers and found them to be stable for hours (data not shown). In addition, aptamers can be stabilised by chemical modifications, i.e. at their sugar moieties, or by using elegant approaches such as spiegelmer technology [24], rendering them stable enough for manifold applications not only in diagnostic biosensors, but also in medical systems. Especially for medical applications, an aptamer could not possibly be active without extensive stabilising modifications to fight off the manifold RNases and DNases in plasma or cellular fluids and to keep the aptamer in circulation long enough for its effects to unfold.

So far, our field sensor system can only qualitatively indicate the presence of TNT and can only give a raw estimate of possible concentrations. We are working on its extension for quantification analysis. Nevertheless, the high sensitivity and specificity of our system as it is now mean that it reliably detects already trace amounts of TNT in the parts-per-billion range and is not prone to false signalling.

Owing to their high specificity and sensitivity and their advantageous chemical properties, we believe that amongst biosensor systems, nucleic acid aptamer systems will

become increasingly important especially for the detection of low molecular weight compounds such as TNT. Our field aptamer fibre-optic system is capable of detecting contamination within minutes on-site.

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