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Analysis of Microbial Biofilms on Medical Devices

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Analytical Challenges of Microbial Biofilms on Medical Devices

Microbial colonization of medical devices is a widespread problem that tests the limits of conventional analytical methods. Successful analytical endeavors require collaboration between clinicians, microbiologists, biomedical engineers, and analytical chemists.

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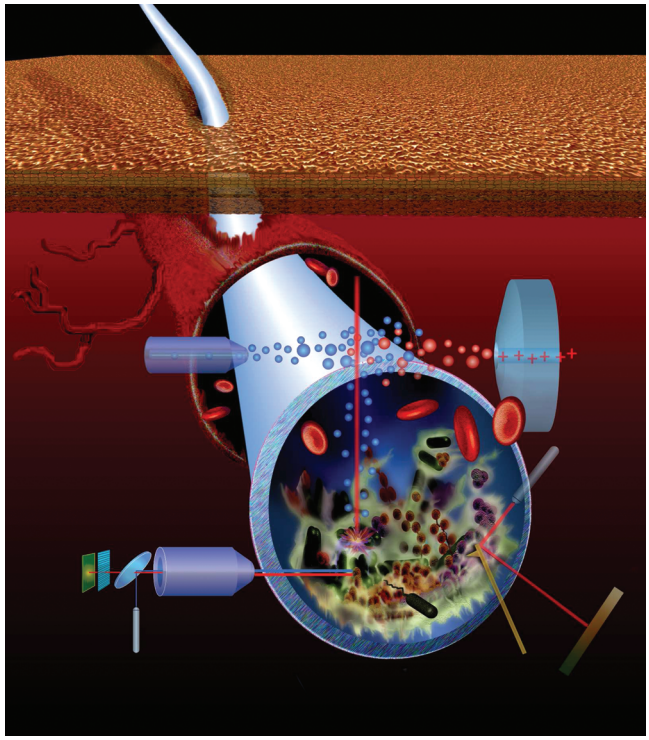
Supporting Information



Robert Gates

The use of medical devices is one of the fastest growing areas of medicine and an increasing source of healthcare associated infections (HAIs). In a recent study, 1.7 million HAIs occurred in the United States in one year, resulting in 99 000 deaths.[1] The costs associated with HAIs were estimated to range from $28−45 billion per year, and medical device associated infections account for upward of 60% of HAIs.[2]

Medical device infections are usually linked to colonization of devices by microbes.[3] Microbial colonization involves at least three complex factors: microorganisms, device, and host environment (tissues, immune cells, etc.) It is often difficult to detect microbial colonization, and in some cases it can go undetected for years, whereas in others it can have life-threatening urgency. The location of a device in the body (Supporting Information, Table S1) can affect the means of colonization. An indwelling device is one that acts as a "bridge" between the nonsterile outside environment and the sterile inside of the patient. Indwelling devices such as urinary catheters are frequently associated with microorganisms which originate from the skin of the patient or healthcare providers (Figure 1A). The longer an indwelling device remains in a patient, the more likely the device interface will be colonized, often by multiple species. For implanted devices, the risk of colonization may be due to other causes such as nonsterile presentation of the device during surgical implantation or hematogenously (blood borne)-derived bacteria from dental caries and urinary tract infections. The first line of defense against colonization of indwelling and implanted devices is the use of sterile techniques when handling and inserting new devices. Once in use, colonization of a medical device surface can be difficult to treat if the bacteria have become resistant to antibiotics, and in many cases successful treatment of persistent infection may require surgical removal of the device.

Many devices that have been explanted after issues with microbial colonization have slimy "biofilm" coatings on them produced by colonizing microbes.[4] These biofilms are self-assembling multicellular communities that behave differently from their free floating (planktonic) counterparts.[5] This Feature will introduce a few of the unique features and challenges of biofilms. In recent years, increased research has led to an improved understanding of biofilms on devices, yet much work remains. Although clinicians are increasingly recognizing biofilms as a medical threat, at present there is no clinical definition for the term biofilm and the quantitative association between biofilm and probability of infection is poorly understood. Medical devices with biofilm resistant technologies such as drug eluting coatings, bactericidal coatings, and adhesion resistant chemistries and nanotopologies are being developed, but it is not always clear how these technologies work *in vivo* or how they affect clinical outcomes.

Better analytical instrumentation is needed to detect and study device colonization and biofilms *in vivo*, and clinically relevant methods are needed for *in vitro* assays of antimicrobial technology. In the sections that follow, we will first discuss basic biofilm biology before delving into analytical methods to detect, quantify, and characterize them.

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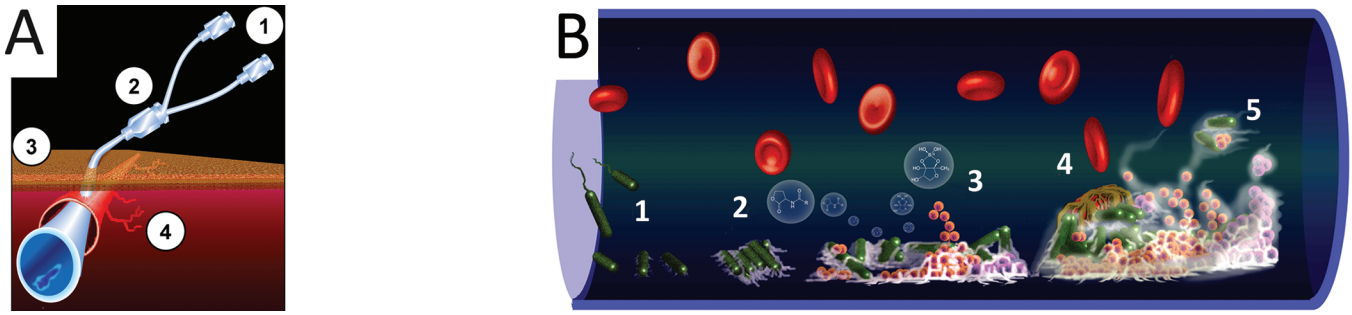

Figure 1. (A) Potential infection sources of a percutaneous intravascular device. Medical devices introduce a vulnerable biointerface into normally well protected organs and vasculature. Contamination can come from (1) infusate (2) from nonsterile catheter materials, (3) the skin, or (4) from distant hematogenous infections. (B) Dynamic biofilm life cycle on a medical device: (1) transport and initial attachment of microbes, (2) irreversible adhesion or attachment, (3) microcolony formation, (4) maturation of the biofilm, and (5) detachment and dispersion of the cells.

BIOFILM BASICS

Microorganisms associated with biofilm infections range from Gram negative bacteria, such as *Pseudomonas aeruginosa*, *Escherichia coli*, and *Proteus mirabilis* to Gram positive, such as *Staphylococcus aureus*. Many of these bacteria are found on the skin (*S. epidermidis* and *S. aureus*), in the water (*E. coli* and *Ps. aeruginosa*), or in improperly cleaned and sterilized equipment, such as infrequently cleaned water lines for ventilators and dental offices. Yeast, such as *Candida albicans* and *C. parapsilosis*, is another common cause of nosocomial infections that can form biofilm on devices and lead to clinical infections.[3]

There are five major stages in biofilm colony formation (Figure 1B): (1) transport and initial attachment of microbes, (2) irreversible adhesion or attachment, (3) microcolony formation, (4) maturation of the biofilm, and (5) detachment and dispersion of the cells.[4−6] In the first stage, planktonic bacteria, single cells that float or swim in a fluid environment, are transported to the biointerface. The first cells adhere to the surface initially through weak, reversible adhesion by van der Waals and electrostatic forces. In stage two, the first cells attract other microorganisms to attach by providing adhesion sites and building the extracellular matrix to hold the biofilm together. Communication between cells occurs via cell signaling molecules and quorum sensing. In the third stage of colony formation, the biofilm grows by a combination of attracting other microorganisms and division of existing cells. The biofilm is considered "mature" (stage four) when it develops both intracellular and intercellular signaling. Finally, cells spread and colonize new surfaces by swarming and seeding-, clumping-, and surface-dispersal.

The viability and growth of adherent microorganisms have a strong dependence on chemical properties at the biointerface such as the type of metal, plastic, etc.[7] The surface texture and the shape of the device, components of the surrounding media (e.g., pH and ionic strength), and prevailing local hemodynamic conditions can also affect bacterial adhesion. In addition to material factors, the rate and extent of biofilm formation on indwelling devices is impacted by biofouling, the number of microorganisms initially contaminating the device, the genus and species of the microorganisms, the biological environment (host serum and platelets, temperature, and circulating drugs), and the host's immune system. The complex interplay of these factors is poorly understood, and statistically significant clinical data is lacking.

BIOCHEMICAL COMPOSITION OF BIOFILMS

Small populations of bacterial cells in isolation are a challenging target for chemical analysis because they contain miniscule volumes of lipids, proteins, nucleic acids, and other biomolecules. In contrast, the extracellular matrix (ECM) produced by a biofilm community can be 10 times the mass of individual cells and is thus an attractive target for chemical detection and quantification methods that are faster and less labor intensive than plating and culturing. Extracellular polymeric substances (EPS) that make up the ECM are insoluble and protect microbial members of the community against desiccation, UV damage, metals, and other harmful molecules. EPS serve as a base for adhesion, a "glue" to keep cells in a community close together, and a scaffold for further growth. The physicochemical properties of the matrix vary depending on the species and environmental conditions such as temperature, shear, and nutrient availability.[8] Biofilm EPS are complex polymers, making them difficult to isolate and characterize. Techniques such as centrifugation, filtration, complexation, and precipitation are adapted uniquely for diverse biofilm compositions. These methods require large or thick biofilm samples and likely introduce sample bias for water-soluble components.[9]

Three main EPS components have been found in biofilms: polysaccharides, DNA, and proteins. While polysaccharides have an important influence on EPS properties, a number of proteins are also identified as EPS and can far exceed polysaccharides on a mass basis. Proteins serve important roles as nonspecific adhesins[10] and compose flagella, pili, and fimbriae. Enzymes and toxic amyloid proteins with cross-β structures are also found ubiquitously.[9] In recent years, DNA in biofilms has also been investigated. A key discovery was the fact that a common enzyme, DNase, is able to degrade many clinical biofilm isolates *in vitro*. The origins of DNA in biofilms is not entirely clear, although it may be from a lysed subpopulation of cells.[8] As cells at the outer layer of a biofilm are killed by environmental factors or drugs, the DNA that remains may become part of the matrix. Other biomolecules found in biofilms include humic substances, lipids, and surfactants.[9]

BIOFILM "COMMUNITY BENEFITS"

Microbes living in a biofilm derive benefits that are not available in planktonic life. These include physicochemical advantages, multispecies synergisms, and rapid gene transfer. Biofilms increase bacterial resistance to antibiotics. The structure of the biofilm itself conveys numerous advantages over an unprotected planktonic cell. At the simplest level, the high density of EPS hinders access of immune system defenses such as

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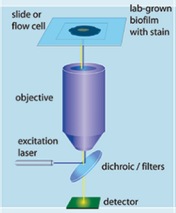
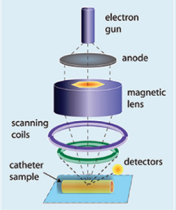
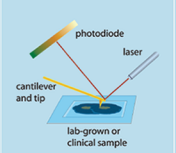
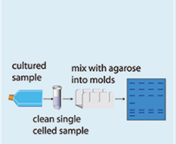
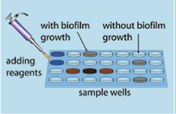
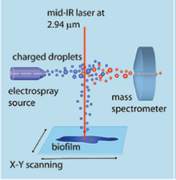
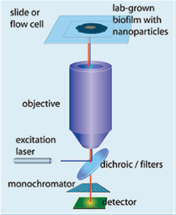
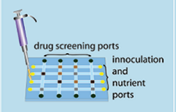
Table 1. Analytical Methods to Explore Colonization and Biofilms

Technique	Schematic	Usage	Benefits	Limitations
Confocal Microscopy		• Lab studies with stains and labels • 3D Profile (confocal laser scanning microscopy) • Diffusion studies	• Sensitive (LOD ~1000 molecules) • High resolution (~0.3 μm) • Works well with flow cells	• Low throughput • Semi-quantitative • Flat, optically transparent
Scanning electron microscopy		• Visualize biofilm on clinical samples • Confirm microscopy on in-vitro tests	• Able to see all bioburden regardless of stain affinity • High x-y resolution (~1 nm)	• Low throughput • Not real-time • May require additional sample prep • Qualitative
Atomic force microscopy		• Explore thickness and morphology of biofilm • Test adhesion forces	• High z-resolution (0.1-1 nm) • Potential to obtain information on forces	• Low throughput • No composition information • Requires open system
Genetic assays		• Clinical diagnostics • Epidemiology • Species determination on wounds and explanted devices	• Potential to give species specific information • More rapid (1-2 days) than culture methods • Finds non-culturables	• Quantitative sampling difficult • No biofilm mass or ECM information • Extensive sample
Microplate assays		• Laboratory screening of biofilm growth	• Potentially higher throughput • Potentially quantitative	• Slower if culturing needed • May not be "realistic"
Mass spectrometry		• Proteomics of biofilm species • Metabolic analysis • Signaling molecules	• 100s to 1000s of compounds identified • High throughput	• Sample preparation cumbersome • Requires lengthy separation
Imaging MS		• Biofilm chemical composition distributions • Living biofilm analysis	• In situ analysis of intact biofilm • Lateral and depth variations can be explored	• Semi-quantitative • Matrix interference • Ion suppression effects
Raman		• Chemical analysis of biofilms • Quantitative mapping and 3D	• Stains or labels not required • Multiple analytes	• Poor sensitivity-may require nanoparticles • Long analysis times
Microfluidics		• High throughput drug screening • Detect signaling molecules • Metabolic profiling	• Small sample requirements (<1μL) • Less biohazard waste (mL vs. L) • Less sample	• No commercial formats • Difficult to test materials (catheter tubing, cement, etc.)

antibodies and macrophages. It also slows the diffusion of antibiotics, possibly giving microbes more time to make genetic and metabolic changes that favor survival.[11] The EPS can bind drugs through sorption or matrix components can chemically react with drugs, resulting in covalent bonds that immobilize the drug and prevent it from reaching its target in cells. Secreted catalase protects microbes against hydrogen peroxide and betalactamase protects microbes against lactam antibiotics.[12] Because of low oxygen and nutrient concentrations deep in the matrix, microbes living there may go into a reduced metabolic state. In the stationary phase, long-lived persister cells appear to be unaffected at concentrations of antibiotics that are far above normal minimum bactericidal concentrations (MBCs).[13]

Clinical investigation of medical device biofilms often shows more than one active species. Gene transfer in biofilms is enhanced and can lead to increased survival, virulence, and even antibiotic resistant strains. In the human body, resistance might be transferred from apathogenic strains found in the oral and intestinal flora to highly virulent strains with which a host is infected.[12] Genetic information can be transferred through conjugative transmission of plasmids (cell−cell contact through specialized pili). A second process, transformation by chromosomal DNA, involves integration via homologous recombination. For this to occur, bacteria must have "natural competence", the ability to enter a genetically programmed state in order to uptake macromolecular DNA from closely related organisms.[14]

MEASURING BIOFILMS AT THE INTERFACE

Analytical chemistry is a crucial tool for understanding biofilms on medical devices. Analytical methods are needed to understand how biofilms form, their biochemical composition, and how much is being formed. For *in vitro* and *in vivo* situations, we need to be able to detect biofilm forming bacteria and their ECM and know how quickly they are forming, how well prophylaxis and antibiofilm technologies work to prevent their proliferation, and the effectiveness of antimicrobial drugs and device coatings. Both conventional and emerging analytical techniques (Table 1) are being employed in these efforts.

Challenges of In Vitro Biofilm Analysis. Compared to the planktonic state of bacteria, biofilms appear at first glance to be an easier target for quantification because their localization on a single colonized surface is similar to a preconcentration step. This perceived advantage, however, evaporates when one considers the complexity and heterogeneity of biofilm structure. The morphology of biofilms presents a challenge for analysis because it falls somewhere between surface-only techniques and bulk material (volume) techniques. Biofilms can vary widely in thickness but are often described on the order of 10−100 μm. Most surface analytical techniques cannot report on the overall composition of these structures when they have thicknesses of up to several hundred micrometers. Conversely, the performance of volume analytical techniques is limited by the low amount of material available in the film.

The complex and rapidly evolving nature of a colony over time is also a challenge for quantification and comparison between samples. In biofilms coexisting populations of microorganisms are held together by an ECM that is a heterogeneous mixture. As the film matures, the composition changes over time and exhibits differences between the developmental stages. Multiple species may be present and the interaction may

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depend on available nutrients and environmental factors. A common problem is that clinical biofilm forming strains become planktonic when cultured repeatedly using techniques that select for planktonic cells. When used in *in vitro* experiments, these strains may no longer produce biofilms with the same properties as their clinical counterpart.

Systematic *in vitro* studies require the reproducible formation of biofilms on medical device surfaces. There are currently four American Society for Testing and Materials (ASTM) standard methods for growing and measuring biofilms: a drip flow reactor (E2647), a flow reactor (E2562), a CDC reactor (E2196), and a modified microplate method (E2799). Each method is used for a different *in vitro* device model. The CDC reactor can hold eight large sample coupons and has been used extensively for testing medical device materials.[15] The microplate method is the first method to incorporate miniaturization for multiplexed studies and relies on an orbital incubator to provide shear flow.[16] The last step of this method requires plating and culturing to obtain quantitative bacterial counts, which is an offline process that will limit the true achievable throughput. While these methods are good for reproducibly growing biofilms, *in vitro* protocols for these formats need to be developed that can better predict biofilm formation on medical devices *in vivo*.

Challenges of Clinical In Situ or In Vivo Biofilm Analysis. The level of difficulty in clinical *in situ* or *in vivo* biofilm analysis depends on the analytical objective. For some cases, such as colonization of an easily exchangeable medical device, it is sufficient to remove the device and directly sample for the presence of a biofilm *in situ*. Under these circumstances, analytical methods can be developed to detect abundant ECM components, such as polysaccharides, proteins derived from bacterial cell appendages, DNA, or even signaling molecules. For implanted devices, obtaining access to the biointerface *in vivo* is more difficult and clinicians currently rely on diagnostic imaging or body fluid/tissue samples for signs of inflammation and infection to determine if the device needs to be explanted.

More challenging than just detection of biofilm is an in-depth biochemical analysis of clinical samples, which requires surveying, identification, and quantitation of the major microorganism subpopulations in the biofilm. Such an endeavor needs to be undertaken for all major device biofilms and will likely require combined use of multiple analytical methods.

Microscopy in Research and Explanted Device Investigations. Microscopy is a commonly used tool for analyzing structural details of biofilms *in vitro* (in flow cells and on coupons).[17] Confocal laser scanning microscopy (CLSM) with fluorescent stains, antibodies, and lectins is ideal to characterize biofilms up to ∼60 μm thickness. Scanning electron microscopy (SEM) has been used to verify that other types of assays are correlated with a real physical change in morphology, density, and substructures of biofilms. An important limit of SEM is that sample preparation for high vacuum involves fixing the cells, which may damage intricate biofilm structures. Cryo-SEM and environmental SEM (ESEM) have been used to study unfixed biofilms without collapse of the extracellular matrix. Ruthenium red, which binds strongly to negatively charged polysaccharides, can provide increased contrast in ESEM.[18] Atomic force microscopy (AFM) is also a useful tool for measuring physical properties of biofilms. It has been used for monitoring bacterial adhesion on different surfaces, interactions between cells, and measuring the strength of adhesion by bacterial adhesins and other macromolecules. Together with SEM, it can provide additional topological information about the biofilm ECM.

There are a number of other imaging methods, including magnetic resonance imaging and scanning transmission X-ray microscopy, that are being used for successful assessment of biofilms.[19]

Genetic Assays for Clinical Diagnostics and Epidemiology. Clinical investigators favor genetic assays because they are a relatively universal format, and the information obtained on rDNA allows for extensive epidemiological characterization. The genes for virulence traits, toxins, adhesins, and antibiotic resistance are most commonly targeted. This type of information is an advantage in device-related infections because it can help distinguish epidemic strains from less threatening ones. Pulsed field gel electrophoresis (PFGE) is the gold standard for genetic assays but suffers from lack of reproducibility between laboratories.[20] Polymerase chain reaction (PCR) is one of the most commonly used methods in clinical settings and has about 63−100% sensitivity (a value used in clinical diagnostics that is related to a test's ability to identify positive results) for detecting prosthetic joint infections. Drawbacks of PCR are low specificity, a high false positive rate, and challenges with contamination.[21] Fluorescence *in situ* hybridization (FISH) is another commonly used method. While FISH allows for detection of multiple species biofilms, the preparation procedure is quite extensive and the dehydration may result in poor quantitation. Other genetic methods used include ribotyping, high-resolution melting analysis, DNA sequencing, and DNA arrays.[20] One potential drawback of using genetic information to measure biofilms universally is that it is unlikely that a single genetic factor can be correlated with biofilm growth. For this reason it is less appropriate for quantifying differences between biofilm growth on different materials or measuring the effect of antibiofilm technologies.[22] In cases where rapid results are not necessary, conventional plating methods are also less expensive and more reliable than genetic assays.

Mass Spectrometry for Sample Analysis and Imaging. Various forms of mass spectrometry (MS) (Figures 2 and 3) have the right combination of sensitivity and selectivity to contribute to laboratory and explanted device analysis and potentially clinical diagnostics. Detection modalities can be broken down into bulk sample analysis and imaging techniques.

For bulk sample analysis, diagnostics (detection of the presence of species specific biofilms) might be achieved by identifying a set of biomarkers, whereas in-depth analysis requires the use of the systematic methods of proteomics and metabolomics. The potential to use MS for detection of biofilm forming bacteria and their ECM materials in patient samples is enticing. Extensive efforts have been made to develop low mass biomarkers for planktonic microorganisms. Pyrolysis and protein fingerprints have even been identified using matrix-assisted laser desorption ionization (MALDI) in the high mass region.[23] Unfortunately, because of the presence of different phenotypes and the extensive amount of ECM in biofilms, these approaches have not yet directly translated into a universal laboratory diagnostic test. On the other hand, conventional MS-based proteomics and metabolomic methods are well suited to tackle the complex challenges of microbial biochemistry. Proteomic methods lend themselves well to the analysis of gene expression and post-translational modifications in biofilms. MS-based proteomics methods have been used to assess changes in protein levels at different biofilm stages,[24] to

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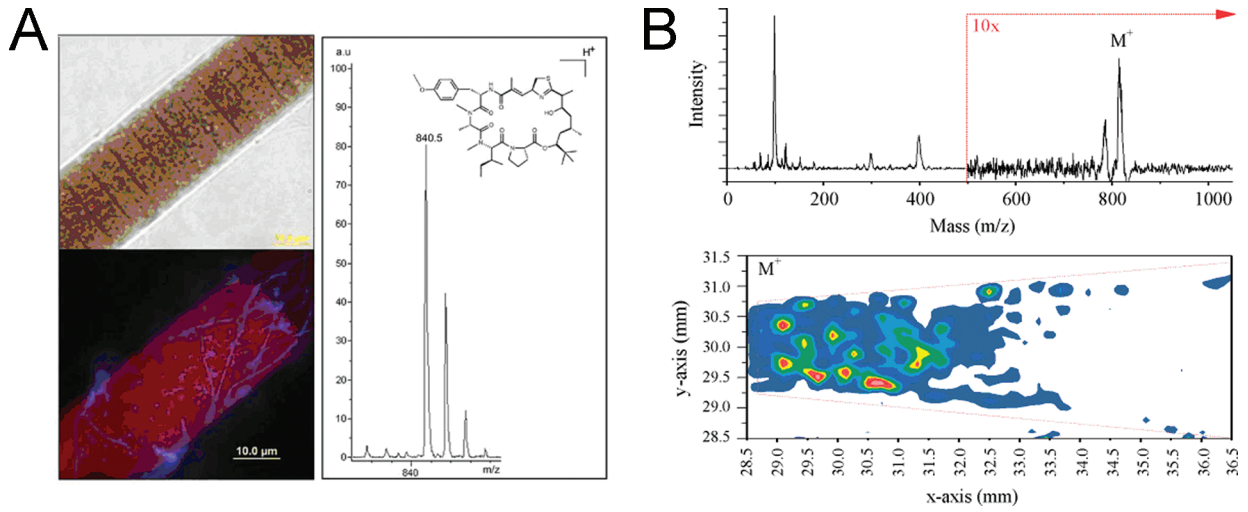

Figure 2. (A) Bright field and fluorescence microscope images (left) and a region of the MALDI mass spectrum with m/z 840 molecular ion (right) of *Lyngbya bouillonii* filaments show the production of apratoxin A.[29] (B) Diffusion of rifampicin in *Staphylococcus epidermidis* biofilms followed by LDPI-MS (top). The amplified section of the mass spectrum shows the rifampicin molecular ion at m/z 822. The image (bottom) indicates the diffusion of rifampicin from the left edge.[33]

identify overexpressed proteins,[25] and indirectly to measure genetic exchange.[26] In metabolomics, mass spectrometry is well suited to identify and quantitate a large percentage of the typical microbe metabolome (500−1 000 distinct chemical species). For example, the consensus metabolic network of *Saccharomyces cerevisiae*, iMM904, consists of 8 compartments, 713 compartment-specific metabolites, and 1402 reactions.[27] MS has also been used in combination with separation techniques to identify metabolites and networks in microorganisms. Using MS to study the colocalization of secondary metabolites is especially useful for identifying natural product drug candidates. More specific studies have taken advantage of unique MS methods such as stable isotope labeling; for example, to look at how bacteria enter dormant growth modes in biofilms during starvation.[28] A combination of fluorescence microscopy and MALDI-MS has revealed the presence of a secondary metabolite and potential cancer cell toxin, apratoxin A, in filamentous cyanobacteria (see Figure 2A).[29]

Currently there are over half a dozen MS modalities available for biofilm imaging, including secondary ion mass spectrometry (SIMS),[30] MALDI, laser desorption with postionization (LDPI), desorption electrospray ionization (DESI), and laser ablation electrospray ionization (LAESI). They are based on the use of primary photon (MALDI, LDPI, and LAESI) or charged particle beams (SIMS and DESI) for sampling and ion production with the optional addition of ionization enhancement via intercepting the produced neutrals by high energy photons (vacuum-UV from laser or synchrotron sources for LDPI) or highly charged particles (electrospray in the case of LAESI). The application of imaging MS to biofilms is a relatively new area of research with most studies focusing on the performance characteristics of the different methods.

Two performance metrics by which MS imaging methods can be compared are spatial resolution and analyte molecular mass limitations. Better spatial resolution allows for clear images of small biofilm structures, whereas the mass limitation describes the uppermost molecular weight limit of biofilm molecules that can be detected. The capabilities of these techniques in terms of spatial resolution and the accessible molecular classes are, in part, complementary. Ion beams can be focused to less than ∼0.05 μm enabling subcellular resolution for SIMS MS,[31] whereas for MALDI and LDPI with low beam divergence and aspherical focusing optics a close to diffraction limited resolution below ∼1 μm can be achieved.[32] In practice, however, because of relatively large matrix crystal sizes, diffusion during sample preparation and to keep the acquisition time at acceptable levels, MALDI imaging is mostly performed with ∼100 μm resolution. For LDPI-MS, the 349 nm Nd:YLF desorption laser beam has a focal diameter of 20 μm for postionization with an excimer laser operating with fluorine gas at 157 nm[33] (Figure 2B) or 300 μm for postionization with a synchrotron in the vacuum-UV.[34] Because of the significant divergence of mid-IR laser beams used in LAESI, focusing and thus the spatial resolution of imaging is limited to ∼200 μm. This limitation can be circumvented by the application of a sharpened optical fiber for the delivery of the laser pulse to ablate cells within a ∼30 μm spot.[35] In DESI, the spatial resolution is determined by the size of the most active area within the spray where most of the sample ions are produced.

Molecular mass limitations for the imaged compounds tend to be most rigorous for the instruments with the highest spatial resolution. For SIMS, the upper limit is 300 Da for conventional primary ions, whereas it is ∼1 500 for the novel cluster ion sources. Many of the single microbial cell analysis and imaging experiments rely on stable isotope labeling. Postionization with vacuum-UV in LDPI is also limited to molecular masses up to ∼1 000 Da. DESI extends the high mass limit for detection to ∼66 kDa,[36] whereas MALDI and LAESI can produce ions up to ∼100 kDa. The latter has demonstrated lateral imaging,[37] depth profiling,[38] as well as 3D imaging.[35]

Because of the relatively recent introduction of imaging MS to biofilm analysis, the strengths and weaknesses of the various approaches are still being explored. Quantitation of the imaged molecular species remains a key challenge.

Living Cell Analysis with Mass Spectrometry. The development of new atmospheric pressure or ambient MS ion sources such as desorption electrospray ionization (DESI) and

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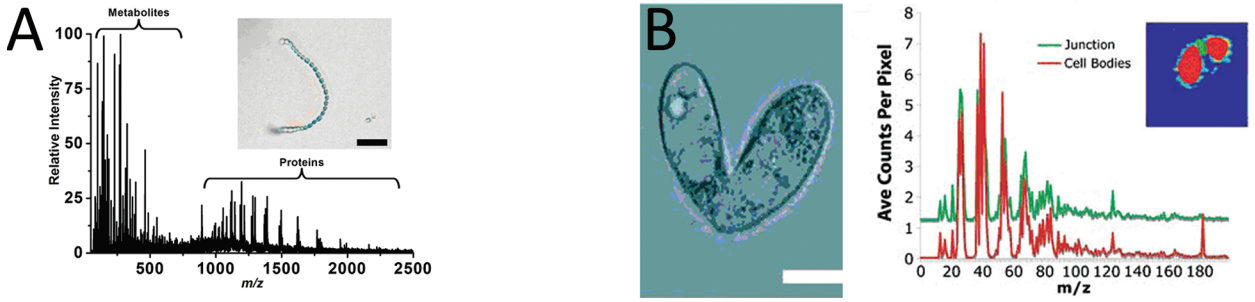

Figure 3. (A) *In situ* mass spectrum of a very small colony ($n < 616 \pm 76$) from functioning *Anabaena* sp. PCC7120 cyanobacteria produced by the LAESI ambient ionization method reveals the composition of the phycobiliprotein complex in the antennae. A cyanobacterial filament is shown in the inset with a 20 μm scale bar.[41] (B) Mating *Tetrahymena thermophila* pair viewed by a differential interference contrast microscope (left, scale bar is 25 μm) and analyzed through SIMS (right). Mass spectra and the image in the inset show the differences in the distributions of phosphocholine and 2-aminoethylphosphonolipid between the cell bodies and the cell-to-cell junction.[42]

direct analysis in real time (DART) offers the ability to study biofilms in their native state. The ability to directly probe microbial systems using some of these approaches has been demonstrated. Imaging by DESI MS was used to explore the distribution of allelochemicals and other secondary metabolites in bacterial cultures,[39] while the relatively scarcely studied microbial volatiles lend themselves to analysis by DART.[40] LAESI MS has been proposed for the investigation of lateral and depth heterogeneity of biofilms. For example, the subunit composition of phycobilisomal antenna proteins and numerous metabolites from a very small population ($n < 616$) of *Anabaena* sp. PCC7120 cyanobacteria were determined by LAESI MS (Figure 3A).[41]

Because of the lack of chemical species amplification in mass spectrometric approaches, the analysis of single cells is volume limited. Hundreds of components in plant or large animal cells (>50 μm) can be directly analyzed, but the analysis of a bacterial cell, typically <1 μm or 0.5 fL volume, by MS remains a challenge. On the basis of molecular ions or their fragments, currently only SIMS and matrix enhanced SIMS are capable of submicrometer resolution chemical imaging enabling the analysis of individual microbial cells and subcellular components. In one notable example, structural changes in the cell membrane of mating *Tetrahymena thermophila* were recognized by time-of-flight SIMS measurements (Figure 3B).[42] With the emergence of enhanced sensitivity techniques, continued development in the field of single cell MS is expected.

Raman Spectroscopic Imaging. In addition to methods already proven for biofilm analysis, there are a number of other analytical technologies with great potential. These technologies can provide new information about biofilms that was either difficult to obtain or simply not possible with current techniques. For example, CLSM is limited because of the need for fluorescent stains. Only a few stains can be used simultaneously, resulting in analysis of 1−2 biofilm components at most. Raman microscopy can overcome this limitation because it provides a spectrum of information related to chemical bonds in a format similar to CLSM. Numerous biofilm chemical groups can potentially be analyzed by Raman. Ideally, Raman might be used to identify biofilms from different species or strains by identification of a chemical fingerprint. One example of this is a group developing Raman-based identification of *S. epidermis* biofilms[43] that was able to distinguish between two strains of *S. epidermis* using principal component analysis. The sensitivity of Raman is a major limitation, and several groups have used surface enhanced Raman spectroscopy (SERS) to improve the analysis of biofilms using two different approaches. In the simplest method, the observation chamber is filled with a colloidal suspension of hydroxylamine hydrochloride reduced silver colloids.[44] Using this strategy, the investigators were able to obtain greatly enhanced signal compared to unenhanced Raman and monitor the biofilm growth for weeks. They identified Raman bands for carbohydrates, proteins, DNA/RNA, carotenoids, and lipids in the biofilm matrix. The limitation of this method is that the Raman signal enhancement is limited to the surface of the biofilm near the colloids. Because of the complex three-dimensional nature of biofilms, the method may oversimplify biofilm measurements. Another strategy is to coculture biofilms with the nanoparticles, allowing surface enhancement throughout the depth of the biofilm (Figure 4).[45]

Microarrays and Microfluidics. Microarrays and microfluidics are changing the way in which biological research is performed, and the field of biofilms is no exception. Microarray tools borrowed from the "omics" are enabling higher throughput testing. A slide based array with 768 distinct

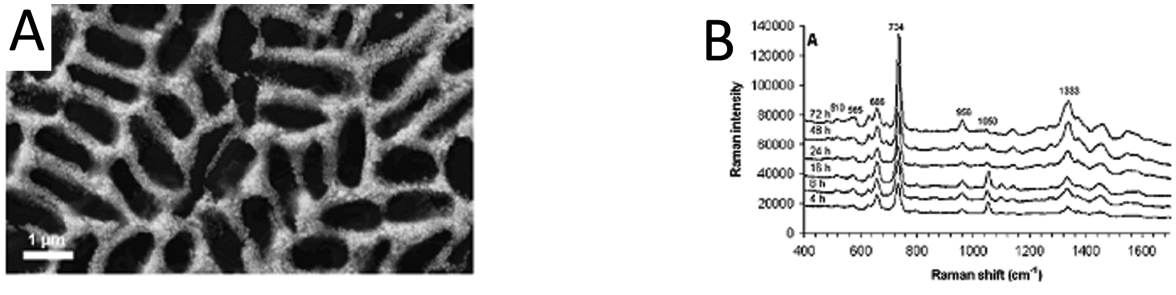

Figure 4. (A) SEM image of *E. coli* biofilm prepared with silver nanoparticles.[45] (B) Time dependent surface enhanced Raman spectra obtained from *E. coli* cultivated with nanoparticles.[45]

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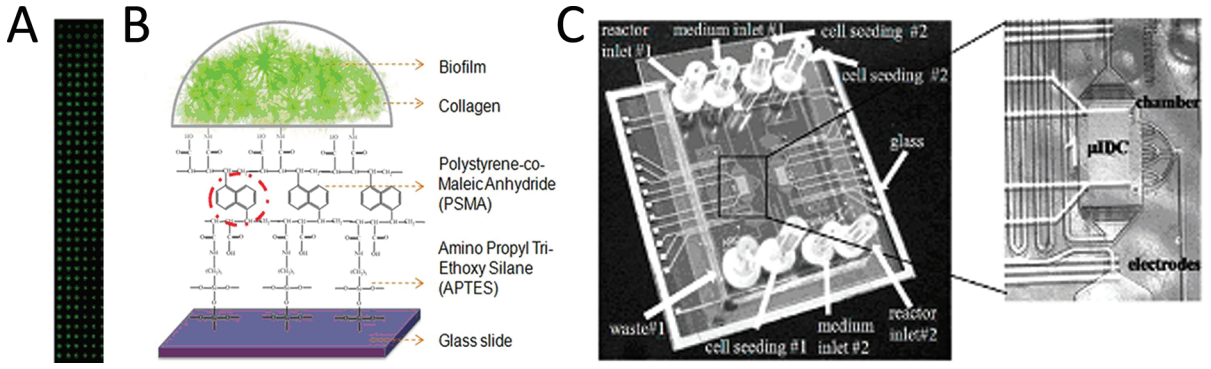

Figure 5. (A) Far left and center left: Crop from fluorescence scan of 768 spot array of fungal biofilms stained with FUN 1.[46] (B) Surface chemistry used to make collagen coated arrays on glass slides for biofilm growth.[46] (C) Photo of microchip for cell population measurements. Blow-up shows proliferation chamber with electrochemical sensors.[50]

biofilm patches was developed to monitor fluorescence changes in response to antifungal compounds (Figure 5A,B).[46] The method takes advantage of established surface chemistry and arraying techniques to fabricate collagen patches on which biofilms form.

Microfluidics can be used to observe biofilms, measure some physical parameter, or apply physical/chemical stimuli and capture response characteristics. Semimicrofluidic flow cells are commonly used for cultivation of biofilms. More advanced culture, such as simultaneous coculture of osteoblasts and bacteria, has also been performed in a semimicrofluidic 3D tissue model to better mimic the *in vivo* environment of orthopedic implants.[47] Quorum sensing molecules are easier to detect in small volumes because of limited diffusive dilution, so detection is a good match for microfluidic chambers.[48] Although many microfluidics are closed channel format, open-channel microfluidic devices may be necessary for some biofilm analysis. Open channels allow for combination of microfluidics with powerful instrumental analysis methods such as imaging MS or synchrotron infrared spectromicroscopy[49] to chemically image biofilm dynamics in real time. More sophisticated "lab-on-a-chip" devices with multiple layers and integrated electronics are able to measure multiple physical properties of biofilms. For example, bacterial population dynamics were measured in a device with both optical detection of population numbers and electrochemical measurement of respiration (Figure 5C).[50]

Biofilms have also been probed on microchips with physical and chemical methods. Exposure to gradients generated in microchips allows for instantaneous evaluation of a continuous range of concentrations. Bacteria have been exposed to oxygen gradients generated on-chip and screened for a response.[51] The ability to create unique cellular size-scale architecture also allows for testing of biofilm physical parameters such as adhesion forces, which were studied by varying the flow in a microfluidic device.[52]

FUTURE DIRECTIONS

Despite increasing use of antimicrobials and attention to sterility, microbial threats are not subsiding. Long-term, drug resistance is developing faster than we can invent new drugs.[53] Microbial colonization and the resulting biofilms are a major source of drug resistance, and the biointerface of many medical devices is a vulnerable target.[54]

Detection of biofilms *in vivo* has benefited from modern molecular methods, but there is still great need for more rapid and reliable analysis. Recently developed amplification strategies and instrumental methods could potentially increase the sensitivity and specificity of current clinical tests. Microfluidics can decrease sample requirements and make these techniques more affordable and reliable by integrating on-chip sample preparation and handling. Current molecular diagnostics are limited by the fact that they require sample fluid or tissue, which may not correlate with biofilm on the surface of implanted medical devices. Molecular genomics can hint at the potential of a specific strain based on genetic expression, but they do not prove that the strain is alive and thriving or identify the type and amount of biofilm. If analytical chemists can develop chemical analysis for aspirates from around an implant, it may be possible to confirm biofilm growth more directly without an invasive surgery or removal of the device. This Feature mentions some potential target analytes, such as components of the ECM or signaling molecules produced by colonizing microbes.

For *in situ* analysis of biofilms on explanted devices, the burgeoning development in mass spectrometric methods holds great promise. As new atmospheric pressure techniques are perfected and become commercially available, investigators may be able to perform chemical imaging of live clinical biofilms in order to elucidate the variety and complexity of multispecies communities on a large scale (biochemical architecture) and at the single cell and subcellular level (chemical cytometry).

For *in vitro* analysis, there are many existing techniques ranging from microscopy to microwell assays. Because most of these methods are highly refined, the limits of *in vitro* analysis are related to realism and reproducibility rather than instrumental performance. In particular, relating outcomes of *in vitro* assays to *in vivo* colonization and infection is a major challenge. Multivariate analysis could be used to unravel the numerous variables and make more reliable *in vitro*–*in vivo* correlations. These efforts require standard methods to grow reproducible biofilms and test a multitude of variables related to device, microorganism, and host environment. Microfluidics may play a key role in helping increase the throughput of *in vitro* testing and minimizing the time and cost associated with bacterial culture and plating on a large scale. Because its scale is similar to that of individual cells, the microfluidic environment may also present unique advantages to probe cells and detect low concentrations of analytes.

Increased understanding of the unique scientific, clinical, and regulatory challenges of medical device biofilms will help spur much needed innovation in the field. There are many opportunities for analytical chemists to play a crucial role in this endeavor.

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■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

Biography

Akos Vertes is a Professor of Chemistry and a Professor of Biochemistry and Molecular Biology at the George Washington University in Washington, DC. He is a cofounder and codirector of the W. M. Keck Institute for Proteomics Technology and Applications, a center of strategic excellence at the university. His research interests span from fundamental studies in analytical and physical chemistry to the development of new technologies for biomedical analysis. Recent accomplishments include in situ analysis of single cells and subcellular compartments, the introduction of a new ambient ion source (laser ablation electrospray ionization), and the discovery of photonic ion sources. Vicki Hitchins is a Research Microbiologist in the Division of Biology, Office of Science and Engineering Laboratories (OSEL), Center for Devices and Radiological Health (CDRH), Food and Drug Administration (FDA). She has worked for the FDA since 1980 reviewing device submissions and is involved in drafting national/international standards for sterilization of medical devices. She has over 30 research publications dealing with infections associated with medical devices and nanotechnology. Hitchins received a Ph.D. in Microbiology from Michigan State University and was an NIH Postdoctoral Fellow in Biochemical Sciences (Princeton University) and Biochemistry (University of Kentucky). Lieutenant Commander K. Scott Phillips is a commissioned officer in the United States Public Health Service (PHS) assigned to FDA in the Division of Chemistry and Materials Science, OSEL, CDRH. He performs regulatory research to ensure the safety and effectiveness of medical devices. Phillips has more than 10 years experience as an analytical chemist developing microfluidic biosensors and is now applying these technologies to solve regulatory science challenges at the biointerface of medical devices.

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■ REFERENCES

(1) Klevens, R. M.; Edwards, J. R.; Richards, C. L.; Horan, T. C.; Gaynes, R. P.; Pollock, D. A.; Cardo, D. M. *Public Health Rep.* **2007**, *122*, 160−166.

(2) Scott, R. D. *The direct medical costs of healthcare-associated infections in U.S. hospitals and the benefits of Prevention*; CDC, March 2009.

(3) Estivill, D.; Arias, A.; Torres-Lana, A.; Carrillo-Munoz, A. J.; Arevalo, M. P. *J. Microbiol. Methods* **2011**, *86*, 238−242.

(4) Hall-Stoodley, L.; Costerton, J. W.; Stoodley, P. *Nat. Rev. Microbiol.* **2004**, *2*, 95−108.

(5) Costerton, J. W.; Stewart, P. S.; Greenberg, E. P. *Science* **1999**, *284*, 1318−1322.

(6) Bryers, J. D. *Biotechnol. Bioeng.* **2008**, *100*, 1−18.

(7) Darouiche, R. O. *Clin. Infect. Dis.* **2001**, *33*, 1567−1572.

(8) Dickschat, J. S. *Nat. Prod. Rep.* **2010**, *27*, 343−369.

(9) Flemming, H. C.; Wingender, J. *Nat. Rev. Microbiol.* **2010**, *8*, 623−633.

(10) Karunakaran, E.; Biggs, C. A. *Appl. Microbiol. Biotechnol.* **2011**, *89*, 1161−1175.

(11) Jefferson, K. K.; Goldmann, D. A.; Pier, G. B. *Antimicrob. Agents Chemother.* **2005**, *49*, 2467−2473.

(12) Fux, C. A.; Costerton, J. W.; Stewart, P. S.; Stoodley, P. *Trends Microbiol.* **2005**, *13*, 34−40.

(13) Mah, T. F. C.; O'Toole, G. A. *Trends Microbiol.* **2001**, *9*, 34−39.

(14) Molin, S.; Tolker-Nielsen, T. *Curr. Opin. Biotechnol.* **2003**, *14*, 255−261.

(15) Donlan, R. M.; Piede, J. A.; Heyes, C. D.; Sanii, L.; Murga, R.; Edmonds, P.; El-Sayed, I.; El-Sayed, M. A. *Appl. Environ. Microbiol.* **2004**, *70*, 4980−4988.

(16) Ceri, H.; Olson, M. E.; Stremick, C.; Read, R. R.; Morck, D.; Buret, A. *J. Clin. Microbiol.* **1999**, *37*, 1771−1776.

(17) Hannig, C.; Follo, M.; Hellwig, E.; Al-Ahmad, A. *J. Med. Microbiol.* **2010**, *59*, 1−7.

(18) Priester, J. H.; Horst, A. M.; Van De Werfhorst, L. C.; Saleta, J. L.; Mertes, L. A. K.; Holden, P. A. *J. Microbiol. Methods* **2007**, *68*, 577−587.

(19) Neu, T. R.; Manz, B.; Volke, F.; Dynes, J. J.; Hitchcock, A. P.; Lawrence, J. R. *FEMS Microbiol. Ecol.* **2010**, *72*, 1−21.

(20) Campoccia, D.; Montanaro, L.; Arciola, C. R. *Int. J. Artif. Organs* **2009**, *32*, 642−654.

(21) Peel, T. N.; Buising, K. L.; Choong, P. F. M. *Anz J. Surg.* **2011**, *81*, 32−39.

(22) Arciola, C. R.; Campoccia, D.; Baldassarri, L.; DoNat.i, M. E.; Pirini, V.; Gamberini, S.; Montanaro, L. *J. Biomed. Mater. Res., Part A* **2006**, *76A*, 425−430.

(23) Fenselau, C.; Demirev, P. A. *Mass Spectrom. Rev.* **2001**, *20*, 157−171.

(24) Sauer, K.; Camper, A. K.; Ehrlich, G. D.; Costerton, J. W.; Davies, D. G. *J. Bacteriol.* **2002**, *184*, 1140−1154.

(25) Ram, R. J.; VerBerkmoes, N. C.; Thelen, M. P.; Tyson, G. W.; Baker, B. J.; Blake, R. C.; Shah, M.; Hettich, R. L.; Banfield, J. F. *Science* **2005**, *308*, 1915−1920.

(26) Lo, I.; Denef, V. J.; VerBerkmoes, N. C.; Shah, M. B.; Goltsman, D.; DiBartolo, G.; Tyson, G. W.; Allen, E. E.; Ram, R. J.; Detter, J. C.; Richardson, P.; Thelen, M. P.; Hettich, R. L.; Banfield, J. F. *Nature* **2007**, *446*, 537−541.

(27) Herrgard, M. J.; Swainston, N.; Dobson, P.; Dunn, W. B.; Arga, K. Y.; Arvas, M.; Bluthgen, N.; Borger, S.; Costenoble, R.; Heinemann, M.; Hucka, M.; Le Novere, N.; Li, P.; Liebermeister, W.; Mo, M. L.; Oliveira, A. P.; Petranovic, D.; Pettifer, S.; Simeonidis, E.; Smallbone, K.; Spasic, I.; Weichart, D.; Brent, R.; Broomhead, D. S.; Westerhoff, H. V.; Kirdar, B.; Penttila, M.; Klipp, E.; Palsson, B. O.; Sauer, U.; Oliver, S. G.; Mendes, P.; Nielsen, J.; Kell, D. B. *Nat. Biotechnol.* **2008**, *26*, 1155−1160.

(28) Bajad, S. U.; Lu, W. Y.; Kimball, E. H.; Yuan, J.; Peterson, C.; Rabinowitz, J. D. *J. Chromatogr., A* **2006**, *1125*, 76−88.

(29) Grindberg, R. V.; Ishoey, T.; Brinza, D.; Esquenazi, E.; Coates, R. C.; Liu, W. T.; Gerwick, L.; Dorrestein, P. C.; Pevzner, P.; Lasken, R.; Gerwick, W. H. *PLoS One* **2011**, *6*, e18565.

(30) Wagner, M. *Annu. Rev. Microbiol.* **2009**, *63*, 411−429.

(31) Guerquin-Kern, J. L.; Wu, T. D.; Quintana, C.; Croisy, A. *Biochim. Biophys. Acta: Gen. Subj.* **2005**, *1724*, 228−238.

(32) Spengler, B.; Hubert, M. *J. Am. Soc. Mass Spectrom.* **2002**, *13*, 735−748.

(33) Akhmetov, A.; Moore, J. F.; Gasper, G. L.; Koin, P. J.; Hanley, L. *J. Mass Spectrom.* **2010**, *45*, 137−145.
(34) Gasper, G. L.; Takahashi, L. K.; Zhou, J.; Ahmed, M.; Moore, J. F.; Hanley, L. *Anal. Chem.* **2010**, *82*, 7472−7478.
(35) Shrestha, B.; Patt, J. M.; Vertes, A. *Anal. Chem.* **2011**, *83*, 2947−2955.
(36) Shin, Y.-S.; Drolet, B.; Mayer, R.; Dolence, K.; Basile, F. *Anal. Chem.* **2007**, *79*, 3514−3518.
(37) Nemes, P.; Barton, A. A.; Li, Y.; Vertes, A. *Anal. Chem.* **2008**, *80*, 4575−4582.
(38) Nemes, P.; Woods, A. S.; Vertes, A. *Anal. Chem.* **2010**, *82*, 982−988.
(39) Watrous, J.; Hendricks, N.; Meehan, M.; Dorrestein, P. C. *Anal. Chem.* **2010**, *82*, 1598−1600.
(40) Pierce, C. Y.; Barr, J. R.; Cody, R. B.; Massung, R. F.; Woolfitt, A. R.; Moura, H.; Thompson, H. A.; Fernandez, F. M. *Chem. Commun.* **2007**, *8*, 807−809.
(41) Parsiegla, G.; Shrestha, B.; Carriere, F.; Vertes, A. *Anal. Chem.* **2012**, *84*, 34−38.
(42) Kurczy, M. E.; Piehowski, P. D.; Van Bell, C. T.; Heien, M. L.; Winograd, N.; Ewing, A. G. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 2751−2756.
(43) Samek, O.; Al-Marashi, J. F. M.; Telle, H. H. *Laser Phys. Lett.* **2010**, *7*, 378−383.
(44) Ivleva, N. P.; Wagner, M.; Szkola, A.; Horn, H.; Niessner, R.; Haisch, C. *J. Phys. Chem. B* **2010**, *114*, 10184−10194.
(45) Kahraman, M.; Keseroglu, K.; Culha, M. *Appl. Spectrosc.* **2011**, *65*, 500−506.
(46) Srinivasan, A.; Uppuluri, P.; Lopez-Ribot, J.; Ramasubramanian, A. K. *PLoS One* **2011**, *6*, e19036.
(47) Lee, J. H.; Wang, H. J.; Kaplan, J. B.; Lee, W. Y. *Tissue Eng., Part C: Methods* **2011**, *17*, 39−48.
(48) Janakiraman, V.; Englert, D.; Jayaraman, A.; Baskaran, H. *Ann. Biomed. Eng.* **2009**, *37*, 1206−1216.
(49) Holman, H.-Y. N.; Miles, R.; Hao, Z.; Wozei, E.; Anderson, L. M.; Yang, H. *Anal. Chem.* **2009**, *81*, 8564−8570.
(50) Gottschamel, J.; Richter, L.; Mak, A.; Jungreuthmayer, C.; Birnbaumer, G.; Milnera, M.; Bruckl, H.; Ertl, P. *Anal. Chem.* **2009**, *81*, 8503−8512.
(51) Skolimowski, M.; Nielsen, M. W.; Emneus, J.; Molin, S.; Taboryski, R.; Sternberg, C.; Dufva, M.; Geschke, O. *Lab Chip* **2010**, *10*, 2162−2169.
(52) De la Fuente, L.; Montanes, E.; Meng, Y. Z.; Li, Y. X.; Burr, T. J.; Hoch, H. C.; Wu, M. M. *Appl. Environ. Microbiol.* **2007**, *73*, 2690−2696.
(53) WHO. Global Strategy for Containment of Antimicrobial Resistance, 2001; http://www.who.int/drugresistance/WHO_Global_Strategy_English.pdf
(54) Reid, G. *Int. J. Antimicrob. Agents* **1999**, *11*, 223−226.

Indwelling device	Example materials	Example species	Example colonization outcomes	Example antimicrobial
urinary catheter	• silicone • latex	• E. Coli	• blockage from thick films • urinary tract infection (CAUTI) • bacteruria/ funguria (CABF)	• silver • nitrofurazone • catheter lock
central venous catheter	• PVC • polyethylene • polyurethane • silicone	• Staphylococci • S. Aureus • C. Albicans	• blood infection (CRBSI)	• silver • chlorhexidine • benzalkonium chloride
dialysis equipment	• silicone tubing	• Pseudomonas Spp. • Staphylococci	• endotoxin exposure	• silver
contact lens	• polymer hydrogels	• P. Aeruginosa • Fusarium	• microbial keratitis	

Implanted device	Example Materials	Example Species	Example Colonization Outcomes	Example Antimicrobial
vascular system (artificial heart valves, vascular grafts, pacemakers)	• teflon • metals • polyester • pyrolytic carbon	• S. Aureus • S. Epidermis • Streptococcus Spp. • E. Coli	• healing complications	• silver • drug releasing • chlorhexidine
orthopedic implants (hip and knee replacements)	• metals • polyethylene	• S. Aureus	• chronic infection	• drug releasing • silver

Table S1. Some indwelling (top) and implanted (bottom) devices with example species, possible outcomes, and antimicrobial technologies.[5,12]