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Detection of methicillin-resistant staphylococci by biosensor assay consisting of nanoscale films on optical fiber long-period gratings

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ABSTRACT

Methicillin-resistance among *Staphylococcus* species is a major health problem in hospitals, communities, and animals. There is a need for culture-free diagnostic assays that can be carried out rapidly, and maintain a high degree of sensitivity and specificity. To address this need an ionic self-assembled multilayer (ISAM) film was deposited on the surface of a long-period grating (LPG) optical fiber by immersion alternately in poly-allylamine hydrochloride and in poly-1-[p-(3′-carboxy-4′-hydroxyphenylazo) benzenesulfonamido]-1,2-ethandiyl (PCBS), resulting in terminal carboxyl groups on the LPG-ISAM. The terminal carboxyl groups were covalently conjugated to monoclonal antibodies (MAb) specific to penicillin-binding-protein 2a of methicillin resistant (MR) staphylococci. After exposure of the LPG-ISAM to 10^2 colony forming units (CFU)/ml of MR *S. aureus* (MRSA) for 50 min., light transmission was reduced by 19.7%. In contrast, after exposure to 10^6 CFU/ml of methicillin-sensitive *S. aureus* (MSSA) attenuation of light transmission was less than 1.8%. Exposure of the LPG-ISAM to extracts of liver, lungs, or spleen from mice infected with MRSA attenuated light transmission by 11.7–73.5%. In contrast, exposure of the biosensor to extracts from MSSA-infected mice resulted in 5.6% or less attenuation of light transmission. When the sensor was tested with 36 strains of MR staphylococci, 15 strains of methicillin-sensitive staphylococci, 10 strains of heterologous genera (all at 10^4 CFU/ml), or tissue samples from mice infected with MRSA, there was complete agreement between MR and non-MR bacteria determined by antibiotic susceptibility testing and the biosensor assay when the cutoff value for attenuation of light transmission was 6.3%. Thus, the biosensor described has the potential to detect MR staphylococci in clinical samples with a high degree of sensitivity and specificity.

1. Introduction

Staphylococcus species are responsible for skin, soft-tissue, and systemic infections in humans and animals. In general, one in three (33%) people carry staphylococci in their nose, usually with no illness (Gorwitz et al., 2008). *Staphylococcus aureus* is a dynamic and adaptable bacterium that has a remarkable ability to acquire antibiotic resistance quickly. In the pre-antibiotic era, *S. aureus* was associated with a high incidence of mortality. Penicillin, first introduced in the early 1940s, quickly lowered mortality associated with *S. aureus* infections. However, by the mid-1940s, strains of penicillin-resistant *S. aureus* were detected in hospitals. By 1960, *S. aureus* resistance to penicillin was commonplace in both community-acquired and hospital-acquired strains. Methicillin, a penicillinase-resistant semi-synthetic penicillin, was introduced to treat patients infected with penicillin-resistant *S. aureus*. However, in 1961 methicillin-resistant *S. aureus* (MRSA) isolates were reported (DeLeo et al., 2010). MRSA has become established in healthcare facilities throughout America, Asia, and Europe (DeLeo et al., 2010; Molton et al., 2013). Compared with infections due to methicillin-sensitive (MS) *S. aureus* (MSSA), infections by MRSA strains are associated with increased morbidity and mortality in affected patients (Cosgrove et al., 2003). Once considered predominantly a hospital-acquired infection (HA-MRSA), community-

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acquired MRSA (CA-MRSA) has emerged as a major public health threat (David and Daum, 2010). According to CDC data, there were estimated to be 75,309 cases of MRSA infections in the U.S. in 2011, with 11,285 deaths (CDC, 2012). A delay in initiation of appropriate therapy is associated with increased mortality (Lodise and McKinnon, 2005). Therefore, an assay to detect MRSA infection rapidly with high specificity and sensitivity is highly desirable to initiate appropriate antibiotic therapy.

Many *Staphylococcus* species other than *S. aureus* are opportunistic pathogens in both humans and animals, and cause serious diseases of the skin and other body tissues and cavities (Kloos and Musselwhite, 1975; Scott et al., 2001). The common staphylococcal species recognized as animal pathogens include *S. aureus, S. pseudintermedius, S. hyicus, S. sciuri,* and *S. schleiferi* subspecies *coagulans* (Devriese et al., 2005; Frank et al., 2003; Nemeghaire et al., 2014; Werckenthin et al., 2001). In humans, *S. aureus* is the predominant pathogen, whereas *S. pseudintermedius* and *S. schleiferi* are the primary pathogens in dogs (Devriese et al., 2005; Frank et al., 2003; May et al., 2005; Oehler et al., 2009; van Duijkeren et al., 2011). *S. pseudintermedius, S. schleiferi,* and *S. aureus* have been described as both commensal and pathogenic species of cats (Abraham et al., 2007; van Duijkeren et al., 2011). Pet animals are believed to acquire *S. aureus* infections from humans, and bi-directional transmission of *S. aureus* has been reported (Weese, 2005; Weese et al., 2006). Methicillin resistance also occurs in *S. pseudintermedius* (MRSP), *S. sciuri,* and *S. schleiferi* (MRSS), and the incidence of methicillin resistance in these and other *Staphylococcus* species is increasing (Nemeghaire et al., 2014). Methicillin-resistant staphylococci have also been isolated from domestic livestock (cows, chickens, horses, and pigs) (Leonard and Markey, 2008). As in humans, methicillin-resistant staphylococci colonize skin, nasal, and the oral mucosa in healthy animals, and infections in animals have been associated with risk factors such as hospitalization, surgery, wounds, chronic disease, and immunosuppression.

A biosensor detects, records, and transmits information regarding a physiological change or the presence of various biological and chemical materials in the environment. Specifically, biosensors are probes that integrate biological components (such as enzymes, antibodies, nucleic acids, etc.) with a physico-chemical transducer (optical, electrochemical, thermometric, piezoelectric) to yield a measurable signal (Ramsden, 1997). Key advantages of optical fibers for use as biosensors include light weight, long interaction length, low cost, and the ability to excite the target molecules and capture the emitted light from the targets. In biosensing applications, an optical fiber can produce a signal that is proportional to the concentration of a chemical or biochemical to which the biological element reacts. Optical fiber grating devices, in which a periodic variation is induced in the refractive index of the optical fiber core, operate by inducing a large decrease in the transmittance of light through the fiber at a specific wavelength. This wavelength (or in the case of turnaround point long-period gratings, the transmitted power) can be modified by temperature, pressure, or binding events (Kersey, 1997).

We have previously shown that optical fibers with long-period gratings (LPGs) exhibit exceptional sensitivity to adsorption of ionic self-assembled multilayers (ISAMs) on the surface of the fiber cladding (Wang et al., 2005a, 2005b). ISAM films (also commonly referred to as layer-by-layer (LBL) films) are a class of materials that allow detailed structural and thickness control at the nanometer level, combined with straightforward manufacturing and low cost (Decher, 1997; Decher and Schmitt, 1992). The ISAM method simply involves the alternate immersion of a charged substrate into an aqueous solution of a polycation and an aqueous solution of a polyanion at room temperature. The LPG causes a strong attenuation at a specific wavelength in the single-mode fiber transmission spectrum due to coupling of light from the fundamental guided mode to a high loss higher order cladding mode. Since LPGs couple light into the cladding, the coupling wavelength can be highly sensitive to material external to the cladding. The ISAM film adsorbed onto the cladding surface causes large wavelength shifts of >1.7 nm per nm of film thickness (Wang et al., 2005a, 2005b). The sensitivity of the sensor has been increased by orders of magnitude by utilizing novel turnaround point (TAP) LPGs. In addition to their exceptional sensitivity, TAP-LPGs offer the additional attractive feature that they exhibit a broadband attenuation that shifts in magnitude rather than wavelength (Wang and Ramachandran, 2003). TAP-LPGs have strong, broadband attenuation peaks that are highly sensitive to changes on the exterior of the optical fiber cladding, providing a highly sensitive, robust, inexpensive biosensor platform where the presence of target materials is detected simply by changes in the transmitted intensity at a particular wavelength. The ISAM film amplifies the sensitivity by providing a high refractive index, large surface area, and a nanoscale coating on the cladding that can be readily coupled to a vast array of receptor molecules such as antibodies and DNA.

We have previously demonstrated that the ISAM TAP-LPG biosensor platform can be used to measure the binding of streptavidin to biotin immobilized on the ISAM surface (Wang et al., 2009). We now present results utilizing a nanoscale-film optical fiber sensor (NOFS) consisting of an optical fiber with nanoscale self-assembled film coatings bound to monoclonal antibodies (MAb) specific to penicillin-binding-protein 2a (PBP2A), which is specific for MR staphylococci. Our results show that the NOFS system provides a promising, culture-free assay for rapid and specific detection of MRSA and other methicillin-resistant staphylococci.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains used and their sources are listed in Tables 1 and 2. All experiments with cultures were carried out in biosafety level (BSL)-2 facilities in an approved biosafety cabinet. Bacteria were grown in brain heart infusion (BHI) (Becton-Dickinson and Sigma-Aldrich, St. Louis, MO) broth, or on BHI agar supplemented with 5% sheep blood (BHIB). Bacterial strains were cultured from -80 °C milk stock suspensions into BHI broth or onto BHIB agar, and incubated at 35 °C. For culture in broth, strains were grown with shaking (175 rpm) in BHI broth at 35 °C. Bacteria grown to mid-log phase in broth cultures were washed once with phosphate buffered saline (pH 7.4) (PBS) and resuspended in PBS to a Klett unit absorbance value of 120, which corresponds to 10^9 colony forming units (CFU)/ml. Serial dilutions of cultures in PBS were streaked onto BHIB agar and incubated overnight at 35 °C to confirm the number of CFU/ml.

All of the animal isolates were obtained from the Veterinary Teaching Hospital at Virginia Tech, and were confirmed to be resistant or sensitive to methicillin by procedures outlined by the Clinical Laboratory Standards Institute (CLSI, 2013). Methicillin-resistant isolates of *S. aureus* (MRSA), *S. epidermidis* (MRSE), *S. haemolyticus* (MRSH), *S. pseudintermedius* (MRSP), and *S. schleiferi* (MRSS) were used. Bacterial species that were *mec*-negative were used to determine the specificity of the assay. The *mec*-negative bacterial species included MSSA, MS *S. hyicus* (MSSH), MS *S. pseudintermedius* (MSSP), *Streptococcus pyogenes, Enterococcus faecalis, E. faecium, Pseudomonas aeruginosa, Pasteurella multocida,* and *Escherichia coli* (Table 1).

Table 1
Growth (methicillin resistance) of bacterial strains on BBL CHROMagar™ MRSA and attenuation of light transmission by the NOFS assay.

Bacterial strains used[a]	Growth on BBL™ % CHROMagar™ MRSA	Attenuation of light transmission (average ± standard deviation)[b]
MS- or MR isolates of S. aureus from ATCC		
MSSA ATCC 29213	–	0.8 ± 1.0
MRSA ATCC 1556	+	23.5 ± 0.1
MR isolates of S. aureus from livestock or pet animals		
MRSA 1573	+	16.8 ± 0.1
MRSA 1961B	+	18.7 ± 0.1
MRSA 9122	+	31.9 ± 1.5
MRSA (resistant to Triclosan)	+	27.8 ± 0.4
MRSA 12–1746	+	30.5 ± 0.1
MRSA 11–0803	+	17.8 ± 1.5
MRSA 94045	+	10.0 ± 0.1
MRSA 1171	+	34.6 ± 0.3
MRSA 12–0057/ 0058	+	9.1 ± 1.3
MRSA 1565	+	18.9 ± 0.1
MRSA 3376.3	+	19.3 ± 0.2
MS or MR isolates of other Staphylococcus species from livestock or pet animal species		
MRSP 2–16–09	+	15.5 ± 0.3
MRSP 09–2908	+	23.9 ± 1.1
MRSP 12–0220	+	13.5 ± 2.5
MRSP 11–2934	+	33.8 ± 2.9
MRSPgr 3–24–10	+	32.5 ± 1.1
MRSPgr 4–6–10	+	9.6 ± 0.1
MRSP 11–2716	+	25.1 ± 0.0
MRSP 11–3222	+	21.0 ± 0.6
MRSP 12–1316	+	27.9 ± 0.8
MRSS 2754	+	27.0 ± 0.1
MRSS 10–2737	+	18.7 ± 0.4
MRSS 12–1309	+	23.1 ± 0.4
MRSE 4–13–10	+	31.4 ± 1.3
MRSH 5–5–09	+	20.7 ± 0.5
MRSS 12–0504	+	10.6 ± 0.6
MSSP 12–1290	–	2.7 ± 0.1
MSSP 12–1789	–	2.0 ± 0.2
MSSP 12–1853	–	2.0 ± 0.1
MSSH 12–1286	–	−2.0 ± 0.2
Non-staphylococcal strains from animals or commercial sources		
Pseudomonas aeruginosa	–	−0.7 ± 0.3
Streptococcus spp. 12–1308	–	−4.1 ± 2.0
Streptococcus spp. 12–1736	–	−1.9 ± 0.0
Streptococcus spp. 12–1853	–	2.0 ± 0.1
Pasteurella multocida 12–1923	–	1.8 ± 1.6
Enterococcus faecium 12–1864	–	−3.0 ± 0.7
Enterococcus faecalis 12–1857	–	−2.2 ± 0.3
Escherichia coli XL1-Blue	–	1.9 ± 1.2
Escherichia coli S17–1	–	−0.4 ± 0.3
Escherichia coli Top 10	–	−0.1 ± 0.6

[a] MSSA – methicillin sensitive *S. aureus;* MSSP – methicillin sensitive *S. pseudintermedius;* MSSH – methicillin sensitive *S. hyicus;* MRSA – methicillin resistant *S. aureus;* MRSE – methicillin resistant *S. epidermidis;* MRSH – methicillin resistant *S. haemolyticus;* MRSP – methicillin resistant *S. pseudintermedius;* and MRSS – methicillin resistant *S. schleiferi.*

[b] The bacteria were grown in BHI broth to mid-log phase, washed, and suspended in PBS. The LPG-ISAM was exposed to 1×10^4 CFU/ml of each strain to determine the attenuation of light transmission.

2.2. Tissue extracts from mice infected with MRSA or MSSA

Groups of six-week old BALB/c mice (Harlan Laboratories, South Easton, MA) were inoculated by the intraperitoneal (IP) route with 1.0×10^7 CFU/mouse of MRSA strain 1961B or MSSA strain ATCC 29213. All challenge doses were confirmed by viable plate count on BHIB agar. Seven days later, the mice inoculated with strain ATCC 29213 were humanely euthanized with excess carbon dioxide. Livers, lungs, and spleens of the mice were rubbed thoroughly with cotton swabs, and subsequently the swabs were immersed in 2 ml of PBS. Serial dilutions of the cell suspensions were cultured onto BHIB agar to determine the bacterial CFU/ml. The mice inoculated with MRSA strain 1961B either succumbed to infection or became moribund less than 16 h after inoculation and were euthanized as described above. Swab samples or tissue suspensions from these mice were prepared and bacterial CFU were determined as described above. All experiments with animals were approved by the Virginia Tech Institutional Animal Care and Use Committee, under protocol 10-097-CVM, and comply with Directive 86/609/EEC for animal experiments.

2.3. Bacterial cultures from human subjects

Some staphylococcal isolates used in the assays were obtained from healthy volunteers (Table 2). The volunteers touched their ear canal, nostril, palm, or skin (on arm) with a swab, which was then streaked onto BHIB agar. These isolates were tested for sensitivity or resistance to methicillin by culture on BBL™ CHROMagar™ MRSA (Becton-Dickinson, Franklin Lakes, NJ) overnight at 35 °C. Isolates that grew on BBL™ CHROMagar™ MRSA were further tested by coagulase assay (Sperber and Tatini, 1975), β-hemolytic activity on blood agar, and Gram stain morphology. Exemption 4 of the 'Department of Justice Guide to the Freedom of Information Act' were followed in handling samples from human

Table 2
Growth (methicillin resistance) of *Staphylococcus* isolates from human volunteers on BBL CHROMagar™ MRSA, and attenuation of light transmission by the NOFS assay.

Bacterial strain[a]	Growth on BBL™ CHROMagar™ % MRSA	Attenuation of light transmission (average ± standard deviation)[b]
BF-N	–	−3.3 ± 1.3
KF-H	–	−0.4 ± 0.6
AH-H	–	−0.7 ± 0.3
YP-H	–	1.1 ± 0.2
KF-E	–	−1.7 ± 0.4
AB-N	–	−1.4 ± 0.9
AB-S	–	0.0 ± 0.3
HAK-H	–	−0.3 ± 0.1
HAK-E	–	−2.2 ± 0.3
AH-E	–	−4.1 ± 0.2
AC-E	+	21.4 ± 0.1
CG-E	+	10.2 ± 0.9
YP-E	+	17.1 ± 3.8
CG-H	+	20.6 ± 0.1
YP-N	+	9.5 ± 0.2
AB-E	+	20.7 ± 0.7
AC-H	+	11.1 ± 0.9
AC-S	+	18.2 ± 0.6
CG-N	+	20.1 ± 2.8

[a] The last letter of the strain represents the body part from which the culture was obtained (E-ear canal, H-hand/palm, N-nostril, S-skin). The resistance or sensitivity of each strain to methicillin was determined by culture onto BBL CHROMagar™ MRSA. Coagulase assay and hemolytic activity on blood agar presumptively identified isolates CG-H and CG-N as MRSA.

[b] The bacteria were grown in BHI broth to mid-log phase, washed, and suspended in PBS. The LPG-ISAM was exposed to 1×10^4 CFU/ml of each strain to determine the attenuation of light transmission.

donors. No data were recorded or are available on the human subjects from which bacterial cultures were collected.

2.4. MRSA-specific monoclonal antibody

Beta-lactam resistance in MRSA is attributed to the presence of an additional penicillin-binding protein (PBP2a), which is encoded for by the *mecA* gene (Murakami et al., 1991; Ryffel et al., 1990). PBP2a is not present in MS isolates. Mouse monoclonal antibody (MAb) to PBP2a (Cat. # 6G10) (CalBioreagents, San Mateo, CA) was used for detection of methicillin-resistant staphylococci in the biosensor assay.

2.5. Immunoblotting

Immunoblotting with *S. aureus* strains ATCC 29213 and ATCC 1556 was carried out as described (Ward et al., 1998) to confirm the specificity of the MAb for reactivity with MRSA. MAb to MRSA PBP2a (1:1000 dilution) and goat anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (heavy and light chains; Cappel, Durham, NC) (1:2000 dilution) were used. The membrane was developed using the TMB Membrane Peroxidase Substrate System (KPL, Gaithersburg, MD).

2.6. Turnaround point long-period gratings

The TAP-LPGs are UV-induced on TrueWave RS^{TM} (OFS) single mode optical fiber with a period of 116 μm and couple to the $LP_{0,14}$ cladding mode of the fiber. A 248 nm excimer laser was used to write the grating through a chrome-plated amplitude mask. The output from a white light source (FiberLabs, Inc. model SLD-11OESL003) was coupled to the optical fiber and spectral readings were recorded by an optical spectrum analyzer (ANDO AQ6317)) subsequent to deposition of materials on the TAP-LPG.

2.7. Fabrication of the ISAM film

Adsorption of nanoscale films onto the surface of the LPG optical fiber by the ISAM method simply involved the alternate exposure of the fiber to an aqueous solution of a polycation and an aqueous solution of a polyanion at room temperature (Wang et al., 2005a, 2005b). Briefly, a freshly rinsed fiber was immersed in an aqueous poly-allylamine hydrochloride (PAH) (10 mM, pH 7.0) solution for 5 min, followed by rinsing three times in distilled water. The fiber was then immersed in an aqueous solution of poly-1-[p-(3′-carboxy-4′-hydroxyphenylazo) benzenesulfonamido]-1,2-ethandiyl (PCBS) (10 mM, pH 7.0) for 5 min, and rinsed. In the present work, these two steps were repeated once, yielding two bilayers. The final layer on the film was PCBS so that terminal carboxyl groups were present on the ISAM film (Wang et al., 2005a, 2005b).

2.8. Coupling of MAb to the ISAM film and detection of MR staphylococci

The MAb was suspended in 0.1 M phosphate buffer, pH 6.1, at approximately 90 μg/ml. Equal volumes (~0.5 ml) of *N*-hydroxysulfosuccinimide (NHSS at 10 mg/ml in 0.1 M PBS, pH 6.1) and *N*-(3-dimethylaminodipropyl)-*N*′-ethylcarbodiimide (EDC at 25 mg/ml in 0.1 M PBS, pH 6.1) were mixed together in a microcentrifuge tube. The MAb was then added at a final concentration of 100 μg/ml. The mixture was transferred to the LPG-ISAM and incubated for 1 h. The solution on the LPG-ISAM was stirred using a pipette at 10 min intervals. The solution was removed, and non-specific binding to the fiber was blocked by the addition of 1% dry milk for 10 min before washing. The LPG-ISAM was then immersed in PBS for 3 min, rinsed with distilled water, and again immersed in PBS for 20 min. The rinsing step removes loosely bound material that has not been bound to the surface through specific adsorption.

Serial dilutions of bacterial suspensions or mouse tissue extracts in blocking buffer were added to the ISAM/antibody film and incubated for 1 h at room temperature. The binding of the target antigen to the sensor was detected as a change in the transmitted power through the fiber at the wavelength of peak attenuation of the TAP-LPG (1550 nm). Each assay was done at least three times with duplicate samples, and included positive and negative controls in each assay. The signal to background level was determined from positive control samples (MRSA strain ATCC 1556) compared to samples containing all reagents except the target bacterium (negative control). Background levels were also determined from assays containing heterologous genera or MS staphylococci. The limit of detection of the assay was determined by viable plate count of dilutions of cell suspensions. Culture and immunoblotting were also used as controls for this assay, and for determination of sensitivity and specificity. Fig. S1 in Supporting information depicts a diagram outlining the ISAM deposited on the LPG fiber, coated with antibody, and bound to antigen.

An example of the progression of the TAP-LPG transmission spectra through the stages of each experiment is shown in Fig. 1. The fiber was first tuned away from the turnaround point by etching the cladding in hydrofluoric acid. As material (ISAM film, MAb, antigen) adsorbs to the surface of the fiber, the attenuation peak becomes progressively stronger as the system moves towards the TAP. This effect is quantified through reporting the transmittance change at 1550 nm near the attenuation peak. We note that although we have used a broadband light source and optical spectrum analyzer to measure the spectrum over a wide range of wavelengths, a highly valuable aspect of using a TAP-LPG is that one could simply use a monochromatic source and photodiode to quantify the change in transmittance near the peak attenuation, thus significantly reducing the cost of and simplifying the system. The change in the attenuation strength over a broad range of wavelengths, as opposed to a shift in the wavelength of the attenuation, is a key, distinguishing feature of a TAP-LPG compared to a conventional LPG. After measurement in each bacterial suspension, the fiber surface was regenerated by immersing it for one hour in a mixture consisting of 95% sulfuric acid and 5% nitric acid heated to 80 °C. This removes all of the organic layers without modifying the silica fiber. The subsequent test involves a fresh deposition of the ISAM film and MAb followed by exposure to the appropriate bacterial suspension. A given fiber can be used for >100 tests using this procedure.

2.9. Statistical analyses

Standard deviations were calculated from the means of assays done at least four times. The sensitivity of the assay compared with culture or immunoblotting was calculated by the equation [true positives/(true positives+false-negatives)] × 100. Specificity was calculated by the equation [true negatives/(true negatives+false-positives)] × 100. Analysis of variance was used to compare the transmission attenuation among different culture dilutions of MRSA and MSSA (Lentner and Bishop, 1993). The mean difference in transmission attenuations between groups with $P<0.05$ was considered significant. The light attenuation value used to distinguish positive from negative samples was 6.3%, which was determined by multiplying the largest standard deviation of the true negative isolates (2.1%) by 3.

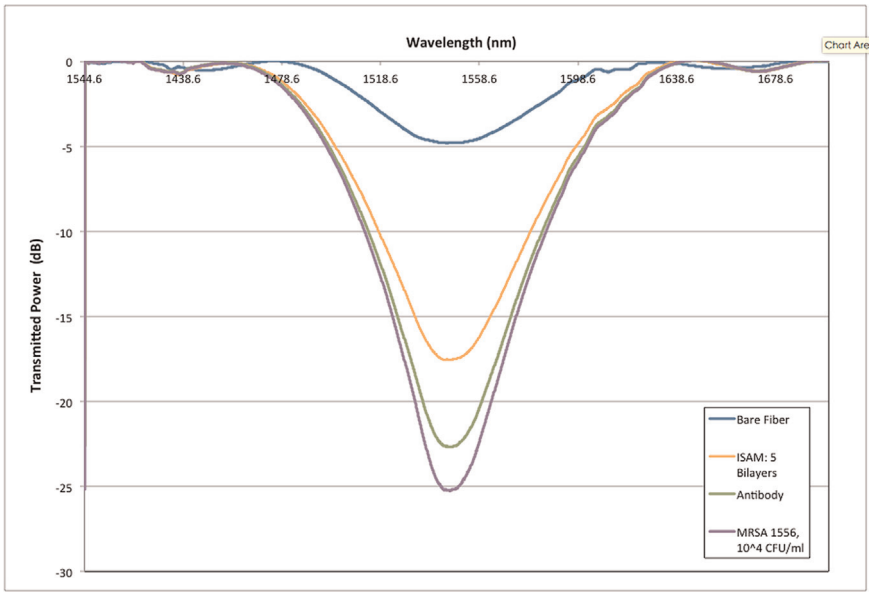

Fig. 1. Transmitted power of (top to bottom) a bare TAP-LPG, after deposition of five bilayers of a PAH/PCBS ISAM film, immobilization of MAb to MRSA PBP2a, and exposure to MRSA ATCC 1556 at 10^4 CFU/ml, respectively.

3. Results

3.1. Specificity of the MAb for methicillin-resistant staphylococci

The MAb to PBP2a of MRSA reacted with 10^8 and 3.5×10^8 cells of MRSA by immunoblotting. In contrast, this MAb did not react with 9.9×10^8 cells of MSSA (Fig. S2), confirming the selected MAb to PBP2a was specific for MR staphylococci.

3.2. Limit of detection of NOFS assay for cultured MRSA

The LPG-ISAM was exposed to serial dilutions of MRSA or MSSA in PBS, and the transmission of light through the fiber at a wavelength of 1550 nm was recorded. Light transmission was reduced by 19.7%, 23.4%, or 50.3% when the LPG-ISAM was exposed to 1×10^2, 1×10^4, or 1×10^6 CFU/ml of MRSA strain ATCC 1556 (Fig. 2). In contrast, exposure of LPG-ISAM to similar concentrations of MSSA strain ATCC 29213 produced less than 1.8% decline of light transmission through the fiber (Table 1; Fig. 2).

3.3. Use of NOFS assay to detect MRSA isolated from animals

Eleven isolates of MRSA collected from livestock or pet animals from a local Veterinary Teaching Hospital were used. The LPG-ISAM was exposed to 0.5 ml of each isolate at 1×10^4 CFU/ml. Exposure to two isolates (MRSA 12-0057/0058 and MRSA 94045) produced 9.1 and 10.0% attenuation of light transmission through the LPG, respectively, whereas exposure to the other MRSA isolates produced a decline of light transmission between 16.8% and 30.5% (Table 1: Fig. S3). These results indicated that the NOFS assay was capable of detecting MRSA isolates from a wide variety of hosts.

3.4. Use of NOFS assay to differentiate MR and MS strains of different Staphylococcus species isolated from animals or commercial sources

Nine strains of MRSP, four strains of MRSS, and one strain each of *MR S. epidermidis* (MRSE), and *MR S. haemolyticus* (MRSH), all collected from livestock or pet animals were tested. Exposure of the LPG-ISAM to one of the MRSP isolates produced 9.6% transmission attenuation, and exposure to an MRSS isolate resulted in 10.6% transmission attenuation (Table 1; Fig. S3). Exposure of the LPG-ISAM to the remaining 13 MR *Staphylococcus* isolates resulted in 13.5–33.8% attenuation of light transmission through the fiber (Table 1; Fig. S3). Thus, the NOFS assay was capable of detecting the PBP2a protein from all *Staphylococcus* species tested.

One isolate each of MSSA and MSSH, and three isolates of MSSP, all collected from livestock or pet animals were also tested. Exposure of the LPG-ISAM to these MS isolates attenuated light transmission by less than 2.7% (Table 1; Fig. S3), indicating the NOFS assay was highly specific for MR isolates of *Staphylococcus* species. The LPG-ISAM was also exposed to three isolates of *S. pyogenes*, three isolates of *E. coli*, and one isolate each of

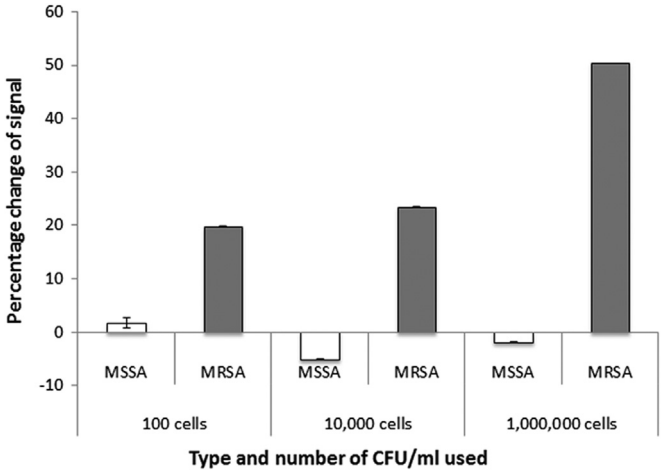

Fig. 2. Capability of the NOFS assay to distinguish MRSA from MSSA. Serial dilutions of MRSA or MSSA cultures were incubated with the LPG-ISAM. The signal attenuations (percent change of signal) resulting from exposure of the ISAM-MAb to various concentrations of MSSA (open bars) compared to MRSA (gray-filled bars) were significantly different ($P < 0.002$, < 0.00001, and < 0.00001 for 1×10^2, 1×10^4, or 1×10^6 cells/reaction, respectively.).

E. faecalis, E. faecium, P. aeruginosa and *P. multocida.* Exposure of the LPG-ISAM to these isolates resulted in 2% or less transmission attenuation (Table 1; Fig. S3), further supporting that the NOFS assay was highly specific for detecting MR staphylococci.

3.5. Use of the NOFS assay to detect MR Staphylococcus strains isolated from humans

Nineteen *Staphylococcus* isolates from human volunteers were tested for methicillin resistance. Nine of these isolates grew and were mauve in color on BBL™ CHROMagar™, supporting that these isolates were resistant to methicillin. Two of the MR isolates (CG-H and CG-N) produced firm clots in the coagulase test and were hemolytic on blood agar, and were therefore presumptively identified as MRSA. The remaining isolates were likely methicillin-resistant *S. epidermidis* (Table 2). When the LPG-ISAM was exposed to the nine MR *Staphylococcus* strains, three isolates produced 9.6%, 10.2%, and 11.1% transmission attenuation, whereas the other six MR *Staphylococcus* isolates produced between 17.1% and 21.4% transmission attenuation (Table 2: Fig. S3). Ten of the *Staphylococcus* isolates from human volunteers did not grow on BBL™ CHROMagar™ MRSA, and generated less than 1.1% attenuation of light transmission (Table 2: Fig. S3). Thus, the NOFS assay was highly specific for detecting MR *Staphylococcus* strains from human specimens.

3.6. Use of the NOFS assay to detect MRSA in mouse tissues

To determine if the biosensor would be capable of detecting MRSA in clinical specimens, swabs of infected mouse tissues were tested. Exposure of the LPG-ISAM to one swab of the liver of a mouse infected with MSSA strain ATCC 29213 resulted in 5.6% transmission attenuation. However, less than 2.6% transmission attenuation resulted from exposure of the LPG-ISAM to all other swabs of the livers, lungs, and spleens from mice inoculated with the MSSA strain. In contrast, exposure to swabs of the same organs of mice inoculated with MRSA strain 1961B resulted in transmission attenuations between 11.7% and 73.5% (Table 3; Fig. S4).

4. Discussion

S. aureus is responsible for skin, soft-tissue, and systemic infections in humans and animals, and rapidly develops antibiotic resistance. In addition to MRSA, methicillin resistance has also been identified in other species of staphylococci (Cohn and Middleton, 2010). As a result, methicillin resistance in staphylococci has become a major concern in both human and veterinary medicine (Cohn and Middleton, 2010). Rapid diagnosis of methicillin resistant staphylococci is essential to identify those patients or animals requiring isolation, and for initiation of effective therapy. Culture and susceptibility require a minimum of 48 h to complete. Therefore, culture-free diagnostic assays that can be carried out rapidly, but are highly sensitive and specific are needed. Several rapid agglutination assays that have high sensitivity but somewhat poor specificity for identification of *S. aureus*, and are based on detection of clumping factor of capsule by hemagglutination or latex agglutination, are available (Evangelista et al., 2002), but still require culture of the organism. Methicillin resistance in staphylococci results from the bacteria acquiring the *mecA* gene, which encodes for penicillin-binding protein PBP2a. PBP2a has such a low affinity for beta-lactam antibiotics, that the bacteria harboring this gene are resistant to all classes of beta-lactams, including cephalosporins (Evangelista and Truant, 2002). Agglutination assays and immunoassays, and PCR are also available for detection of either PBP2a or the *mecA* gene, respectively, but still require culture of the organism (Evangelista and Truant, 2002a). However, MRSA isolates may express heterogeneous resistance to beta-lactams, resulting in false-negative results (Weist et al., 2006), and variable expression of PBP2a due to steric interference by surface components, such as capsular polysaccharides, clumping factor, or protein A (Fournier et al., 1987; Kuusela et al., 1994). Furthermore, variation in inoculum size, incubation time, temperature, medium pH, salt concentration of the medium, and exposure to beta-lactam antibiotics may also influence phenotypic expression of PBP2a (Babel and Decker, 2008). Selective and differential media, such as BBL™ CHROMagar™ MRSA II, is approved for detection of MRSA in nasal swab samples (Farley et al. 2008; Flayhart et al., 2005), but still requires an incubation time of 24–48 h (Farley et al., 2008).

Real-time (RT)-PCR is well established for same day identification of MRSA (Kolman et al., 2010; Soderquist et al., 2012). These assays have been used successfully in detecting the *mecA* gene of MRSA as well as coagulase-negative staphylococci (Wang et al., 2014). However, RT-PCR is generally only available in large laboratories that can afford the equipment and staff to maintain and operate this equipment, and is not suitable for small labs or bedside use.

Wang et al. have detected MRSA by labeling specific DNA probes with paramagnetic and ferromagnetic nanoparticles to concentrate the hybridized DNA molecules under a magnetic field, which can then be detected and quantified using a spectrophotometer (Wang et al., 2011a). However, the use of magnetic particles hinders the sensitivity of the biosensor, and the magnetic particles or beads used to label the DNA probe may react with the buffer solution used (Palecek and Fojta, 2007).

Biosensors for detection of MRSA DNA amplified by PCR have been developed using electrodes and impedance spectroscopy (Corrigan et al., 2012; Wang et al., 2011b), and piezoelectric gold electrode (Tombelli et al., 2006). Following amplification of the DNA by PCR or loop-mediated isothermal amplification, the limit of detection for MRSA has been lowered to 1×10^2 CFU/ml, and the

Table 3
Detection and differentiation of MRSA from MSSA in tissues of BALB/c mice by NOFS assay.

Mouse #	*S. aureus* strain used in inoculations	Attenuation of light transmission (%)[a] (± standard error)		
		Liver (CFU/ml)	Lung (CFU/ml)	Spleen (CFU/ml)
1	MSSA ATCC 29213	$5.6 \pm 0.0 (2.1 \times 10^1)$	$-4.4 \pm 2.2 (0)$	$2.6 \pm 1.7 (3.2 \times 10^2)$
2	MSSA ATCC 29213	$0.7 \pm 1.2 (3.0 \times 10^1)$	$-0.91 \pm 1.4 (0)$	$1.6 \pm 0.9 (8.2 \times 10^1)$
3	MRSA 1961B	$67.0 \pm 0.1 (4.4 \times 10^3)$	$38.2 \pm 0.1 (1.6 \times 10^3)$	$47.2 \pm 2.6 (6.8 \times 10^3)$
4	MRSA 1961B	$50.1 \pm 14.5 (8.4 \times 10^3)$	$11.7 \pm 3.7 (3.7 \times 10^2)$	$19.2 \pm 7.2 (7.9 \times 10^3)$
5	MRSA 1961B	$23.4 \pm 0.4 (5.2 \times 10^3)$	$73.5 \pm 0.9 (7.7 \times 10^2)$	$72.9 \pm 3.7 (9.3 \times 10^3)$

[a] Groups of mice were inoculated IP with MSSA strain ATCC 29213 or MRSA strain 1961B. Liver, lung, and spleen from the infected mice were swabbed, and the swab immersed into PBS. Serial dilutions of the suspensions were cultured to determine the CFU/ml of bacteria from each specimen (shown in parentheses). The LPG-ISAM was exposed to the cell suspensions to determine the attenuation of light transmission.

detection time has been reduced to 1–2 h (Ceylan Koydemir et al., 2011). However, such biosensors may cost more than $10,000, and require trained personnel, which may be unsuitable for small hospitals or clinics. House et al. (House et al. 2010) developed a lab-on-a-chip (LOC) device that integrates a micro-fluid system onto a micro-PCR chip, which precisely replicates the thermal cycling process of PCR. However, the test results need to be interpreted manually, and the required CCD detection of the fluorescent light needs to be added to the system. Optical transduction methods such as surface plasmon resonance (SPR) have also been developed for detection of MRSA (Yang et al., 2012). Optical biosensors have been shown to be rapid and sensitive. However, analyte detection by this assay involves the use of LED and spectroscopy to generate exciting light and receive a signal. In addition, the use of such biosensors requires expertize in optics and expensive equipment, and is therefore not suitable for many clinical applications.

The distinguishing feature of the biosensor platform described in our work from prior reports on LPG biosensors is the combination of ISAM films, which allow deposition of a variety of materials (including polymers and nanoparticles) into multiple layers each just a nanometer thick, with TAP-LPGs covalently bound to a MAb specific to the PBP2a protein of MR staphylococci. Antigen binding to the MAb alters the thickness and refractive index of the attached thin film, which then immediately modifies the transmission characteristics of the fiber and produces an observable output indicating the presence of target antigen. This binding resulted in a significant decrease ($P < 0.002$) in the optical power transmitted through the fiber. Furthermore, antibodies are very stable and have maintained binding activity in agglutination assays after at least 1 year in storage at 4 °C (Inzana, 1995). Therefore, antibodies can be conjugated to individual fibers, which can be stored at 4 °C until needed, and the sensitized fiber then coupled to the sensor as needed. Of 36 known methicillin-resistant bacterial strains used, 33 strains resulted in greater than 10% attenuation of light transmission through the fiber. Moreover, exposure to MRSA-infected mouse tissues attenuated transmission by greater than 11.7%. Using a light attenuation breakpoint value of 6.3% (three times the largest standard deviation value), both the sensitivity and specificity of the assay was 100% for the samples examined. These results indicate that the NOFS assay described is capable of detecting methicillin-resistant staphylococci from cultures or infected tissues.

5. Conclusions

Our results demonstrate that the LPG-ISAM system provides a promising culture-free assay for specific detection of methicillin-resistant staphylococci in one hour. This assay can be operated by non-specialist personnel, and allow accurate determination of MRSA with minimal sample treatment. The assay can also be applied to a variety of other infectious agents. The portable sensing method would be useful for a small clinic, and bedside applications where compact equipment (smaller than a laptop) can be combined to simply include a monochromatic light source, optical fiber, photodiode, and computer, in which the optical fiber segment containing the TAP-LPG and functional coating is replaced after each use.

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Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.03.041.

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