

Self-Assembled Modular TNT Biosensor

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Introduction: Increasing demand is being placed on the biosensor community to deliver sensors for military and security purposes. The Navy and Homeland Security are particularly interested in detecting explosives underwater, especially in marine and port environments. Rather than developing a unique sensor for each analyte of interest, a generalized sensing strategy that is readily adaptable to a variety of small analytes in many challenging environments would be extremely useful, but this has been lacking until now. We developed a general biosensing strategy that is based on a modular design and employs multifunctional surface-tethered components. Biosensors built on this strategy are designed to be fully reversible, reagentless, and capable of self-assembly on surfaces.¹ The first prototype targeted the nutrient sugar maltose and utilized *E. coli* maltose binding protein and a flexible biotinylated DNA oligonucleotide that contained both a fluorescent dye and an analog of maltose.¹ A key criterion in the overall sensor design is modularity with the idea of facile adaptation to target other analytes. We demonstrate here that this same self-assembled modular-sensing strategy can be applied for the sensitive and specific detection of 2-, 4-, and 6-trinitrotoluene (TNT) in aqueous environments.²

Sensor Self-Assembly and Function: The TNT sensor consists of a dye-labeled, TNT-binding, single-chain antibody fragment (α -TNT scFv), which interacts with a multifunctional DNA arm. The arm contains a TAMRA dye attached to a modified internal base and is terminally labeled with the TNT analog 1,3,5-trinitrobenzene (TNB), (Fig. 7). The α -TNT fragment, dye-labeled with AlexaFluor 532 dye on a unique cysteine residue, is allowed to bind the TNB analog on the DNA arms' terminus, bringing both the AlexaFluor and TAMRA dyes into close proximity and establishing a baseline level of fluorescence resonance energy transfer (FRET). In this configuration, selective excitation of the AlexaFluor donor dye results in significant energy transfer to the TAMRA acceptor dye. This complex is then self-assembled on a NeutrAvidin (NA) covered microtiter well. Addition of TNT to the sensor solution displaces the TNB from the α -TNT fragment binding site, altering the proximity of the dyes and resulting in a concentration dependent change in FRET, which is monitored for signal transduction. The sensor can be washed free of analyte and regenerated for subsequent detection events.²

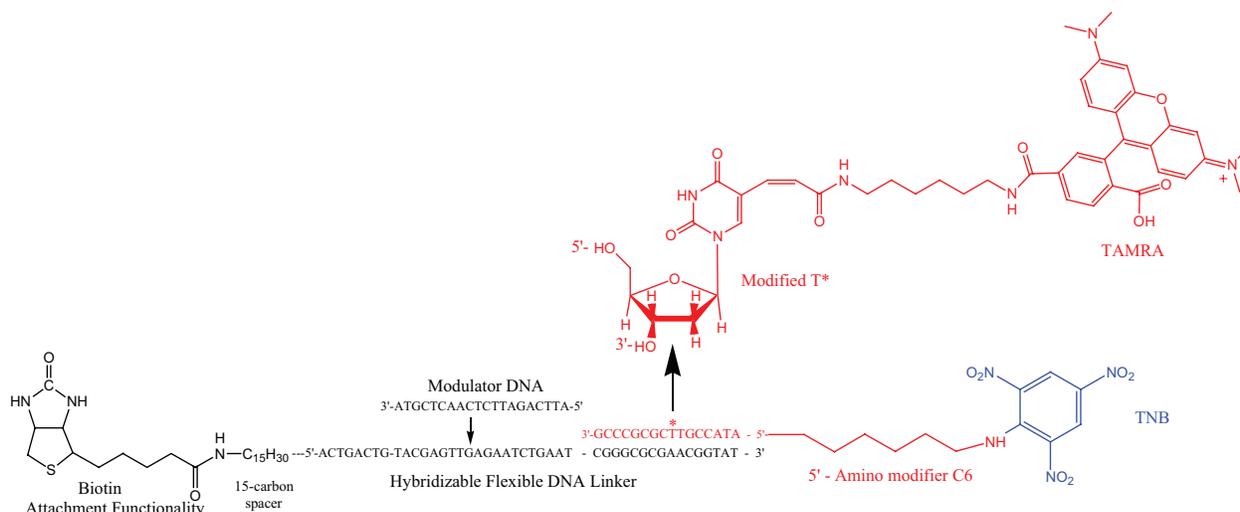
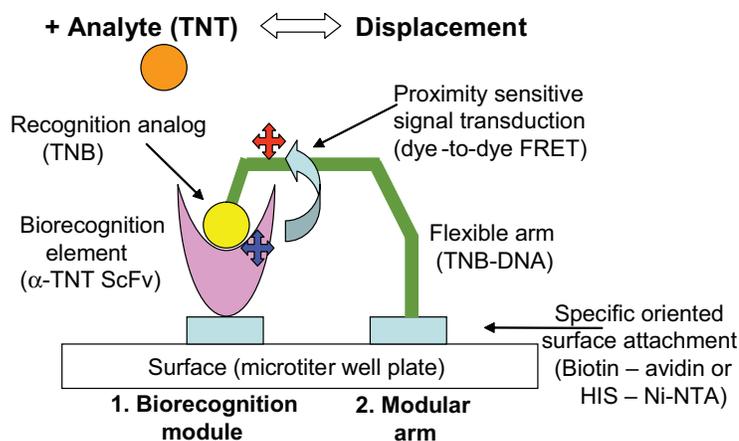
Sensor Testing and Specificity: TNT was tested for in phosphate buffered saline (PBS), a matrix more saline than seawater (Fig. 8). The resulting loss of FRET-based fluorescence at 600 nm was plotted against the concentration. A lower limit of detection of 1 mg/L TNT (1 ppm) was noted for this sensing assembly. The binding curve appears as a relatively linear function over the concentrations tested. The sensor assembly was washed using 10 volumes of PBS and then regenerated in PBS. A second TNT titration was performed; the sensor was washed, regenerated for 1 hr at room temperature, and then a third titration was performed. Similar control experiments were performed using sensor assemblies, incorporating a dye-labeled DNA arm, lacking the TNB moiety by substituting a control myoglobin protein, which resulted in no change in fluorescence upon TNT addition.

To determine specificity and cross-reactivity, sensor assemblies were tested against several TNT structural analogs (Fig. 9). TNB, used for the original antibody fragment selection, elicited the highest sensor response and was the most effective competitor for binding sites. Other explosives tested included RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) and 2-amino 4,6 DNT (2-amino-4,6-dinitrotoluene), which caused significantly less response. The least response was seen with 2,4 DNT. The dynamic sensing range of this sensor assembly was modulated by "stiffening" the tether arm through the addition of a DNA complementary to the DNA linker (Fig. 7(b)). Although the binding constant essentially remained the same, the binding curve of the modulated prototype became broader, significantly increasing the useful sensing range. The lower limit of TNT detection dropped approximately tenfold from 1 mg/L to 0.1 mg/mL (100 ppb). This sensitivity approaches that of whole antibodies, selected specifically against TNT, but whose bulk size has precluded use in the current sensor configuration.

Discussion: The TNT sensing assembly demonstrated here has several desirable features. These include the use of robust avidin-biotin chemistry for surface tethering, ease of self-assembly in a microtiter plate format, and analysis by readily accessible fluorescent plate readers. The sensor can be regenerated for subsequent reuse and an upper limit of 6 to 8 regenerations was obtained. This also suggests that real-time sensing in a flow cell is feasible. The current results validate the modular nature and adaptability of the sensor design. Comparison to the original maltose sensor¹ confirms the design can easily be adapted to target another analyte and yet remain functionally robust. The many choices of biorecognition elements available, including other binding proteins, receptors and antibody fragments that can fit directly into the current format, suggest that this design could be easily adapted to

FIGURE 7(a)

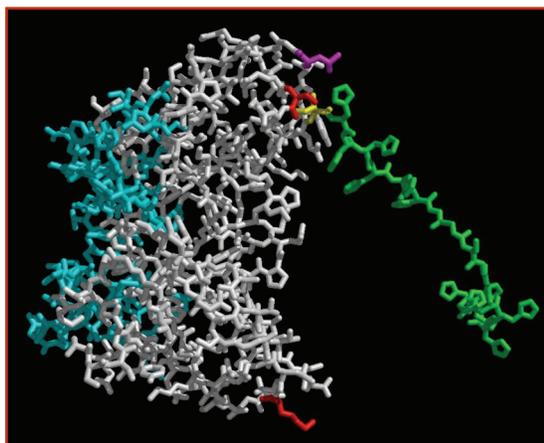
Schematic of the modular biosensor illustrating its targeting of the explosive TNT. The sensor consists of two modules: (1) the biorecognition module, a single-chain antibody fragment specific for TNT (α -TNT scFv); and (2) a modular arm (DNA). Both modules are specifically attached to a surface in a particular orientation using biotin-avidin chemistry and metal-affinity coordination. The surface of the microtiter well plate is coated with NeutrAvidin (NA), and the dye-labeled TNB DNA arm (2. modular arm) is attached to the NA via complementary hybridization to a biotinylated flexible DNA linker. The dye-labeled α -TNT scFv (1. biorecognition module) is attached to the surface with Bio-X-NTA (Biotin-spacer-nitrilotriacetic acid), coordinating the fragments 12-histidine residues and orienting the protein on the NA. Both modules are added together in equimolar amounts and then self-assembled on the surface to form the final sensor. ScFv binding of the TNB analog brings the protein-located dye and DNA-located dye into close proximity, establishing efficient FRET. Addition of TNT displaces the TNB analog and DNA arm disrupting FRET in a concentration dependent manner. The sensor is washed and regenerated for subsequent detection events. Rigidifying the DNA arm can modulate or usefully improve the dynamic sensing range.^{1,2}

**FIGURE 7(b)**

Structure of the dye-labeled TNB DNA arm. The precursor DNA modified with a TAMRA dye on an internal T* and the 5' amino modifier C6 is shown in red. The amine was subsequently modified with TNB (blue). The flexible DNA linker and modulator DNA aligned with its complement are shown in black. Hybridization of the linker to the TNB containing oligonucleotide completes the arm and allows it to be attached to the NA surface by the biotin.²

FIGURE 7(c)

Model structure of the α -TNT scFv fragment. The (His)₆-spacer-(His)₆ carboxy terminus is shown in green. The sites of cysteine mutations tested are shown in yellow, red, and magenta. Only the magenta site yielded a viable fragment. The "TNT binding region" as defined by the hypervariable regions of the scFv is colored cyan.²



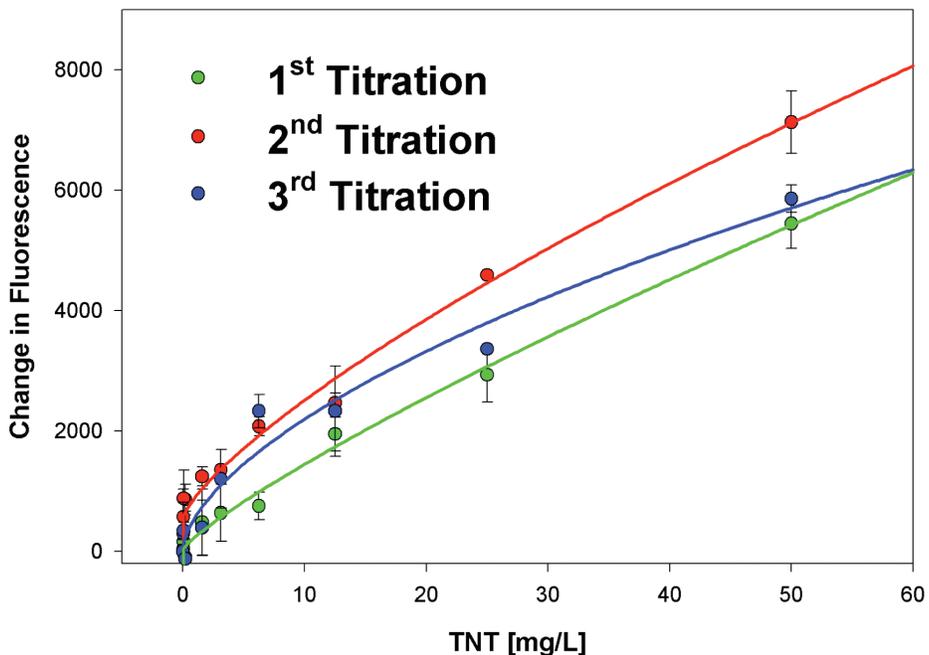


FIGURE 8 Repetitive testing of the sensor assembly with TNT. The sensor was self-assembled, challenged with TNT, washed, regenerated, and tested again. Samples were excited at 510 nm and emission monitored at 600 nm. The change in fluorescence is plotted on the vertical axis versus TNT concentration in the horizontal axis. TNT concentrations are in mg/L, which corresponds to ppm. In this configuration, a maximum of 6 to 8 consecutive sensings were attained.²

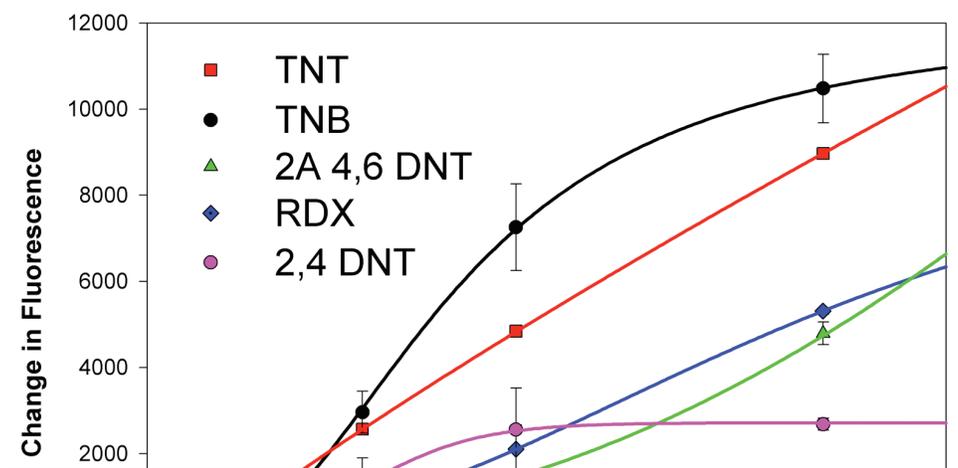


FIGURE 9 Sensor specificity. Sensor assemblies were tested against the indicated explosives. Only TNT and its closest analog TNB resulted in significant changes in sensor fluorescence. As the antibody fragment was generated against TNB it resulted in the largest changes.²

target further analytes. Experiments with the original maltose-sensing prototype showed that sensor assemblies could be dried, stored, and reconstituted for later use. This would allow sensors to be manufactured in one location and reconstituted for use at other locations or remote field sites. Multianalyte or “multiplex” sensing formats that screen for multiple analytes may also be possible. Current research is focused on adapting this sensor to target other analytes of priority to the Navy.

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providing the original single-chain antibody fragment. Figures reprinted with permission from the American Chemical Society.

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