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Fluorescent homogenous immunosensors for detecting pathogenic bacteria

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Abstract

We developed a straightforward antibody-based assay for rapid homogeneous detection of bacteria. Our sensors utilize an antibody recognizing cell-surface epitopes of the target cell. Two samples of the antibody are prepared each labeled via nanometer size flexible linkers with short complementary oligonucleotides that are modified with fluorochromes that could participate in Fluorescence Resonance Energy Transfer (FRET). The length of the complementary oligonucleotide sequences was designed such that very little annealing occurred in the absence of the target cells. In the presence of the target cells the two labeled antibodies bind to the surface of the cell resulting in a large local concentration of the complementary oligonucleotides that are attached to the antibody. This in turn drives the annealing of the complementary oligonucleotides which brings the fluorescence probes to close proximity producing large FRET signal proportional to the amount of target cells. Long flexible linkers used to attach the oligonucleotides to the antibody enable target-induced oligonucleotide annealing even if the density of surface antigens is only modest. We used *E. coli* 0157:H7 and *Salmonella typhimurium* to demonstrate that this design produced sensors exhibiting rapid response time, high specificity and sensitivity of detecting the target bacteria.

Keywords

Fluorescence; FRET; antibody; sensor; bacteria; pathogen

Introductory Statement

Pathogenic bacteria are responsible for over fifty infectious diseases. Detection of pathogens is thus of great importance for health and safety reasons. Food industry, clinical, water, and environment are the area of principle interest in pathogen detection [1;2]. Currently, the most commonly used methods for pathogen detection involve traditional methods based on cell culture and colony counting [3] and PCR-based methods [4;5;6;7]. Traditional methods offer reliability and selectivity but are very time consuming. PCR-based methods, similarly to traditional methods, can produce conclusive results but at much reduced time (hours instead of days). Disadvantages of PCR-based methods include their relative complexity, a relatively high rate of false-positives, and that they still require hours to perform the analysis. Traditional immunological methods (ELISA) have been also very useful in pathogen detection [2;8;9;10]. The major common limitation of the above-described techniques is relatively long time

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(hours to days) required for the analysis. For obvious reasons, short analysis time (minutes) is very desired in pathogen detection. The goal of reducing the time of the analysis has been a major driving force behind the development of variety of biosensors for detecting the pathogens [2]. It is predicted that the use of biosensors will rapidly grow as these methods are improved. There is a continued need for new methodologies for pathogenic bacteria detection that will be rapid and simple to use.

Our laboratory has recently developed new sensor design that allows rapid and sensitive homogenous detection of proteins in solution [11;12]. A pair of antibodies (or other specific binders, for example, aptamers) recognizing non-overlapping epitopes of the protein is labeled with a pair of short complementary signaling oligonucleotides using a long flexible PEG-based crosslinker, respectively. Oligonucleotides are modified with a pair of fluorophores which could function as a donor and an acceptor in Fluorescence Resonance Energy Transfer (FRET) [13]. In the presence of the target protein both antibodies bind to the target protein resulting in a great increase of the local concentration of signaling oligonucleotides. This in turn leads to annealing of the oligonucleotides bringing the fluorophores to close proximity resulting in efficient FRET that can be used as a signal for target protein detection[12].

Molecular pincer design for detecting proteins described above have inspired an idea for simple, rapid, homogenous sensors for detecting pathogenic bacteria that we describe in this paper (Fig. 1). We hypothesized that by analogy to the target-protein induced annealing of signaling oligonucleotides, a similar effect could be produced by binding of signaling oligonucleotide-labeled antibodies to the epitopes on a surface of a bacterial cell (Fig. 1). Taking into account the dimensions of the bacterial cell (~500 – ~3000 nm) and the length of flexible linkers used to attach the oligonucleotides to the antibodies (10–50 nm), we expected that even a moderate density of cell surface epitopes recognized by the antibody should be sufficient to bring the antibodies to the proximity that would allow annealing of the signaling oligonucleotides that would in turn bring the donor and acceptor fluorochromes to the proximity producing FRET signal in the presence of target cells. We describe here the data obtained with *E. coli* O157:H7 and *Salmonella typhimurium* that validate the design illustrated in Fig. 1.

Materials and Methods

Materials

E. coli O157:H7 and *Salmonella typhimurium* antibodies and the corresponding heat-killed bacteria were purchased from KPL (Gaithersburg, MD). NHS-PEO₈-maleimide and Traut's reagent were from Pierce (Rockland, IL). Oligonucleotides were obtained either from Keck Oligonucleotide Synthesis Facility at Yale University or from IDT (Coralville, IA). The following oligonucleotides were used (names in parenthesis):

(A1) 5' C6 amino- TAGGTGCTCGACGCTGAC

(A2) 5' C6 amino-TAGGAGAGAGAGAGAGGA

(A3) 5'-Fluorescein-GCTCATTGTCAGCGTCGAGCACCTA

(A4) 5'-Cy5- ATGAGCTTCCTCTCTCTCTCCAT

A3 and A4 oligonucleotides contain short sequences (underlined) that are used in target-induced annealing to generate FRET signal. Fluorescein and Cy5-labeled oligonucleotides were purified by reversed-phase HPLC[14]. Concentrations of oligonucleotides were

calculated from UV absorbance at 260 nm, after correction for fluorophore absorbance at 260 nm.

Antibody modification and purification

Antibodies were labeled with signaling oligonucleotides using a previously described procedure[12] (method “c” in Fig. 1B from ref. 12). A1 or A2 oligonucleotides were first attached to the antibodies via long linkers followed by annealing of A3 and A4 oligonucleotides to produce antibody-A1/A3 and antibody-A2/A4 conjugates, respectively. The first step of the procedure involves preparation of a thiol-reactive oligonucleotide that is subsequently used to react with thiolated antibody. 200 μ l of 5'-amine containing oligonucleotides (A1 or A2) at ~250 μ M in 20mM NaH₂PO₄ (pH-7.4), 150mM NaCl and 2.5mM EDTA buffer (conjugation buffer) were mixed with 5 μ l of ~250mM of NHS-PEO₈-maleimide dissolved in DMF. The reaction mixtures were incubated for 1–1.5hr at room temperature. Oligonucleotide was purified from the excess of the crosslinker by ethanol precipitation in the presence of 1mg/ml of glycogen. Precipitated oligonucleotides were dried in Speed-Vac and were stored at –20° C until they were used for antibody modification. 50–75 μ l antibody solutions containing 0.3–0.4 mg of the protein were run on a spin column (Zeba™, Pierce, Rockford, IL)) equilibrated with the conjugation buffer. Antibodies were thiolated for 1.5hrs at room temperature with 40 molar excess of Traut's Reagent added as ~14mM stock solution in DMF. The excess of Traut's Reagent was removed on Zeba™ spin column equilibrated in the conjugation buffer. The thiolated antibody was then reacted with a 15–20 molar excess of linker-conjugated oligonucleotide (calculated assuming that ~50% of the oligonucleotides were conjugated with the crosslinker). Reaction mixtures were incubated for 4 hrs at room temperature followed by an overnight incubation at 4° C.

Modified antibodies were purified from the excess of the oligonucleotides by size exclusion FPLC chromatography using 10/30GL Superdex™ 200 column (Pharmacia) equilibrated with 10 fold-diluted 20 mM Tris (pH 8.0), 100 mM NaCl, 10 μ M EDTA buffer. Fractions containing modified antibodies were pooled and concentrated 10-fold in the Speed-Vac. The protein concentration was estimated using Bradford assay. Labeling of the antibodies with oligonucleotides was confirmed (and the extent of the labeling estimated) by analyzing the UV spectra of purified final product. Observed spectra were fitted by a linear combination of the spectra of free antibody and free oligonucleotide to determine relative amounts of the protein and oligonucleotide in the sample. The A1 and A2-labeled antibodies were annealed with fluorescent A3 and A4 oligonucleotides by incubating 100 nM antibodies with equimolar amounts of A3 or A4 for 30 min at room temperature.

Fluorescence experiments

All fluorescence measurements were performed in 20 μ L of binding buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl and 10 μ M EDTA) in 384-well low-volume black microplates (Corning cat #3676) at 25 °C. The donor (fluorescein; excitation at 485 nm, emission at 535 nm) and sensitized acceptor emission (Cy5; excitation at 485 nm, emission at 665 nm) signals were read with Analyst AD plate reader (LJL Biosystems, Sunnyvale, CA) or Spectra FluorPlus microplate reader (Tecan, Research Triangle Park, NC). Reaction mixtures (typically containing 20 nM A1/A3-labeled antibody and 25 nM A2/A4-labeled antibody diluted from the 100 nM stocks prepared as described above) were incubated for 30 min before fluorescence measurements were made (with the exception of kinetic experiments where incubation time varied).

Results of fluorescence measurements were expressed as a fold change of FRET signal:

$$\text{FRET (fold change)} = (F_{SA}/F_D)/(F_{SA}^0/F_D^0) \quad (1)$$

where F_{SA} , F_D , F_{SA}^0 and F_D^0 are sensitized acceptor and donor emission intensities in the presence and absence of the bacteria, respectively. Buffer background was subtracted from the measured fluorescence intensities before FRET values were calculated. Using the ratio of sensitized acceptor and donor emission intensities for calculating FRET according to eq. 1 reduces the variability of FRET measurements due to dilution and instrumentation errors.

Fluorescence microscopy

Confocal images of *E. coli* O157:H7 incubated with labeled antibodies were obtained on Leica SP5 inverted microscope. Glass slides were coated with 1% agar. *E. coli* O157:H7 ($\sim 1 \times 10^5$ cells) were incubated with 20 nM A1/A3 labeled antibody or with a mixture of 20 nM A1/A3 labeled antibody and 25 nM A2/A4 labeled antibody in 20 μ l of the binding buffer. The mixtures were incubated at room temperature for ~ 30 min, were layered on the agar-coated slide, were covered with a coverslip and imaged on the microscope.

Results and Discussion

We first used *E. coli* O157:H7 as a target to test the functionality of sensor design illustrated in Fig. 1. It would seem that similarly to the molecular pincer sensors for protein targets[12] it should be necessary to have two antibodies (labeled with complementary signaling oligonucleotides, respectively) to two distinct cell surface epitopes for the assay depicted in Fig. 1 to work. However, we hypothesized that it will be sufficient to use mixture of the same antibody labeled with two complementary signaling oligonucleotides, respectively. The reasons for that are illustrated in Fig. 2A. Complementary oligonucleotides provide additional favorable binding energy when the antibodies labeled with complementary oligonucleotides bind next to each other. This energy is absent when the two neighboring antibodies are labeled with the same signaling oligonucleotide (an arrangement that does not generate FRET signal). Thus, when a mixture of the same antibody labeled with two complementary signaling oligonucleotides is used, distribution of the antibodies on the surface of the cell will be such that antibodies containing complementary signaling oligonucleotides should be preferentially located near each other. Thus, our design has a built-in self-organizing process that assures maximizing FRET signal generated in the presence of target cells.

We prepared two samples of labeled *E. coli* O157:H7 antibodies. One was labeled with A1/A3 oligonucleotides that contain 5'-fluorescein-GCTCAT unpaired signaling sequence and the other was modified with A2/A3 oligonucleotides that contain 5'-Cy5-ATGAGC unpaired signaling sequence. When a mixture of these antibodies was incubated with increasing amounts of *E. coli* O157:H7 cells, a large dose-dependent FRET signal (emission at 670 nm with the excitation at 488 nm) was observed (Fig. 2B, lower panel). In contrast, no FRET signal was observed when the same amounts of *E. coli* K12 cells were used demonstrating the specificity of the sensor. Large increase of sensitized acceptor (FRET) signal was accompanied by a small decrease of the donor (fluorescein) emission (Fig 2B, upper panel). The data shown in Fig. 2B validated the design of the sensors shown in Fig. 1.

Sensor design as illustrated in Fig. 1 implies that FRET signal is generated in the presence of target cells by annealing of complementary oligonucleotides attached to the antibodies co-binding to the same cell. The other possibility could be that the FRET signal could be produced by annealing of signaling oligonucleotides attached to the antibodies bound to different cells (i.e. resulting in antibody-induced aggregation of the cells). To investigate the nature of the FRET signal generated in the presence of the target cells, we have obtained confocal

microscope images of *E. coli* O157:H7 cells incubated with the mixture of antibody labeled with fluorescein and Cy5-modified complementary oligonucleotides (Fig. 3) and the same cells incubated only with the antibody labeled with fluorescein-modified signaling oligonucleotide that lacks the ability of crosslinking via annealing of the complementary oligonucleotides (not shown). In both cases individual cells and cells aggregates could be found. No increase in cell aggregation was detected in donor/acceptor sample compared to donor-only sample arguing against annealing of signaling oligonucleotides between different cells as a mechanism for FRET signal. Furthermore, large FRET signal could be seen on the surface of individual cells (Fig. 3B). These observations indicate that the major route for the FRET signal produced in the presence of target cells is the process schematically illustrated in Fig. 1.

To test the robustness and reproducibility of detecting target cells with the sensors, we performed three independent repeats of the assay with a wide range of target cells amounts (Fig. 4A). Under the assay conditions employed (20 nM A1/A3 labeled antibody and 25 nM A2/A4 labeled antibody) few thousands of target cells were easily detectable with high reproducibility (mean CV was 1.7%; range: 0.7–3.9%). No signal with negative control (*E. coli* K12) has been detected at any amount of the cells tested. At very high amounts of the target cells, the FRET signal started to decrease most likely due to the decrease in the average amount of antibodies bound to each cell due to the depletion of the free antibody in the reaction mixture. Such decrease of the signal at high numbers of cells in practical applications will almost never be a complicating issue since a simple option of diluting the sample would be always available. If measurement of high numbers of cells would be desired, the increase of concentration of the antibodies will allow extending the useful range of the assay to higher cell numbers.

To demonstrate the generality of the sensor design illustrated in Fig. 1, we used the same procedure as used in the case of *E. coli* O157:H7 sensor to prepare a sensor for a different bacteria (*Salmonella typhimurium*). Fig. 4B shows that as in the case of *E. coli* O157:H7, a large dose-dependent FRET signal was observed with *Salmonella* cells. In contrast, very little FRET signal in the presence of *E. coli* K12 (negative control) was observed demonstrating the specificity of the sensor for *Salmonella*. As in the case of *E. coli* O157:H7 sensor, the reproducibility of *Salmonella* sensor was excellent (mean CV was 1.0%; range: 0.3–2.2%). FRET signal in the case of *Salmonella* was smaller compared to *E. coli* O157:H7 most likely reflecting differences in properties of corresponding antibodies (for example, different surface density of the epitopes recognized by the antibodies or lower affinity of *Salmonella* antibodies compared to *E. coli* O157:H7). Nevertheless, the fact that the same design blueprint applied to two different bacteria cells produced functional sensors provided a strong indication of the generality of the design illustrated in Fig. 1.

We expected that one of the important advantages of our sensor design would be the uncomplicated rapid manner by which the assay could be performed due to its homogenous nature. We thus measured the kinetics of FRET signal appearance after addition of *E. coli* O157:H7 (Fig. 5A) or *Salmonella* (Fig. 5B) to the labeled antibody mix. Maximum FRET signal was obtained after ~ 30 min incubation in case of *E. coli* O157:H7 or ~ 40 min incubation in case of *Salmonella*. The faster kinetics of FRET signal appearance in the case of *E. coli* O157:H7 was most likely due to higher binding affinity of *E. coli* O157:H7 antibodies (consistent with the higher total FRET signal in the case of *E. coli* O157:H7 compared to *Salmonella*). After only ~5 min incubation > 80% of the maximal FRET signal was produced in the case of *E. coli* O157:H7 (Fig. 5A) suggesting that it would be possible to perform this assay with only a 5 min incubation time. The data shown on Fig. 5 confirm the rapid response time and simplicity of target detection that are enabled by our sensor design.

Several aspects of sensor design could have an important effect of sensor performance. The quality of the antibodies used will most certainly have an effect on assay performance. Tight binding and highly specific antibodies will result in better performing sensors. The length of complementary single-stranded signaling oligonucleotides could also affect the sensor performance. If the length is too long, the oligonucleotides will anneal in the absence of the target bacteria producing elevated background and decrease in sensor performance. If the length is too short, the annealing in the presence of target bacteria will be inefficient negatively affecting assay performance. We tested a series of *E. coli* 0157:H7 antibodies that were labeled with oligonucleotides differing the length of complementary single-stranded signaling sequence (data not shown). Signaling oligonucleotide sequence that we used in all experiments was found to be optimal by this analysis. Antibody concentration is another important determinant of sensor performance. We expected that reducing antibody concentrations could increase assay detection limits since a bigger fraction of the total antibody concentration would be bound to cells at a given target cell density (provided that the antibody concentrations are not reduced below the K_d of antibody-antigen complex or below the sensitivity of fluorescence instrumentation used). To test this notion we measured the response of the sensor at two (lower) antibody concentrations (Fig. 6). As predicted, lowering antibody concentration shifted the response curve toward lower cell numbers (compare the data in Fig. 4A (20 nM and 25 nM antibody mix with data in Fig. 6 (10 nM and 12 nM or 5 nM and 6.2 nM antibody mix)). Thus, lower cell numbers could be detected with sensors employing lower antibody concentrations. For example, we estimated that using a mixture of 5 nM A1/A3 labeled antibody and 6.2 nM A2/A4 labeled antibody the limits of detection were ~300 cells/20 μ l assay. Changing antibody concentration will be thus a simple way to tailor the assay to a particular range of cell numbers that need to be detected.

Conclusions

A straightforward antibody-based assay for detecting pathogenic bacteria was developed. Proof-of-principle experiments described here were performed with bacteria suspended in a buffer. In real applications various possibly interfering substances could be present in a sample. High concentrations of unrelated nucleic acids or proteins do not affect significantly our antibody-based sensors (data not shown). Any fluorescent compound present in a sample could be the most obvious interfering substance. If such compounds would be present, a different set of FRET probes with excitation and emission peaks that do not overlap with the fluorescence of the interfering substances might need to be used.

We believe that our new sensors will have several important advantages. First, the assay is extremely simple. This is because the assay is homogenous and thus requires only the addition of the sample to the sensor mix. No complicated sample manipulation is necessary. Only simple fluorescence intensity readout is required to perform the assay making the assay adaptable to simple portable instrumentation that will allow its use in the field. Second, the assay, due to self-organizing property illustrated in Fig. 2A, requires only a single antibody to a cell surface antigen. Third, the assay is fast. We showed that as little as ~ 5 min might be sufficient to detect the target cells. Fourth, the assay is inexpensive since it is amenable to miniaturization limited only by technical capabilities of the instrumentation used to detect the fluorescence. Since the antibodies are used in our sensor at low (nanomolar) concentrations, only very small amounts of antibodies are used per each measurement. Fifth, the assay is sensitive. We showed that our sensors could readily detect as little as ~300 cells. In many practical applications this sensitivity will still require initial sample enrichment step (cell culture or sample concentration on semi-permeable membrane). Sample enrichment step in such cases will add considerable extra time that would be required for the analysis. This extra time could range from minutes (if rapid concentration on semi-permeable membrane is used) to hours or days (if cell culture enrichment is applied). Even if a sample enrichment step would be necessary for a given application, the

uncomplicated homogenous nature of our sensors would make it a preferable approach compared to currently used ELISA assays. We are currently working on improving assay limits of detection by employing better fluorescence probes and by utilizing signal amplification procedures. Sixth, the assay is quantitative. With appropriate standard, it could be used for quantitation of the target bacteria. Seventh, the sensor design blueprint is not specific to any target bacteria (or limited to bacteria; the assays for other cell types should be feasible) and thus could be used to develop sensors for any bacteria of current or future interest. Our recent analysis of the properties of ligands containing long flexible linkers[15] suggested that linkers up to at least 50 nm length should be compatible with sensor design depicted in Fig. 1. This suggests that functional sensors for target cells characterized by a wide range of cell surface antigens density should be feasible. Furthermore, we believe that the same design could be also used to develop sensors for other cell types to, for example, detect specific cell surface molecules.

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Fig. 1.
Design of homogeneous sensors for detecting pathogenic bacteria.

Fig. 2.

(A) Increased stability of the pair of antibodies bound to neighboring sites on the surface of the cell due to additional favorable free energy derived from hybridization of complementary signaling oligonucleotides. (B) Fluorescence images of 96-well microplate wells containing indicated amounts of *E. coli* O157:H7 cells or control cells (*E. coli* K12). Upper and lower panels show the donor emission image (excitation at 488 nm, emission at 530 nm) and sensitized acceptor (FRET) emission (excitation at 488 nm, emission at 690 nm), respectively.

Fig. 3.

Fluorescence microscope images of *E. coli* O157:H7 incubated with a mixture of anti-*E. coli* O157:H7 antibodies labeled with fluorescein and Cy5 modified signaling oligonucleotides. Left panel: donor emission (excitation at 488 nm, emission at 530 nm); middle panel: sensitized acceptor emission (FRET) (excitation at 488 nm, emission at 690 nm); right panel: light transmittance image.

Fig. 4. Response of the sensors to the indicated amounts of *E. coli* O157:H7 (A) and *Salmonella typhimurium* (B). Experiments were performed using a mixture of 20 nM A1/A3 and 25 nM A2/A4-labeled antibodies. Insets: part of response curves at low amounts of target cells. Grey circles represent FRET signals observed at indicated amounts of negative control (*E. coli* K12). The average and standard deviation of three measurements are shown.

Fig. 5. Kinetics of sensor response. Indicated amounts of *E. coli* O157:H7 (A) or *Salmonella typhimurium* (B) were added to a mixture of 20 nM A1/A3 and 25 nM A2/A4-labeled antibodies and FRET signal was measured as a function of time.

Fig. 6. Dependence of sensor response on antibody concentration. Circles: sensor containing 10 nM A1/A3 labeled antibody and 12 nM A2/A4 labeled antibody; triangles: sensor containing 5 nM A1/A3 labeled antibody and 6.2 nM A2/A4 labeled antibody.