Development of Fiber Optic Biosensors for the Rapid Detection of Methicillin-Resistant *Staphylococcus aureus*

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is known for skin infections and is potentially life threatening if not treated immediately. Its presence in livestock and farm workers suggest agriculture industries may have an impact in spreading the infection. Its methicillin-resistance, encoded by *meca*, protects it from beta-lactam antibiotics. Current diagnostic techniques are very slow (48-72 hours) and performed only by trained personnel. We are developing a rapid detection method for MRSA based on tapered fiber optic biosensors, which we have recently demonstrated for detection of binding between immunoglobulin-G (IgG) and anti-IgG. This method does not require any fluorescent labels and data acquired in real time allows rapid detection in less than 3 hours. MRSA is detected by a hybridization reaction between immobilized *meca* probes (oligonucleotides) on the tapered fiber sensor surface and target complimentary *meca* (prepared by heat-treatment and lysostaphin cell lysis of *S. aureus*). The protocol and the reagents used to bind *meca* probes to the sensor surface were the same as the one used in our previous IgG-anti-IgG binding studies. Typical buffers used in hybridization reactions include sodium dodecyl sulfate (SDS) and standard saline citrate (SSC). In our preliminary experiments, we found that both *meca*+ and *meca*- generated large sensor signals. We hypothesized these signals were contributed by the reagents of the buffer and not the hybridization reaction. Through multiple control experiments, we discovered by removing the SDS from the buffer we were able to show difference in the sensor response to *meca*+ and *meca*-. 

**Summary**

With the past success of functionality of our biosensors immobilized with IgG in rapidly detecting anti-IgG, we have modeled the biosensor functionalization protocol to detect the *meca* gene in MRSA extracted DNA samples (Miller et al., 2014). The DNA samples were prepared referencing functional DNA extraction parameters. Immobilization of the *meca* oligonucleotide probe was also referenced from the same experiment. Our IgG-biosensor functionalization protocol adapted to the new *meca* oligonucleotide-biosensor functionalization protocol with ease due to identical bio-chemicals used with the exception of the reagent probe (IgG to *meca* oligonucleotide) in immobilization. Testing parameters were recreated to suit the conditions required for *meca* hybridization between the biosensor and DNA samples (Jenison et al., 2000). *meca* oligonucleotide probes were referenced from an updated recent study of the same team (Jenison et al., 2014).

However, due to inconsistent data when testing against extracted *meca*+/- samples, we devised several troubleshooting experiments. Through various trials using unfunctionalized biosensors, we discovered that the sodium dodecyl sulfate (SDS) in the hybridization buffer was causing the inconsistent shifting.

To validate that the detected shifting was due to DNA hybridization between the *meca* probe
and target DNA, a series of additional experiments using meca complementary DNA (cDNA) as a sample instead of extracted DNA samples. Even with removal of SDS from hybridization buffer, irregular shifting still occurred. We suspect that the shifting is being caused by “microbubbles” forming on the sensor’s surface. To confirm this, we have implemented a sonication step prior to data collection to remove these bubbles. Further experiments still need to be performed for confirmation.

**Materials and Methods**

*Silica fiber optical sensor fabrication and Refractive Index*

The optical silica fiber was tapered by using a butane torch (Fig. 1-A, 1-B). The final product was then fabricated onto a glass slide in a U-shape using tape. As the refractive index around the sensor is changed, the transmission spectrum will undergo a phase shift (Fig. 1-C). This phase shift can be quantified by tracking the location of peaks in the spectra (Miller et al., 2014).

*meca oligonucleotide probes*

The meca oligonucleotide probes were ordered from Integrated DNA Technologies (IDT) with modified 5’ and 3’ amino ends:

- meca1:
  /5AmMC12/GTCATTTCTACTTCACCATT ACCAAC
- meca2:
  /5AmMC6/GTCATTTCTACTTCACCATT CCAAC
- meca3:
  GTCATTTCTACTTCACCATTACCAAC/3AmMO/

The optical silica fibers were functionalized using the meca1 oligonucleotide probes (Jenison et al., 2000).

**Biosensor functionalization**

The fused silica fiber surface was treated with plasma produced by a high-frequency generator for 2 minutes to deposit hydroxyl groups on the glass surface. The surface was then coated using a silane reagent with an amine functional group (APTES=3-Aminopropyltrimethoxysilane). We used BS3 (Bis[sulfo succinimidyl] suberate) as our amine-amine crosslinker. Finally, the meca oligonucleotide is immobilized via its amino groups forming an amide bond with the free end of the crosslinker. We used bovine serum albumin (BSA) protein for blocking to prevent non-specific binding.

**MRSA DNA sample preparation**

MRSA samples were prepared through standard cell culture inoculation. Grown samples underwent DNA extraction by first inoculating a single colony in reaction buffer (75 mmol/L NaCl, 25 mmol/L EDTA, 20 mmol/L Tris, pH 7.5) followed by vortex mixing. The solution was then treated with 2µl of lysostaphin (10 mg/L) solution for cell lysis, incubated at room temperature for 20 min, 95° for 10 min, and mixed via vortex. Lysostaphin treated samples were inoculated onto agar plates and incubated to test for cell death. The sample solution was then centrifuged for 20 min at 13,000 rpm (Jenison et al., 2000). The supernatant of the samples were tested for purity using a NanoDrop1000 spectrophotometer.

**Experimental setup (Fig. 1D)**

Amplified spontaneous emission from a semiconductor optical amplifier (SOA) was used as a broadband light source (1450-1600 nm). A 1x5 optical switch allowed us to measure the source and the sensor response ~5 seconds apart and up to 4 different sensors can be automatically interrogated with one channel reserved for reference measurement to account for any fluctuations in the source power. An optical spectrum analyzer (OSA) was
used to determine the optical power of the SOA as a function of wavelength (Miller et al., 2014).

**DNA Hybridization**
The DNA sample was diluted (50:50) in hybridization buffer [10X Standard Saline Citrate (SSC)] and heated to 95°C for 10 min. The biosensor was then introduced to the solution and incubated at 57°C for 2 hours. The biosensor and solution were then incubated at 23°C for 10 min, followed by washes with 0.1X SSC, 1 g/L SDS and 0.1X SSC solutions (Jenison et al., 2000).

Troubleshooting with un-functionalized and plasma treated sensors
To determine why the data produced by the hybridization experiments behaved unpredictably, troubleshooting tests were devised to break down testing parameters. This allowed isolation of specific areas in each test and control which element in the testing procedure likely caused this unpredictable factor. Un-functionalized sensors were tested against diH2O and hybridization buffer (no DNA) to observe potential shifting in the refractive index. Identical parameters to the hybridization experiments without functionalized biosensors or the presence of DNA in hybridization buffer.

Hybridization with single-stranded complementary DNA (cDNA)
Single stranded cDNA (5'-GTTGGTAATGGTGAAGTAGAATGAC-3') was purchased from IDT. 1 µM cDNA sample was prepared by diluting in 6X SSC. Sample solution was tested in room temperature (23°C) conditions for hybridization. meca probe functionalization remained the same. (Fig. 1B)

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**Fig. 1-1. Silica fiber optical sensor fabrication and Refractive Index.** (A) Tapering with a butane torch. (B) Diagram depicting immobilization of IgG antibodies on sensitive region of silica fiber surface following tapering. (C) Transmission spectrum undergoing a phase shift, indicating changes in the optical properties of the experimental sample. (D) Experimental set up of fabricated biosensor attached to semiconductor optical amplifier (SOA) and optical spectrum analyzer (OSA) for testing in sample.
Results

IgG/Anti-IgG detection

Three different concentrations of anti-IgG were tested with the results shown in Fig. 2A. Fig. 2A.1-A.3 show the peak shift vs time for different concentrations. Typical diameter of the tapered region is about 8-10 mm. However, in a recent experiment, we made the taper even thinner leading to enhanced sensitivity, and the average shift at 50 ng/mL was as high as that at 500 ng/mL. As shown in Fig. 2A.4, we get measurable difference even in the first few minutes, which suggests that we might use the rate of shift with time to determine concentration.

mecA hybridization with functionalized and unfunctionalized sensors

Two strains of MRSA DNA samples and two strains of non-MRSA DNA samples were tested. Preliminary results are shown in Fig. 2B. Both functionalized (Fig. 2B.1, B.2) and unfunctionalized (Fig. 2B.3, B.4) biosensors were tested with positive and negative strains. The functionalized biosensors observed shifting to the right similar to the IgG/anti-IgG binding experiments, indicating potential DNA hybridization between the mecA oligonucleotide probe immobilized to the biosensor surface and mecA gene contained in the MRSA DNA sample. The negative control test showed no shifting, indicating no interaction between the biosensor and sample. The un-functionalized biosensors observed no shifting when tested with the mecA gene containing MRSA DNA sample. However, the negative control test showed shifting similar to the functionalized biosensor positive control test. This implies that the un-functionalized biosensor’s refractive index was not affected by DNA hybridization.

Fig. 1-2. DNA hybridization diagram. mecA oligonucleotide probes are functionalized by the 5′ NH2 end onto the surface of the tapered region of the silica optical fiber (1). The fiber optic biosensor is introduced to solution containing single-stranded complimentary DNA (2). DNA Hybridization has occurred (3).

Fig. 2A. IgG/anti-IgG (peak shift vs. time) in different concentrations. (1) 50 µg/mL (2) 5 µg/mL (3) 0.5 µg/mL (4) Comparison of 50 µg/mL, 5 µg/mL, and 0.5 µg/mL.
**B. meCA gene detection**

![Graphs showing meCA gene detection](image)

**Fig. 2B. meCA detection using functionalized vs. un-functionalized sensors (peak wavelength vs. time).** (1, 2) Functionalized biosensors testing against meCA positive (1) and meCA negative samples. (3, 4) Un-functionalized biosensors testing against meCA positive (3) and meCA negative (4) samples.

**A. Test against diH2O**

![Graphs showing test against diH2O](image)

**Fig. 3A. Troubleshooting using un-functionalized sensors against diH2O (peak wavelength vs. time).** (1, 2) Trials show no shifting.
B. Unfunctionalized sensors

C. Plasma-treated sensors

Fig. 3B. Troubleshooting using un-functionalized sensors against hybridization buffer (peak wavelength vs. time). (1, 2) Trials show minor shifting. Fig. 3C. Troubleshooting using plasma treated sensors against hybridization buffer (peak wavelength vs. time). (3, 4) Trials show more apparent shifting.

Troubleshooting with un-functionalized and plasma treated sensors against varying solutions

The un-functionalized sensor trials tested against the diH₂O (Fig. 3A) behaved similar with no shifting. The tests using hybridization buffer (Fig. 3B, 3C) on the other hand behaved differently between trials and when compared to the tests against the diH₂O. Removal of SDS in the hybridization buffer demonstrated more consistent results compared to hybridization buffer containing SDS.

Hybridization with single-stranded complementary DNA (cDNA)

Initial tests when testing functionalized sensors against cDNA did not demonstrate large variation of shifting amongst tests, but still showed inconsistencies. As a result, Un-functionalized sensors were tested in varying solutions (cDNA, 6X SSC, diH₂O). Tests in 6X SSC and diH₂O brought to the attention of upward shifting when transferring the sensor between two different mediums.

Discussion

Through binding of IgG and anti-IgG, we have demonstrated the ability for real-time biosensing of proteins with tapered optical fibers. The sensor limit of detection is estimated to be less than 50 ng/mL. We anticipated successful nucleic acid immobilization to the sensor silica fiber surface due to the identical reagents used in functionalization. We were able to modify the
functionalization procedure using mecA oligonucleotide probes in place of IgG reagent for rapid detection of extracted MRSA DNA samples. The mecA oligonucleotide probes were expected to hybridize with complimentary mecA gene sequences and display peak shifts in the refractive index. Through our preliminary experiments using nucleic acid based detection, we have found inconclusive results. While significant shifting was found in mecA gene containing MRSA DNA samples in functionalized biosensors, failure to pass negative control tests for un-functionalized biosensors implies the need to troubleshoot.

With the results obtained from the troubleshooting experiments, we can look further into the effects that the Hybridization Buffer might be having on the tapers. In the plasma treated sensors, we observed an odd occurrence of shifting happening after the 30 min mark. The spectrum drops and continues in a downward progression for the full two hours. It can be noted that in the un-functionalized sensor test, we can still see this effect happening only that it is less obvious.

We determined that the 1 g/L SDS in the hybridization buffer affected the refractive index by producing bubbles in solution which we infer to be micelles. After removing 1 g/L SDS from the hybridization buffer, which only consisted of 0.1X SSC, we found a significant difference between mecA + and mecA - detection.

However, our results still remained inconclusive with inconsistent results following the mecA detection experiments. We developed a validation experiment to detect single-stranded complementary DNA by adjusting the concentrations to 1 µM, increasing the 0.1X SSC to 6X SSC and testing in room temperature. When testing un-functionalized sensors in hybridization buffer only contained 6X SSC and diH₂O, we noticed a trend when transferring the sensor between the two mediums. There is an apparent upward shifting progression which we suspected to be caused by the need for the sensor to acclimate between each medium. By using a sonication device prior to testing, we are in the preliminary stages of investigating whether micelles are the culprit to the inconsistent data. It appears that conditioning the environment for hybridization is the difficult issue.

Considering we have been exclusively working with un-functionalized sensors, the mecA probe could not cause the irregular shifting. By removing 1 g/L SDS from the hybridization buffer, the results began to show consistencies. Nevertheless, the ongoing occurrence of upward shifting when transferring the sensor between diH₂O and 6X SSC hints at the idea that something besides the functionalization protocol, sensor itself, and hybridization buffer are not likely to cause the irregular shifting. We suspect the cause for this is a physical factor on the surface of the sensor.

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References

