

Electrical detection of pathogenic bacteria via immobilized antimicrobial peptides

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Edited* by Charles Lieber, Harvard University, Cambridge, MA, and approved September 3, 2010 (received for review June 21, 2010)

The development of a robust and portable biosensor for the detection of pathogenic bacteria could impact areas ranging from water-quality monitoring to testing of pharmaceutical products for bacterial contamination. Of particular interest are detectors that combine the natural specificity of biological recognition with sensitive, label-free sensors providing electronic readout. Evolution has tailored antimicrobial peptides to exhibit broad-spectrum activity against pathogenic bacteria, while retaining a high degree of robustness. Here, we report selective and sensitive detection of infectious agents via electronic detection based on antimicrobial peptide-functionalized microcapacitive electrode arrays. The semi-selective antimicrobial peptide magainin I—which occurs naturally on the skin of African clawed frogs—was immobilized on gold microelectrodes via a C-terminal cysteine residue. Significantly, exposing the sensor to various concentrations of pathogenic *Escherichia coli* revealed detection limits of approximately 1 bacterium/ μL , a clinically useful detection range. The peptide-microcapacitive hybrid device was further able to demonstrate both Gram-selective detection as well as interbacterial strain differentiation, while maintaining recognition capabilities toward pathogenic strains of *E. coli* and *Salmonella*. Finally, we report a simulated “water-sampling” chip, consisting of a microfluidic flow cell integrated onto the hybrid sensor, which demonstrates real-time on-chip monitoring of the interaction of *E. coli* cells with the antimicrobial peptides. The combination of robust, evolutionarily tailored peptides with electronic read-out monitoring electrodes may open exciting avenues in both fundamental studies of the interactions of bacteria with antimicrobial peptides, as well as the practical use of these devices as portable pathogen detectors.

bacterial sensing | bioelectronic sensors | biorecognition | water monitoring | biomimetic devices

Bacterial infections remain the leading cause of death in developing nations, accounting for an estimated 40% of deaths (1). For instance, the strain O157:H7 of *Escherichia coli* is considered to be one of the most dangerous food-borne pathogens (2, 3). In developed countries, bacterial contamination is also of critical concern, particularly in the pharmaceutical industry. Indeed, the most reliable test for contamination is the limulus amoebocyte lysate (LAL) test, based on the detection of endotoxins via coagulation of horseshoe crab blood (4, 5). Microbial infections and drug-resistant supergerms are also a leading cause of military deaths, particularly in soldiers with burn injuries, and are considered potential biowarfare agents (6–8). Although containment strategies—such as vaccination and “broadband” antibiotic usage in hospitals—have helped reduce the severity of bacterial infections, these strategies have also inadvertently promoted the emergence of antibiotic resistance. Thus, the development of a sensor that can detect the presence of an infectious outbreak from a broad spectrum of pathogenic species would be highly desirable.

Current methods for detecting pathogenic bacteria include ELISA and PCR (9, 10). In the former case, the assays exploit antibodies as molecular recognition elements due to their highly specific targeting of antigenic sites. However, antibodies lack the stability needed to detect pathogenic species under harsh envi-

ronments, reducing the shelf life of antibody functionalized sensors. The high specificity of antibody–antigen interactions also requires a one-to-one pairing of antibody-based sensors for each target to be detected. Nucleic acid probe-based techniques such as PCR can reach single-cell detection limits, yet require the extraction of nucleic acids and are limited in portability.

By contrast, the ease of synthesis and intrinsic stability of antimicrobial peptides (AMPs) render them particularly interesting candidates for use as molecular recognition elements in electronic biosensing platforms (11, 12). AMPs appear in multiple niches in nature including the skin of higher organisms and the extracellular milieu of bacteria as the primary line of defense against bacteria and fungi (13). AMPs are much more stable than typical globular proteins—explaining how they can be continually exposed to the natural environment—and are exceptionally efficient at fending off bacterial infection (14). Indeed, some cationic antimicrobial peptides have shown activity toward pathogenic bacteria under harsh environmental conditions such as thermal (boiling/autoclaving) and chemical denaturants (15, 16). The replacement of current antibody-based affinity probes with more stable and durable AMPs in biological sensors may thus help to increase the shelf life of current diagnostic platforms. Finally, a major potential advantage of AMPs as recognition elements stems from their semiselective binding nature to target cells, affording each peptide the ability to bind a variety of pathogens.

The bioactivity of AMPs toward microbial cells is classified into groups according to their secondary structures (13). Many AMPs adopt amphipathic conformations that spatially cluster hydrophobic from cationic amino acids, thereby targeting the negatively charged head groups of lipids in the bacterial membrane. In contrast, the membranes of plants and animals seclude negative charges to the inner leaflet and contain cholesterol that reduce AMP activity (12). By aiming at the very foundation of the bacterial cell membrane, and remaining generically unrecognizable to proteases (17), AMPs as antibiotics have remained remarkably free of acquired resistance. Among AMPs, linear cationic peptides such as magainins are particularly attractive for microbial sensing applications because of their small molecular size and intrinsic stability (18, 19). In particular, the positively charged AMP magainin I (GIGKFLHSAGKFGKAFVGEIMKS) binds most selectively to the bacterial cell *E. coli* O157:H7 as a precursor to bactericidal activity (20). Magainin I also displays broad-spectrum activity toward other Gram-negative bacteria, which comprise the majority of pathogenic infection in humans.

A number of methods have been successful at detecting bacteria, including nanomechanical cantilever sensing (21, 22),

Author contributions: M.S.M., A.J.L., and M.C.M. designed research; M.S.M. and S.Z. performed research; M.S.M., A.J.L., and M.C.M. analyzed data; and M.S.M. and M.C.M. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1008768107/-DCSupplemental.

surface-enhanced Raman spectroscopy (23), and quartz crystal microbalance-based sensors (24). Similarly, recent attempts have utilized AMPs as biorecognition elements in fluorescent-based microbial detection with achievable detection limits of 5×10^4 cells/mL (25, 26). Yet, the development of an “all-in-one” solution that combines a high degree of portability, robustness, sensitivity, and selectivity toward pathogenic strains remains challenging. Among the various label-free signal transduction platforms that have been investigated, impedance spectroscopy is promising due to its simple instrumentation, ease of device assembly, and adaptability to multiplexed lab-on-a-chip applications (27, 28). A microcapacitive sensor detects impedance changes in the dielectric properties of an electrode surface upon analyte binding, where the variation in the impedance is directly proportional to the activity of analyte binding (29). Here, we report a label-free electronic biosensor based on the hybridization of the antimicrobial peptide magainin I with interdigitated microelectrode arrays for the sensitive and selective detection of pathogenic bacteria via impedance spectroscopy. We anticipate that the combination of compact, naturally bioselective AMPs with microcapacitive sensors may represent a highly robust and portable platform for fundamental studies of AMP-bacteria interactions, and for portable infectious disease threat agent signaling.

Results and Discussion

Sensitivity Measurements. As a first step toward the development of an AMP-based, label-free electronic biosensor, the targeting of microbial cells by magainin I was investigated using impedance spectroscopy. Fig. 1 schematically outlines our sensing platform. AMPs are first immobilized on microfabricated interdigitated gold electrodes (Fig. 1A; see *Materials and Methods*). Magainin I was acquired with an additional cysteine residue at the C terminus (Fig. 1B), allowing for facile and site-specific covalent attachment to the gold electrodes. Next, heat-killed bacterial cells were injected and incubated with the AMP-modified electrodes. If the bacteria are recognized by the AMPs, binding will occur (Fig. 1C), leading to dielectric property changes that can be monitored by a spectrum analyzer (see Fig. S1). The impedance was measured over a frequency range of 10 Hz to 100 kHz. Fig. 1D shows an optical micrograph of the device, which is made using standard microfabrication techniques.

Sensitivity of microbial detection is a key determinant for utility of sensors. To this end, the sensitivity of the magainin-functionalized microelectrode array in detecting bacterial cells was first investigated using impedance spectroscopy. Fig. 2 shows the results of measurements performed after incubation of the immobilized AMPs with pathogenic *E. coli* O157:H7 cells in

concentrations ranging from 10^3 to 10^7 cfu/mL. A “blank” device with no immobilized AMPs was also tested for comparison; the impedance of the blank device without immobilized AMPs is found to change negligibly upon exposure to various bacterial concentrations (see Fig. S2). Fig. 2A shows that at low frequencies, the different concentrations of bacterial cells have the effect of increasing the impedance in proportion to the number of cells present in the sample for concentrations greater than 10^2 cfu/mL. As the frequency increases, the contribution to the impedance from the bacterial cells decreases, leaving only the dielectric relaxation of small dipoles including water molecules in the buffer solution to affect the measured impedance. Fig. 2B thus depicts the impedance change at a fixed frequency of 10 Hz. The variation in the impedance is directly proportional to the number of bacterial cells bound to the immobilized AMPs and manifested in a logarithmic increase with respect to serially diluted bacterial concentrations. Significantly, the detection limit of response of the hybrid AMP-microelectrode device to *E. coli* was found to be 10^3 cfu/mL (1 bacterium/ μ L). This lowest limit of detection appears to be limited by the presence of impedance due to the electrical double layer resulting from the electrode polarization effect at low frequencies. Importantly, this sensitivity limit is clinically relevant (30) and compares favorably to AMP-based fluorescent assays (26), antibody-based impedance sensors (27), and to the LAL test (5).

To gain further insight into the activity of magainin I toward *E. coli*, AMPs were immobilized “upside down” via incorporation of a cysteine residue at the N terminus. The binding affinities of magainin I immobilized via cysteine residues at the C terminus and N terminus were compared and coplotted in Fig. 2A and B. Considerably reduced binding activity was observed for magainin immobilized via the N-terminus compared to C-terminal immobilization. This reduction in the binding affinity is likely due to the diminished exposure of the target bacteria to the amine-containing residues near the N terminus. This observation supports the hypothesis that the initial interaction of α -helical AMPs with the membranes of the target bacteria occurs via electrostatic attraction of positively charged amino acids on the AMP with negatively charged phospholipids in the bacterial membrane (20, 31, 32). Indeed, it has been previously shown that amino acid omissions in the N-terminal region of magainin result in the complete loss of antimicrobial activity, whereas analogs with omissions in the C-terminal region exhibited equal or increased activity (33). Finally, the effect of varying the surface density of the immobilized AMPs on the detection of bacterial cells was investigated (see Fig. S3). The response of the biosensor toward target cells was found to increase monotonically with increasing concentration of immobilized magainin.

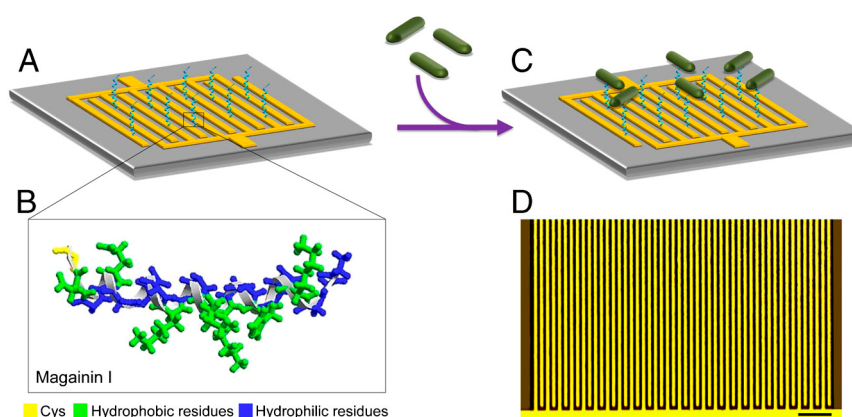


Fig. 1. AMP-based electrical detection of bacteria. (A) Schematic of AMPs immobilized on an interdigitated microelectrode array. (B) Magnified image of the AMP magainin I in helical form, modified with a terminal cysteine residue, and with clearly defined hydrophobic and hydrophilic faces. (C) Detection of bacteria is achieved via binding of target cells to the immobilized AMPs. (D) Optical image of the interdigitated microelectrode array (scale bar: 50 μ m).

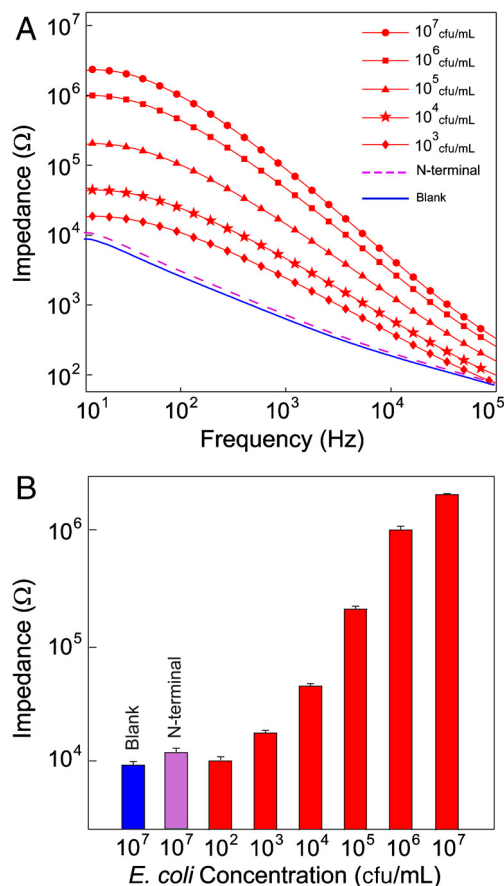


Fig. 2. Sensitivity of the AMP electronic biosensor. (A) Impedance spectra of various concentrations of *E. coli* O157:H7 cells (red), of a nonlabeled sensor (blue), and of a sensor with an N-terminal immobilized AMP (purple). (B) Impedance spectra of various concentrations of *E. coli* with the AMP sensor at 10 Hz. Error bars show standard deviation ($N = 3$).

Selectivity Measurements. As a next step, we investigated the selectivity of the AMP-functionalized biosensors toward various bacterial species. Specifically, the binding behavior of AMPs was probed toward (i) Gram-negative pathogenic *E. coli* O157:H7, (ii) the nonpathogenic *E. coli* strain American Type Cell Culture (ATCC) 35218, (iii) Gram-negative pathogenic *Salmonella typhimurium*, and (iv) *Listeria monocytogenes*, a Gram-positive pathogen. Collectively, these studies elucidate the matrix of selectivity as it depends on Gram-negative vs. Gram-positive species, and pathogenic vs. nonpathogenic strains. The selectivity was first investigated using fluorescent microscopy methods, by staining bacterial cells and optically mapping their binding density to gold films hybridized with AMPs. Fig. 3 shows the discriminative binding pattern of immobilized magainin I to various bacterial cells (all 10^7 cfu/mL) stained with propidium iodide (PI) nucleic acid stain (see *Materials and Methods*), as well as the surface density of the bound bacterial cells. Likewise, Fig. 4A plots the electrical response of the AMP biosensor against these various species as a function of the interrogating frequency, and Fig. 4B plots the response at 10 Hz.

Intriguingly, inspection of the fluorescent images and surface density plots agree qualitatively with the response of the AMP electrical biosensor and reveal the following insights. First, magainin I exhibits clear preferential binding toward the pathogenic, Gram-negative species *E. coli* and *Salmonella*, relative to the Gram-positive species *Listeria*, with a 2 order of magnitude impedance difference at 10 Hz (Fig. 4B) (34). This selectivity was particularly enhanced for pathogenic *E. coli*, which showed a slightly larger response relative to *Salmonella*. The response of

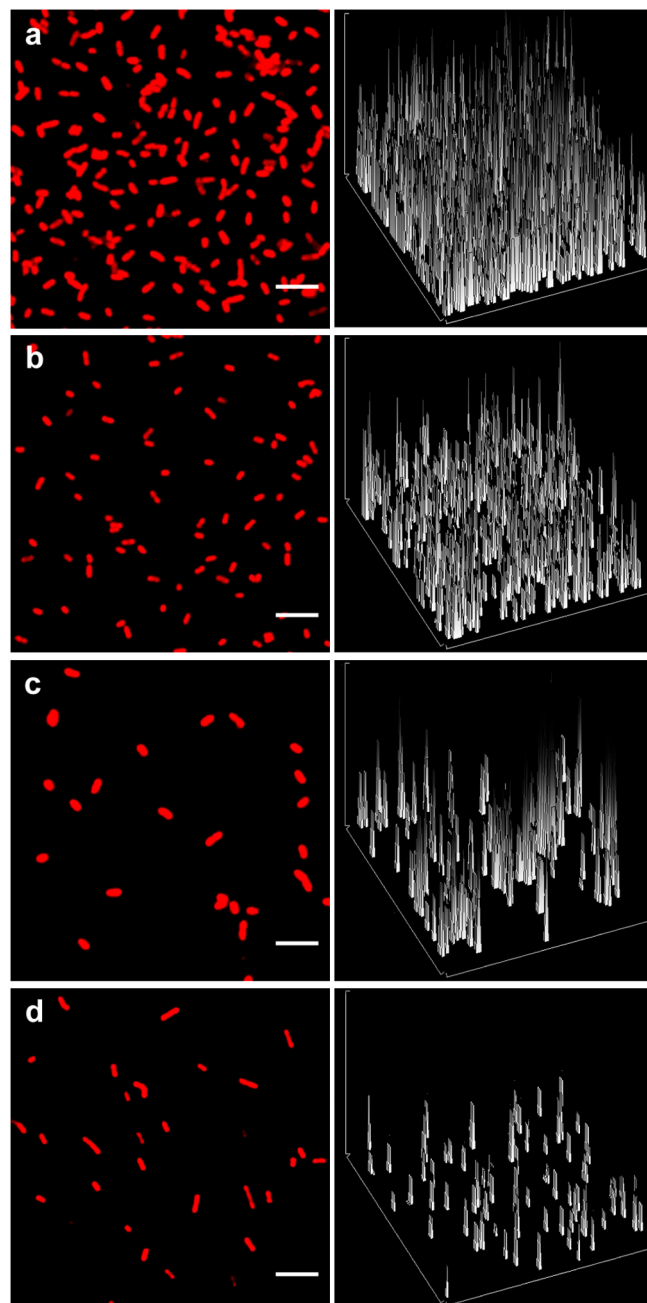


Fig. 3. Optical microscopy of the selectivity of AMPs. (Left) Demonstration of selective binding of the immobilized AMP to various stained bacterial cells (10^7 cfu/mL), including (A) *E. coli* O157:H7, (B) *S. typhimurium*, (C) *E. coli* ATCC 35218, and (D) *L. monocytogenes*. (Right) The corresponding surface density of the bound cells. Scale bars are 10 μ m.

the sensor to a mixture of pathogenic *E. coli* and *Listeria* with a total cellular concentration of 10^7 cfu/mL similarly revealed dominant *E. coli* binding (see Fig. S4). Next, interbacteria strain differentiation between pathogenic and nonpathogenic bacteria is demonstrated by the ability of the sensor to selectively detect pathogenic *E. coli* relative to the nonpathogenic strain, again with a nearly 2 order of magnitude impedance difference at 10 Hz. Interestingly, this preferential binding is mitigated in a highly basic medium (see Fig. S5) (12, 35, 36). Finally, the response of the sensor to all microbial species was larger than the response of the blank biosensor that was not functionalized with AMP.

The observed specificity differences can be explained by noting that a balance between electrostatic and hydrophobic interactions

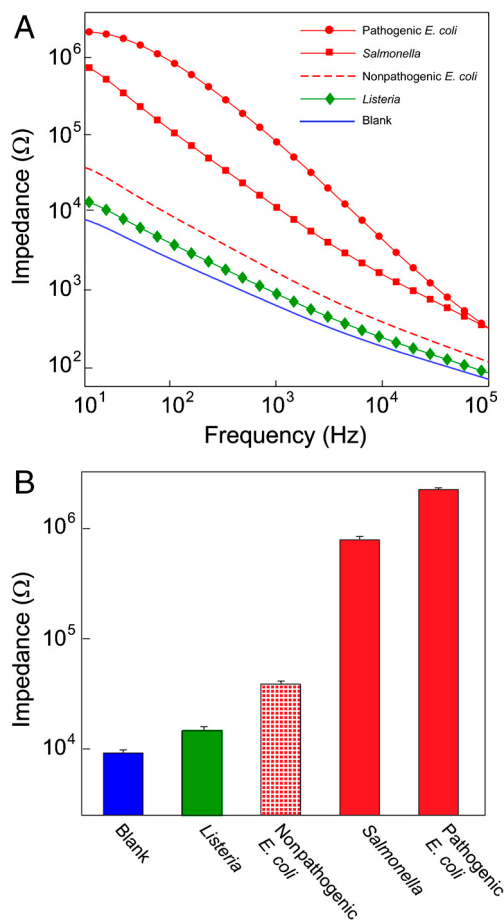


Fig. 4. Impedance spectroscopy of the selectivity of AMPs. (A) Impedance spectra of the AMP-functionalized microelectrode array after interaction with various bacterial samples (10^7 cfu/mL). (B) Impedance changes associated with various bacterial species at 10 Hz. Error bars show standard deviation ($N = 3$).

is believed to underlie the mechanism of bacterial cell binding by AMPs (31, 37). In the case of magainin I, the difference in the membrane structures of Gram-negative vs. Gram-positive bacteria may account for the differential selectivity (38). Gram-negative bacteria possess an outer membrane with negatively charged LPS—the first site of encounter for AMPs—and a thin peptidoglycan layer. In contrast, Gram-positive bacteria lack the LPS-containing outer membrane, instead possessing a thick peptidoglycan layer and teichoic acids. Further, although both pathogenic and nonpathogenic *E. coli* cell walls contain LPS, the LPS of the pathogenic strain includes O antigens, which are hydrophilic branched sugar side chains. These O antigens form the outermost portion of the polysaccharide chain and are thought to enhance electrostatic and hydrogen bonding (39–41). This ability of magainin I to selectively prefer Gram-negative species, and pathogenic vs. nonpathogenic strains of *E. coli*, agrees with other bacteria adhesion studies (20, 35, 42, 43).

Real-Time Detection. To simulate the use of the AMP microelectrodes in everyday applications, such as direct water sampling, the biosensor response was investigated in real time, as shown in Fig. 5. First, a microfluidic cell was bonded to the interdigitated biosensor chip (Fig. 5A), such that the electrodes were perpendicular to the direction of the sample flow (Fig. 5B) (44). Next, fluid was injected using a syringe pump connected to the inlet port and allowed to flow through to the outlet port at a flow rate of 100 $\mu\text{L}/\text{min}$. The flow cell was first flushed with buffer to establish a baseline. Various dilutions (10^4 – 10^7 cfu/mL) of

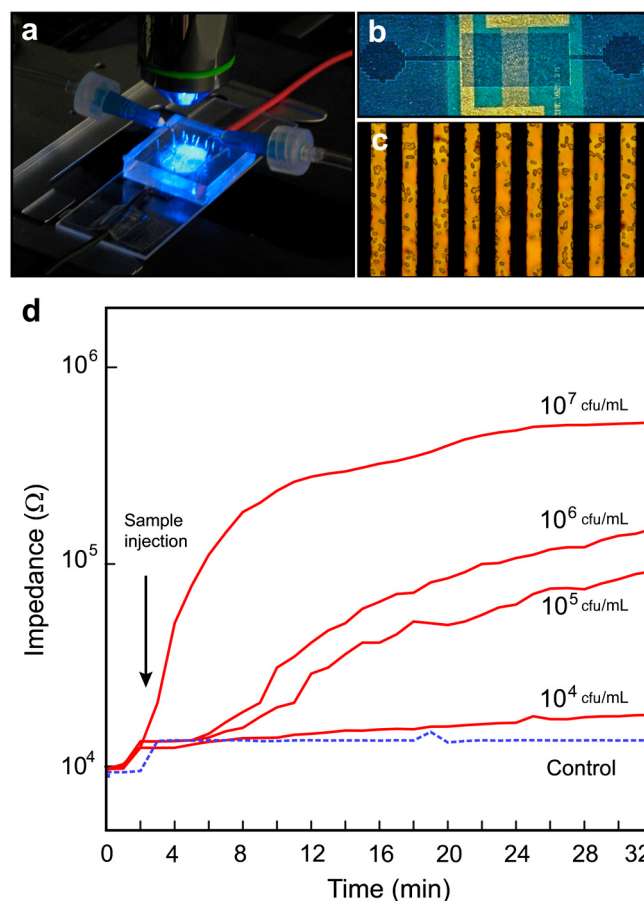


Fig. 5. Real-time binding of bacteria to AMP biosensors. (A) Digital photograph of the microfluidic flow cell. (B) Optical micrograph of the microfluidic channel with an embedded interdigitated microelectrode array chip. (C) Optical image of the embedded microelectrode array after exposure to 10^7 cfu/mL bacterial cells for 30 min. (D) Real-time monitoring of the interaction of the AMP-functionalized sensor (and an unlabeled control chip) with various concentrations of *E. coli* cells.

pathogenic *E. coli* cells in PBS were then injected to the channel at a reduced flow rate of 5 $\mu\text{L}/\text{min}$ for 30 min. For example, Fig. 5C shows the microelectrode array after exposure to 10^7 cfu/mL bacterial cells. Simultaneously, the impedance response was continuously monitored during the sample flow-through process (Fig. 5D). All samples produced a measurable response relative to the control sample within 5 min, with the highest concentration sample yielding a response within 30 s; the responses saturated after *ca.* 20 min. These results bode well for the implementation of this sensor in continuous monitoring of flowing water supplies. Yet, it should be noted that for the same concentration of bacterial cells, the response of the sensor under flow-through conditions was found to be comparatively lower than the response after static incubation. We attribute this to the opposing effects of shear and mixing on the binding kinetics, as reduced binding of AMPs to target cells under flow-through conditions has also been reported in fluorescent-based assays (25).

Conclusion

In summary, coupling of AMPs with microcapacitive biosensors has resulted in the implementation of a portable, label-free sensing platform for the detection of infectious agents. The achievable sensitivity approached 1 bacterium/ μL —a clinically relevant limit—and the AMPs allowed for sufficient selectivity to distinguish pathogenic and Gram-negative bacteria, while retaining broadband detection capabilities. Finally, real-time

sensing results demonstrated the capability of the relatively simple impedance-based transduction architecture to directly detect bacteria, suggesting a promising alternative to traditional antibody-based immunoassays. We anticipate these results could provide a significant positive impact on the use of pathogenic sensors to test and monitor bacteria in reservoir water, or for use as biological threat agent detection systems. Yet, a number of key challenges remain. First, the detection of bacteria in real water samples has not yet been studied. Second, as confirmed with detection of *E. coli* in the presence of *Listeria*, the broadband selectivity of magainin-functionalized sensors complicates scenarios in which there are multiple infectious agents present, or when the concentrations of the target species are unknown. Finally, based on our previous work in coupling peptides to silicon nanowire sensors (45, 46), significantly enhanced sensitivity may be achievable by reducing the sensors down to the nanometer scale.

Materials and Methods

Antimicrobial Peptides and Bacterial Cells. Antimicrobial peptide magainin I (GIGKFLHSAGKFGKAFVGEIMKS), chemically synthesized to contain a C-terminal cysteine residue via standard N-Fmoc solid phase peptide synthesis, was obtained from Anaspec. Magainin I was also synthesized with an N-terminal cysteine to compare the bacterial binding activity. Heat-killed pathogenic bacterial cells of *E. coli* O157:H7, *S. typhimurium*, and *L. monocytogenes* were purchased from KPL. Heat-killed cells of a nonpathogenic strain of *E. coli* (ATCC 35218) was obtained from ATCC for control experiments. The stock solution of AMP was prepared by the reconstitution of the lyophilized product in phosphate buffered saline (Sigma-Aldrich) consisting of 137 mM NaCl, 2.7 mM KCl, 4.4 mM Na₂HPO₄, and 1.4 mM KH₂PO₄ (pH 7.4) (35, 36). The heat-killed bacterial cells were rehydrated in PBS, according to manufacturer recommendations.

Interdigitated Microelectrode Array (IMA) and Microfluidic Flow Cell. Interdigitated capacitive electrodes were microfabricated on 4" p-type silicon wafers (boron-doped, <100>, 10–16 Ω-cm, 550 μm thick). A 1-μm thick silicon dioxide layer was deposited on the wafer by plasma enhanced chemical vapor deposition to form electrical insulation between the Si substrate and the capacitive electrodes. S1813 photoresist was patterned using photolithography, followed by electron-beam evaporation of 10 nm Ti and 300 nm Au. The IMA was then finally developed by liftoff patterning of the metallic layer in acetone with ultrasonic activation. The electrode array consisted of 50 pairs of interdigitated capacitive electrodes with an electrode width and separation of 5 μm. A polydimethylsiloxane (PDMS) microfluidic flow cell consisting of a detection microchamber with an embedded microelectrode array and inlet and outlet ports was formed by bonding the IMA chip to the PDMS channel. The PDMS microchannel formed on the master mold was partially cured, aligned with the microelectrode array, and bonded by permanently curing at 80 °C for 2–3 h. Microfluidic connectors were fixed onto the inlet and outlet ports through drilled holes.

Sensor Surface Functionalization with Magainin. A simple technique for the immobilization of peptides to a gold surface is through the utilization of native thiol groups present in cysteine residues (47–50), and cysteine residues can be synthetically introduced at a specific site of the peptide to form a properly oriented recognition layer (49, 51–53). Previous studies have revealed that the covalent immobilization of AMPs on gold surfaces via C-terminal cysteine leads to adsorption at an angle to the surface (43, 54). Prior to

the immobilization procedure, the gold IMA electrodes were cleaned using acetone, isopropanol, and deionized (DI) water. Stock solutions of the AMPs were prepared in PBS, pH 7.4, consisting of 137 mM NaCl, 2.7 mM KCl, 4.4 mM Na₂HPO₄, and 1.4 mM KH₂PO₄ (35, 36). For the immobilization of the AMPs, 800 μg/mL (unless otherwise mentioned) of magainin I in PBS buffer was injected into the sensing chamber and incubated for 60 min under static conditions. The functionalized electrodes were then rigorously washed with 1 × PBS to remove any unbound AMPs, rinsed with deionized water and dried in liquid nitrogen. Gold surfaces covalently functionalized with magainins have shown antimicrobial binding activity persisting for at least 6 mo (54).

Fluorescent Microscopy. Stock solutions of PI, nucleic acid stain (Molecular Probes) was made from solid form by dissolving PI (molecular weight = 668.4) in deionized water at 1 mg/mL (1.5 mM) and stored at 4 °C, protected from light. Heat-killed bacterial cells rehydrated in PBS were then incubated with 3 μM solution of PI (made by diluting the 1 mg/mL stock solution 1:500 in buffer) for 10–15 min (55). After incubation, the cells were pelleted by centrifugation and removal of the supernatant and were resuspended in fresh 1 × PBS buffer. The samples of stained bacterial cells (*E. coli* O157:H7, *Salmonella*, nonpathogenic *E. coli*, and *Listeria*, all 10⁷ cfu/mL) were then allowed to incubate with the immobilized magainin for 15–20 min in the dark. After incubation, the Au surfaces were washed with PBS buffer and dried under liquid nitrogen. The binding pattern of the different bacterial cells was imaged using a Zeiss axiovert inverted microscope and recorded with a Zeiss axiocam digital camera. Surface density of the bound bacterial cells was analyzed and plotted using the ImageJ software package.

Impedance Spectroscopy. Dielectric property changes due to AMP-bacteria interactions were probed using a fast-Fourier transform spectrum analyzer. The dielectric properties were investigated over a frequency range of 10 Hz to 100 kHz, with 0-V dc bias and 50-mV ac signals using a SRS 785, two-channel dynamic signal analyzer. An in-house LabView program routine was used to collect and record the data through a general purpose interface bus interface. An external op-amp amplifier circuit was used to minimize the noise, and a MATLAB program was used to plot the impedance spectra from the analyzer output (see Fig. S1). For sensitivity measurements, pathogenic Gram-negative *E. coli* O157:H7 bacterial cells were injected into the microfluidic flow channel at various dilutions and incubated with the immobilized magainins for 12–15 min, under static conditions. To ensure the response of the sensor toward bound bacterial cells, the impedance spectrum was taken after the removal of unbound cells by thorough washing in PBS. For real-time measurements, the impedance vs. time data were recorded while buffer solutions or different dilutions of bacterial solutions flowed through the microfluidic channel. The flow cell was first flushed with 1 × PBS buffer at a flow rate of 100 μL/min to establish a baseline. Bacterial detection measurements were performed with the sample flowing at a rate of 5 μL/min. The sensor device was regenerated via a cleaning solution containing 1 M NaCl, 100 mM HCl, and 200 mM CHAPS followed by 1 × PBS buffer. The electrodes were then thoroughly flushed with DI water to remove any salts. The effect of bacterial cells binding to immobilized magainins on the impedance signal is due to the dielectric property of the cell membrane. All experiments were repeated three times.

ACKNOWLEDGMENTS. We thank Ann Mularz and Kellye Cung for valuable discussions and illustrations. M.C.M. acknowledges support of this work by the Air Force Office of Scientific Research via a Young Investigator Grant (FA9550-09-1-0096) and as a fellow of the American Asthma Foundation (09-0038). A.J.L. acknowledges support from the National Science Foundation (CBET-0952875).

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