

Rapid amplification/detection of nucleic acid targets utilizing a HDA/thin film biosensor

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Thin film biosensors exploit a flat, optically coated silicon-based surface whereupon formation of nucleic acid hybrids are enzymatically transduced in a molecular thin film that can be detected by the unaided human eye under white light. While the limit of sensitivity for detection of nucleic acid targets is at sub-attomole levels (60 000 copies) many clinical specimens containing bacterial pathogens have much lower levels of analyte present. Herein, we describe a platform, termed HDA/thin film biosensor, which performs helicase-dependant nucleic acid amplification on a thin film biosensor surface to improve the limit of sensitivity to 10 copies of the *mecA* gene present in methicillin-resistant strains of *Staphylococcus*. As double-stranded DNA is unwound by helicase it was either bound by solution-phase DNA primers to be copied by DNA polymerase or hybridized to surface immobilized probe on the thin film biosensor surface to be detected. Herein, we show that amplification reactions on the thin film biosensor are equivalent to in standard thin wall tubes, with detection at the limit of sensitivity of the assay occurring after 30 minutes of incubation time. Further we validate the approach by detecting the presence of the *mecA* gene in methicillin-resistant *Staphylococcus aureus* (MRSA) from positive blood culture aliquots with high specificity (signal/noise ratio of 105).

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Introduction

Timely administration of appropriate treatment for infectious diseases has been associated with lower treatment costs and improved patient outcomes.^{1–5} Culture-based methods for identification of pathogens and determination of drug resistance profiles are slow, requiring 24–48 hours for results.^{1–5} Slow turnaround time forces clinicians to treat infections with broad spectrum antimicrobial therapy when there is clinical suspicion of infection. This approach may not be effective when dealing with intrinsically resistant organisms and can result in iatrogenic infections.⁶ Due to greater clinical sensitivity and shortened assay times, molecular diagnostic (MDx) tests drive treatment costs savings with improved patient outcomes. Early MDx analyzers were large, complex instruments that required significant user interaction and hands-on time from highly trained personnel. Recently, several simplified approaches have been described for molecular diagnosis in the central laboratory by automating all assay steps in a sample-to-result format.^{7–9} However, while easy-to-do, these systems are still large, complex, and expensive, limiting their usefulness to testing within centralized laboratories.

Moving diagnosis closer to the patient can result in further improvements in patient outcome especially in the point of care (POC) setting. However, the POC environment has limited

bench space and trained personnel as well as the inability to afford the expense associated with tests that require complex instrumentation. Additionally, test turnaround times must be rapid for the physician to provide appropriate treatment before the patient leaves the office (<60 minutes). Antigen-based tests utilizing lateral flow detection are rapid, non-instrumented, and very easy to perform, but suffer from poor sensitivity and inability to multiplex.¹⁰ In an attempt to bring the improved sensitivity of MDx to the POC setting, lab-on-a-chip approaches (LOC) have been described, wherein target sequences are amplified and detected on a glass or silicon chip.^{11–13} LOC approaches incorporate solid-phase PCR (SP-PCR), wherein one or both primers are immobilized on the chip surface as a means to improve amplification specificity and increase multiplexing capability.^{11–13} However, amplification on chips with immobilized primers tends to have poor amplification efficiency leading to poor assay sensitivity and specificity.^{12,13} This may be due to “screening effects” wherein the highly negative charge density of immobilized DNA primers at the surface affects DNA polymerase function, reducing extension efficiency. Also, a low fraction of primer appears to be converted into target DNA during solid-phase amplification, which may be explained by steric hindrance by surface-attached larger amplified target DNA molecules preventing additional DNA molecules from reaching primers on the surface.¹¹ Also, effects of the solid surface materials may inhibit amplification reactions.^{14,15}

To avoid issues such as thermal transfer and cycling associated with PCR, isothermal amplification methods have been

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described, allowing amplification at a single temperature. Several methods have been described that amplify target DNA in an exponential manner including helicase-dependent amplification (HDA, ref. 16), recombinase polymerase amplification (RPA, ref. 17), and loop-mediated amplification (LAMP, ref. 18). HDA, wherein the enzyme DNA helicase unwinds double-stranded DNA targets instead of heat as is done with PCR but is otherwise identical utilizing DNA polymerase to extend DNA primers, is an attractive technique. Design is straightforward, requiring only 2 primers/target sequences. Additionally, HDA has been demonstrated to be tolerant to the presence of blood and feces, simplifying sample preparation as well.^{19,20}

Herein we describe the performance of a simplified approach to molecular diagnosis by performing simultaneous amplification and detection of nucleic acid targets using a thin film biosensor.^{21,22} Thin film biosensors exploit optical situations to allow the unaided human eye to detect thickness changes on the same order as the size of intermolecular interactions of biological molecules on a chip surface.^{21,22} The thickness changes are observed as a color change, gold to purple, to which the human eye is maximally sensitive. Thin film biosensors have been described previously and have been commercially successful in the POC setting for the rapid detection of viral and respiratory pathogens that require rapid, accurate results directly from a clinical specimen with minimal hands-on interaction.²³ While thin film biosensors have excellent reported limits of detection (<100 000 copies of target DNA), they require target or signal amplification methods to detect lower quantities of target present in some clinical specimens.^{21,22}

In the approach described herein, isothermal HDA reactions were performed in solution to exploit optimal kinetic conditions for amplification above the thin film biosensor surface and avoid the limitations of solid-phase based amplification reactions. During the HDA reaction the helicase enzyme is

continuously unwinding double-stranded DNA. Once in single-stranded form, the DNA can either bind to primer sequences and be copied by the action of DNA polymerase or bind to DNA probes immobilized onto the chip surface. At the completion of the amplification step, amplicon immobilized onto the chip surface can be rapidly (5 minutes) detected. By combining the amplification and hybridization step the test is simplified by reducing the number of reagents required as well as speeding up the test by eliminating steps in the procedure. Herein, we have demonstrated rapid and sensitive amplification/detection of nucleic acid targets. The rate of amplification is similar to that in solution and the sensitivity is excellent (10 copies of MRSA genomic DNA). As a proof of concept, we applied this technique to the specific detection of the *mecA* gene in staphylococci present in positive blood cultures.

Results

Characterization of the HDA/thin film biosensor approach

As a first application of the HDA/thin film biosensor system, we developed HDA primers which recognize conserved sequences within the *mecA* gene, which encodes for an altered penicillin binding protein, pbp2a. PBP-2a expression confers resistance to the frontline treatment for staphylococci, methicillin. Isolated genomic DNA was used to characterize the properties of the system. As a baseline to determine the sensitivity of the *mecA* gene primer set, a dose response experiment with methicillin-resistant *Staphylococcus aureus* (MRSA) was performed. Genomic DNA was amplified in solution in a tube and then removed into a separate plate containing a chip coated with an amplicon-specific capture probe for detection by hybridization.¹⁹ The lower limit of detection was determined to be 10 copies of input target MRSA using this primer set (Fig. 2(a)). Quantification of the chip signal by color difference confirms significant signal above background at 10 copies of input MRSA

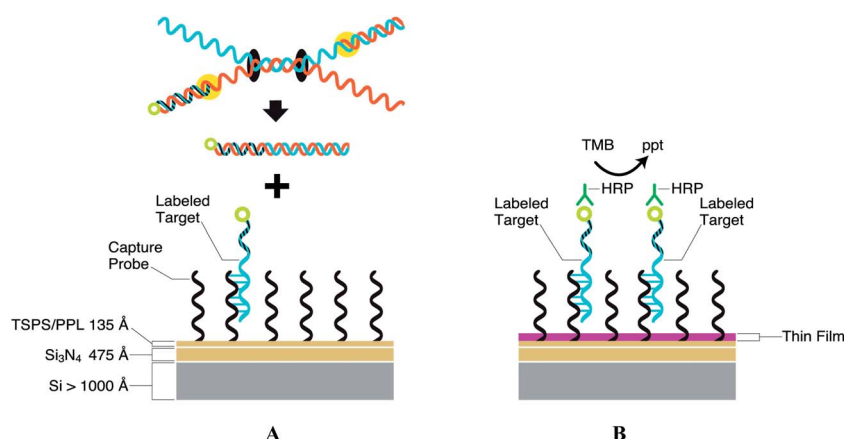


Fig. 1 HDA/thin film biosensor method. HDA/thin film biosensor method. (A) HDA process performed in presence of thin film biosensor. Double stranded DNA is unwound by the action of uvrD helicase (black oval). Resultant single stranded regions can then hybridize to either complementary biotin labeled (light green) DNA primers (black and white stripe) and be copied by the action of DNA polymerase (yellow circle) to create double stranded amplified products (blue represents amplified sequence copied by DNA polymerase) or hybridize to complementary probes on the thin film surface. (B) Upon completion of the HDA reaction, surface-bound, biotin-labeled DNA targets trigger reactions that enzymatically transduce the formation of hybrids on the surface into a molecular thin film. This results in a color change from gold to blue.

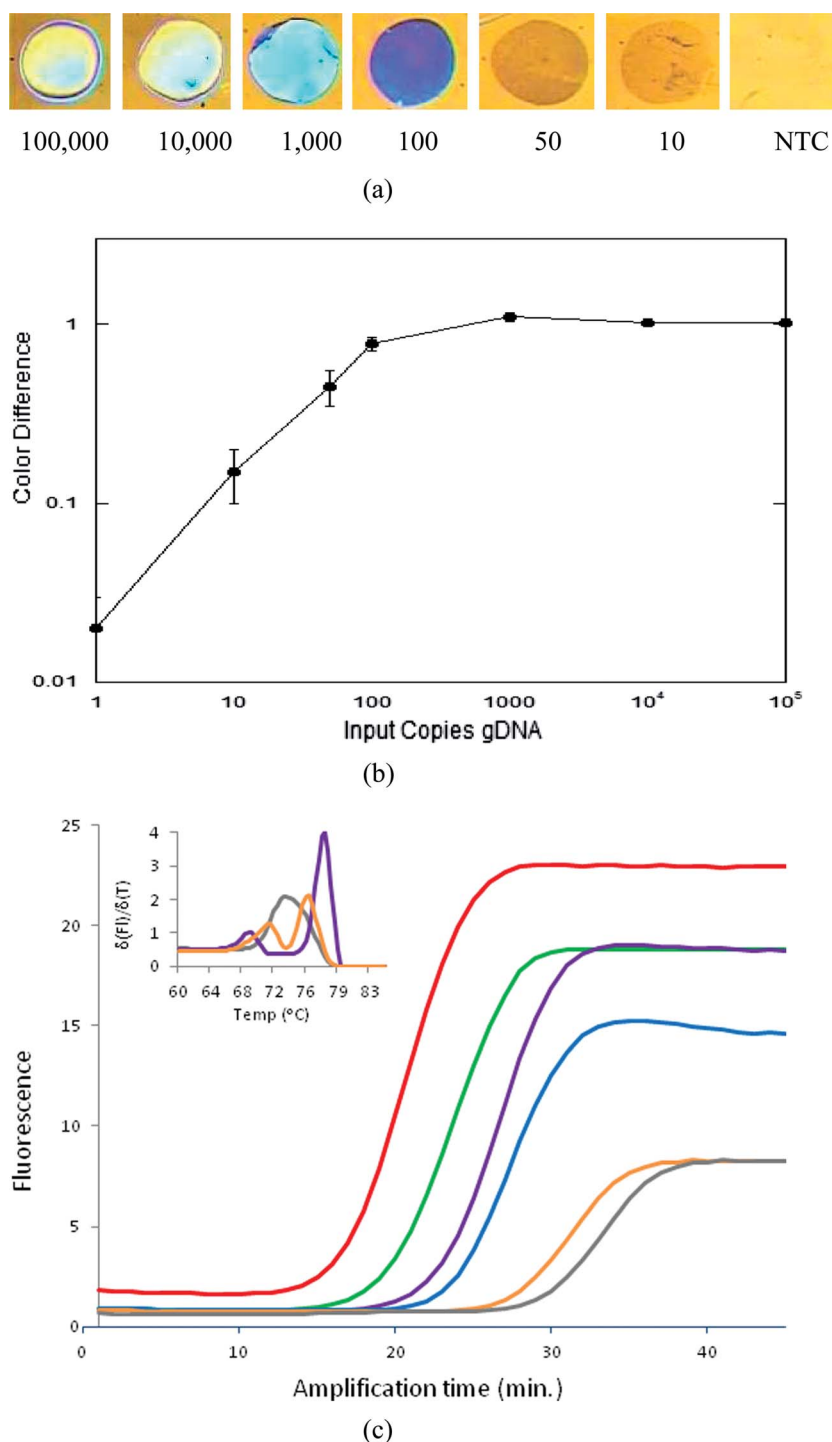


Fig. 2 Sensitivity of detection of MRSA using HDA/thin film biosensor. (a) Sensitivity of detection of MRSA using HDA/thin film biosensor. HDA reactions were performed on a serial dilution (0–100 000 copies of genomic DNA) in individual tubes for 60 minutes and resultant amplicon was hybridized to *mecA* gene specific probe on the thin film biosensor. Chips were imaged using a CCD camera. (b) Color difference plot as a function of input target DNA amount. Color difference was determined as described.²¹ Background (0.01) was not subtracted from any data point. Data from two independent determinations are shown. (c) Real-time HDA amplification curves. Fluorescence (EvaGreen) was monitored as a function of amplification time at 65 °C. Colors represent the quantity of input target MRSA genomic DNA; no-template control (gray), 100 copies (orange), 1000 copies (blue), 10 000 copies (purple), 100 000 copies (green), 10⁵ copies (red). (c), inset: melt profile of amplified products from real-time PCR analysis. The change in fluorescence as a function of temperature is plotted vs. temperature. Colors represent the melt curve for various input MRSA genomic DNA amounts; no-template control (gray), 100 copies (orange), 10 000 copies (purple). The *mecA*-specific amplified DNA target has a T_m of 78.2 °C.

genomic DNA with saturation of color by 1000 copies of input target DNA (Fig. 2(b)). Specificity of the primer set and capture probe was confirmed by tests with genomic DNA isolated from methicillin-sensitive *S. aureus* (SA) and *S. epidermidis* which yielded no detectable signal.

To provide a comparison to a more conventional technique, we also tested limit of detection by real-time HDA on a Light-Cycler. MRSA genomic DNA was serially diluted and amplification progress was monitored with the double-stranded DNA intercalating dye, EvaGreen. Amplification proceeded rapidly with 10 000 copies of genomic DNA amplified to detectable levels within 23 minutes, however the no template control also yielded detectable signal within 30 minutes (Fig. 2(c)). Analysis of the melt curves revealed significant non-specific amplification for input amounts of 1000 copies or less of genomic DNA; a characteristic T_m of 78.2 °C for the *mecA*-specific amplified DNA target was not apparent at these lower input target DNA amounts with primer artifact product detected as a broad peak at a T_m of 74 °C (Fig. 2(c) inset).

To determine the effect of the chip on amplification efficiency, we amplified MRSA genomic DNA for various times either in tubes or on chips and applied products of each time point to gel electrophoresis (Fig. 3). Amplification reactions both in tubes and on chip produced detectable amplicon in 30 minutes suggesting very similar reaction kinetics. Real time PCR studies confirm this observation with a doubling time of 66 seconds for in tube amplifications and 55 seconds for on-chip amplification reactions (data not shown). Amplification reactions performed on the coated chip produced a single full length product as determined by gel electrophoresis and the correct T_m as confirmed by real-time PCR. Sequence was confirmed by hybridization to a target-specific probe on the thin film biosensor. Amplification reactions performed in tubes generated two amplified products; one representing the correct full length amplicon and a shorter product created by either mis-priming or primer artifact. The observation of improved



Fig. 3 Effect of thin film biosensor on HDA amplification rate. effect of thin film biosensor on HDA amplification rate. HDA reactions were performed on 1000 copies of MRSA genomic DNA on the thin film biosensor surface. Aliquots (10 μ L) were removed and subjected to electrophoresis on 2% agarose gel. Lanes 1–11: amplification reactions on thin film biosensor, lanes 12–21: amplification reactions in tubes. Lane 1: 25 bp ladder; lane 2, blank; lanes 3 and 13, negative control at 20 min.; lanes 4 and 14, negative control at 35 min.; lanes 5 and 15, 20 min. reaction; lanes 6 and 16, 22.5 min. reaction; lanes 7 and 17, 25 min. reaction; lanes 8 and 18, 27.5 min. reaction; lanes 9 and 19, 30 min. reaction; lanes 10 and 20, 32.5 min. reaction; lanes 11 and 21, 35 min. reaction.

amplification fidelity for the HDA/thin film biosensor could be related to higher surface/volume reaction conditions.

A time course was performed to determine the time necessary to amplify various quantities of MRSA genomic DNA using the HDA/thin film biosensor. As is seen in Fig. 4, 1000 copies of genomic DNA were readily detected by chip within 25 minutes with 100 copies generating very slight signal. By 30 minutes, sufficient amplification occurred to detect at the limit of detection of the assay. The improved speed of detection compared with gel electrophoresis is a function of the 30-fold improved limit of detection of the thin film biosensor.²⁴ Amplification for longer time did not improve the limit of detection of the assay.

Detection of MRSA in positive blood cultures

We next determined the ability of the HDA/thin film biosensor system to specifically detect the *mecA* gene within MRSA from positive blood cultures. Standard microbiological methods to diagnose staphylococcal bloodstream infections requires 24–48 hours post positive blood culture forcing physicians to treat infections empirically. It has been shown in multiple studies that they provide the incorrect treatment 25–33% of the cases of staphylococci.²⁵ More rapid detection of staphylococci directly from positive blood cultures has been shown to be important in reduction of hospitalization and treatment costs by aiding in the direction of appropriate therapeutic interventions.^{7,8} Blood culture aliquots seeded with either MRSA or SA were subjected to a cell lysis step for 10 minutes at room temperature. Then crude lysates were placed onto the chip surface, subjected to 40 minutes amplification and signal was developed. As is seen

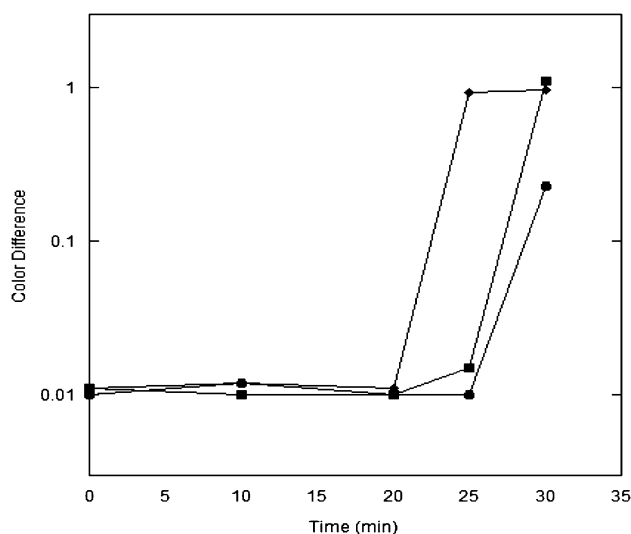


Fig. 4 HDA time course for HDA/thin film biosensor method. Time course for HDA/thin film biosensor method. HDA reactions were performed on a serial dilution (0–1000 copies) of genomic DNA for various times on the thin film biosensor and then detected. Chips were imaged with a CCD camera and color difference values were determined as described.²¹ Color difference is plotted as a function of time: NTC (closed circles), 10 copies input (closed square), 100 copies input (closed diamond), 1000 copies input (closed triangle).

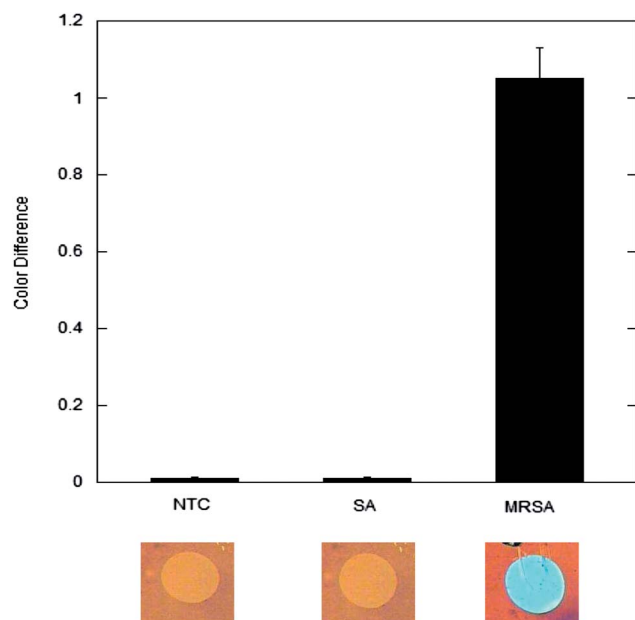


Fig. 5 Detection of *mecA* from MRSA positive blood cultures. Detection of *mecA* from MRSA positive blood cultures. Positive blood cultures containing either methicillin-resistant *Staphylococcus aureus* (MRSA) or methicillin-sensitive *Staphylococcus aureus* (SA) were processed as described and subjected to HDA reactions for 45 minutes on the thin film biosensor surface. A no template control (NTC) was performed as a negative control. Color difference values were determined from 12 independent determinations. Error bars represent ± 1 standard deviation.

in Fig. 5, strong signals were generated by on-chip amplification of the equivalent of a 0.1 μL aliquot of MRSA positive blood culture whereas no signal was observed for SA positive blood cultures. The results show that the HDA/thin film biosensor has a measured signal/background ratio of 105 for both a non-target organism and a no template control. This indicates that this approach performs with both excellent sensitivity and specificity.

Discussion

The HDA/thin film biosensor described here permits integrated target DNA amplification and detection of as few as 10 copies of input target DNA in under 60 minutes with high specificity. To our understanding this is the first description of the integration of HDA into a chip-based amplification approach. The excellent analytical performance of the HDA/thin film biosensor can be explained by a few possible factors. First, amplification specificity and efficiency is ideal when performed in solution, however in order for amplification to proceed efficiently, optimization of materials which may contact amplification reaction components is required.^{14,15} For example, it has been shown elsewhere that Si_3N_4 coated silicon chips are inhibitory to PCR reactions.¹⁴ While the thin film effect occurs due to the presence Si_3N_4 applied to a crystalline silicon chip, the thin film biosensor has an additional coating of the chemically inert polymer TSPS. TSPS is a molecularly flat (surface roughness of

<15 angstroms as measured by surface profilometry) hydrophobic coating providing a barrier between bio-molecules and the Si_3N_4 coating. Additionally the coating is chemically inert, limiting potential non-specific interactions. Also, the sensitivity of the thin film biosensor is critical for good analytical performance. The thin film biosensor has previously been shown to display quantitative hybridization efficiency²¹ with rapid kinetics similar to solution-phase reaction rates.²⁴ This allows for the thin film biosensor to effectively compete with primers for hybridization to amplified DNA targets for subsequent detection. Because the thin film biosensor yields a sequence-specific positive signal with sub-attomole quantities of input target DNA molecules, relatively few hybridization events are required to generate detectable signal.²¹ So while significant primer artifact was observed with HDA amplification in this study below 1000 copies of input target DNA, the thin film biosensor was able to detect amplified target DNA with high specificity in the presence of significant non-specific amplified DNA.

Conclusion

We have developed a chip-based approach that combines isothermal amplification and visual detection to allow for a simple, inexpensive non-instrumented approach to rapidly detect low levels of pathogenic bacteria or viruses in a clinical sample. The system would only require a simple and inexpensive heat block due to the temperature tolerance of HDA ($\pm 2^\circ\text{C}$). All of the reagents are inexpensive, highly stable and robust with measured stability at 37°C for at least 9 months with no loss in activity.²⁶ The HDA amplification reagents may be stored in a dried pellet on the chip surface and all other reagents placed into dropper bottles. These attributes make this system ideal for use in resource poor settings, including developing world laboratory or the POC setting.²⁶

Experimental

Primers and probes

For design of the *mecA* gene amplicon, alignments were performed with multiple *mecA* gene sequences from clinically relevant methicillin-resistant *Staphylococcal* species. Primers were designed targeting conserved regions of the *mecA* gene using previously published parameters for HDA design¹⁶ with Primer 3 software (primer3.ut.33). Primer designs were optimized for speed and limit of detection. Additionally, primer artifact is a significant competing reaction and primer sets were screened for primer artifact formation using real-time PCR (LightCycler, Roche, Indianapolis, IN). The primers used for these studies were *mecA* L FWD, 5'-TGGATAGACGTCATATGAAGGTGTGCT-3', and *mecA* L REV, 5'-BT-ATTATGGCTCAGGTACTGCTATCCACC-3', wherein BT is a 5'-biotin TEG linker. These primers create an 82 base pair amplicon.

DNA capture probes were designed to have T_m values in the range of $52\text{--}58^\circ\text{C}$ using MeltCalcTM (ref. 27 and 28, MeltCalc Software, Verden, Germany). BLAST analysis was performed for all primer and probe sequences to determine any potential

cross reactivity. The probe used for these studies was *mecA* 1703 CP, 5'-ILink12/iSp18/CAAGTGCTAATAATTCACCTGTTG-3', wherein 5'-ILink12 is a 5'-hydrazide with a 12 carbon atom spacer and iSp18 is an 18 carbon atom internal spacer.

All oligonucleotides were synthesized by Integrated DNA Technologies (IDT, Coralville IA).

Blood culture

Bacterial strains were purchased from ATCC (Manassas, VA). All strains were sub-cultured on tryptic soy agar and grown overnight at 37 °C. To prepare spiked blood cultures, bacterial cultures were diluted in sterile saline to a no. 3 McFarland standard and then further adjusted to a final amount of 5 CFU mL⁻¹ in 5 to 7 mL of human blood. The spiked blood mixture was injected into BD BACTEC + bottles. The bottles were placed onto a BD BACTEC 9240 and incubated until microbial activity was detected at the Microbiology Department of the Denver Health Medical Center (Denver, CO).

Chip preparation

Thin film biosensors were prepared as previously described.²¹ Briefly, crystalline silicon wafers were coated in a vapor deposition chamber (Nordiko, Santa Clara, CA) with 475 Å silicon nitride. Thickness is monitored by ellipsometry. The polymer aminoalkyl functional T-structure polydimethylsiloxane (TSPS, UCT, Bristol, PA) was applied using a spin coater (Machine Technologies, Inc., Parsippany, NJ) and cured at 150 °C for 24 h to produce the thin film biosensor. The TSPS coated wafer was further treated by soaking in a 50 mg L⁻¹ solution of poly(lys-phe), (Sigma) in 1× phosphate-buffered saline (PBS, pH 6) containing 2 mol L⁻¹ NaCl overnight at room temperature. Next, the poly(lys-phe) coated wafer was washed and soaked again with 10 μM succinidyl-4-formyl benzoate (SFB, Sigma, St. Louis, MO) for 2 hours at room temperature, washed thoroughly with water, dried with a stream of nitrogen and stored at room temperature.

DNA capture probes were synthesized containing a reactive hydrazide group on the 5'-end designed to interact and attach to the aldehyde-functionalized surface of the silicon wafers with a 12 carbon atom spacer to separate the surface from the probe sequence. Probes in spotting buffer (0.1 M phosphate buffer pH 7.8, 10% glycerol) were applied (75 nL) onto the SFB coated silicon wafer. After incubating for 2 hours, the wafers were washed with 0.1% SDS, dried, scribed into 6.5 mm² chips (DynaTek, Valencia, CA), and stored in nitrogen purged sealed bags prior to use.

HDA amplification

For HDA reactions performed in solution, 2 μL of genomic DNA was added to 38 μL of dilution buffer (20 mM Tris-HCl, pH 8.8, 10 mM KCl, 7.7 mM MgSO₄, 40 mM NaCl, 5 mg mL⁻¹ BSA, 0.02% Tween 20), mixed thoroughly, and then 20 μL of the diluted sample was added to 20 μL of HDA mix (20 mM Tris-HCl pH 8.8, 40 mM NaCl, 200 nM *mecA* L FWD, 200 nM *mecA* L REV, 0.8 mM each dCTP, dGTP, and dTTP, and 6.8 mM dATP, 10 ng μL⁻¹ uvrD helicase (BioHelix, Beverly, MA), 1.6 U μL⁻¹

GST polymerase (New England BioLabs, Beverly, MA), and 4 ng μL⁻¹ ET SSB (New England BioLabs)). Reactions were incubated at 65 °C in an incubator oven (Torrey Pines Scientific, Carlsbad, CA). For real-time HDA reactions, EvaGreen (Roche) was added to the HDA mix. Real-time reactions were performed using a LightCycler (Roche) with the setting of 65 °C for 60 minutes to collect fluorescence signals.

HDA/thin film biosensor amplification and detection method

The HDA/thin film biosensor method described here occurs on a multilayered, optically coated silicon chip that is highly reflective with a gold appearance in white light. Surface color is a consequence of low reflectance in the blue region of the visible spectrum and high reflectance in the orange-red region. This characteristic color is created by coating silicon wafers with 475 Å of the anti-reflective coating silicon nitride (Si₃N₄). To create a more biocompatible surface, an additional coating of TSPS is applied followed by application of two aqueous coatings to create an aldehyde functionalized surface allowing for one step immobilization of capture probes modified with hydrazide on the 5'-end (Fig. 1(a)). Chips were adhered to the bottom of individual wells of a square well 96 well plate (Millipore, Billerica, MA) using double sided tape and 50 μL of dilution buffer pre-warmed to 65 °C. An aliquot from a positive blood culture (10 μL) was added to 90 μL of an achromopeptidase-based extraction reagent (Great Basin, Salt Lake City, UT) and incubated at room temperature for 10 minutes in a microcentrifuge tube, followed by heating at 95 °C for 5 minutes. 1 μL of crude extract was added to 74 μL of HDA mix pre-warmed to 65 °C on the chip surface. After incubation at 65 °C in an incubator oven, the solution was removed from the chip surface and replaced with 100 μL of conjugate solution (1 μg mL⁻¹ anti-biotin antibody/HRP (Great Basin, Salt Lake City, UT) in 5× SSC, 10% fetal calf serum, 0.5% ATC) and incubated at room temperature for 3 minutes. The chip was then washed with wash solution (0.1× SSC, 0.05% Tween 20) using a squirt bottle (~1 mL total). Finally, 100 μL of Membrane TMB (Surmodics, Eden Prairie, MN) was added to the chip surface and incubated at room temperature for 2 minutes wherein a molecular thin film was deposited on the surface in the vicinity of the probe/amplicon complex. The additional mass on the surface alters the path-length of light reflecting from the optical layers, effectively attenuating specific wavelengths through destructive interference, resulting in an apparent surface color change from gold to blue exploiting a color change to which the human eye is maximally sensitive (Fig. 1(b)). Chips were washed with water followed by methanol and allowed to air dry. Charge-coupled device (CCD) images were then taken of each chip using a SSC-DC54 CCD camera (Sony, Norbain, Redding, Berkshire, UK) with an illuminator (Dolan Jenner Industries, Inc., Lawrence, MA). Color difference of the surface was quantitated as described previously.²¹

Doubling time determination

10 000 copies of genomic DNA were applied to HDA/thin film biosensor and amplified for various times as described.

Reaction products were removed from chip surface and diluted 100-fold in molecular grade water to stop then reaction. An aliquot was added to 2× SYBR Green I mastermix (Roche), 500 nM each of *mecA* L FWD and *mecA* L REV primers and amplified in a LightCycler (Roche). At the same time known amounts of MRSA genomic DNA was amplified to generate a standard curve. Amplification conditions were as follows: 95 °C for 5 minutes, followed by 40 cycles of 95 °C for 20 s/60 °C for 15 s/72 °C for 15 s. Using the standard curve, numbers of copies of target DNA generated during each time point of amplification on the HDA/thin film biosensor was determined. HDA/thin film biosensor amplification time was plotted against number of copies of target DNA produced. Using an exponential curve fit $A = A_0(1 - e^{-kt})$, the amplification rate (k) is determined by the slope of the curve. Doubling rate was calculated by the relation, $T_d = \ln(2)/k$, wherein T_d is doubling time.

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