Review of immobilized antimicrobial agents and methods for testing

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Antimicrobial surfaces for food and medical applications have historically involved antimicrobial coatings that elute biocides for effective kill in solution or at surfaces. However, recent efforts have focused on immobilized antimicrobial agents in order to avoid toxicity and the compatibility and reservoir limitations common to elutable agents. This review critically examines the assorted antimicrobial agents reported to have been immobilized, with an emphasis on the interpretation of antimicrobial testing as it pertains to discriminating between eluting and immobilized agents. Immobilization techniques and modes of antimicrobial action are also discussed. © *2011 American Vacuum Society*. [DOI: 10.1116/1.3645195]

I. INTRODUCTION

Approximately two million cases of hospital associated infections (HAIs) occur each year in the United States. These infections, which involve bacterial, fungal, and viral agents, contribute to approximately one hundred thousand deaths annually.[1] Bacterial and fungal pathogens often enter patients via invasive elements employed in supportive measures such as intubation, intravascular lines, and urinary catheters.[1] The frequency, severity, and cost of HAIs have driven the development and implementation of increasingly involved and rigorous aseptic, disinfection, and sterilization procedures. In addition to improving best aseptic practices in clinical settings, medical device makers are introducing devices with antimicrobial and antifouling properties as part of overall infection control technologies designed to help reduce HAIs.[2]

Medical applications of antimicrobial agents have leveraged soluble agents[3–7] such as benzalkonium chlorides, cetylpyridinium chloride, aldehydes, anilides, diamidines, silver, chlorhexidine, triclosan, N-halamines, and povidone-iodine. Although such agents are known to be efficacious and appropriate for specific applications, their extension to some medical device applications might be hindered by the elution of the agent, due to a limited reservoir capacity or potential side effects caused by unwanted exposure. Irreversible immobilization of the antimicrobial agents in the device offers an alternative motif that eliminates patient exposure to elutable active agents and potentially increases the duration of antimicrobial efficacy.[8,9]

A wide range of antimicrobial agents have been immobilized, including small molecules (e.g., quaternary ammonium silanes),[10–17] quaternary ammonium polymers,[18–35] polyamines,[36–42] chitosan,[43–49] enzymes,[50–55] peptides, and peptide mimetics.[56–65] These agents have been immobilized on a host of surfaces, including metals, plastics, and natural and man-made fabrics.

The scope of this review includes literature wherein the authors demonstrate immobilization and use some methods to demonstrate efficacy with immobilization. In that context, the modes of action, method of efficacy measurement, immobilization strategies, and chemistry of the antimicrobial agents are all discussed, with an emphasis on understanding and interpreting the antimicrobial performance.

In many real world applications, the immobilization state of the agent is irrelevant, and only the efficacy is of interest. However, if there is a need to demonstrate that the antimicrobial agent (AMA) is efficacious *and* immobilized, for regulatory or mechanistic reasons, then this paper should help to clarify what others have done, as well as pointing the way for future researchers to direct their efforts.

II. ANTIMICROBIAL MODE OF ACTION

When considering the immobilization of an antimicrobial agent, it is valuable to consider the likely antimicrobial mode of action and how this mode of action will be impacted by the immobilization. Factors such as the chemical composition and dimensions of the extra-cytoplasmic bacterial components (membranes, peptidoglycan wall, capsule, fimbriae, and flagella, if present) are expected to be relevant to the performance of surface tethered AMAs. For example, the immobilization of an antibiotic like a tetracycline via a short tether (5 nm) would seem to be a pointless venture, given that the mode of action for tetracycline involves disruption of the binding between 16 S ribosomal ribonucleic acid and transfer ribonucleic acid.[66] This immobilization of tetracycline would severely restrict its access from the cell interior, thereby dramatically reducing, if not eliminating, the AMA's efficacy. AMAs with modes of action that require only external contact, or even charge induced membrane interactions, might be more appropriate choices for immobilized antimicrobial agents.

Many papers have discussed theoretical modes of action for immobilized antimicrobial agents (iAMAs).[2,67–74] Among the common mechanisms are (1) physical lysing of the membranes, (2) charge induced disruption of the membrane potential, (3) solubilization of the membrane phospholipids creating physical holes, and (4), in the case of peptides, a wide range of interesting supramolecular assemblies. However, often very little is known about the detailed mode of action for a specific agent, especially for newly developed molecules, and in many cases the mode of action might be completely unknown. This lack of information

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TABLE I. Immobilization and test methods for various iAMAs.

	Zone of inhibition	Immersion	Direct inoculation	Surface growth	Luminescence	Other
Small quaternary ammonium compounds	11,12,14,17	11–13,15,17	12,14,15	17	16	11–13,15,16
Quaternary ammonium polymers	18,20,21,28,31	19,24,26–31,33–35		20,21,30,31,33,35	24,26,29,30	19,30,33,34
Polyamines (1° to 3°)	37,40	37,39	40	39,40	40	37,40
Chitosan derivatives		48	45			
Enzymes	51	50–53	55			52,53
Peptides and mimetics		56,59,60,65			56,57,60,65	58,59

should not inhibit the research, but perhaps this could guide the choice of immobilization and measurement strategies.

A. Efficacy testing

When it comes to immobilized antimicrobial agents, the choice of an appropriate antimicrobial efficacy test method and interpretation of the results can often require a sophisticated understanding of disparate scientific disciplines. For example, it is valuable to understand the potential mode of action, the mass transport of the agent from the sample, the potential interferents in the test medium, and the manner in which the bacteria will sample the available surfaces.

Mass transport is one of the topics that are rarely discussed, as standard methods are applied to immobilized AMAs, specifically, planar diffusion with and without convection.[75] When the immobilization state of the AMA is unclear, or when a microbiological test method is to be used to discern the immobilization state, then it is useful to consider how mass transport of the AMA from the sample will manifest in the test method. For example, does it develop a concentration gradient at the surface, perhaps in a stagnant layer? What is the dimension of that layer, and how does the concentration of the agent in that layer compare to the bulk concentration outside the stagnant layer? If cells enter this region, and perhaps adhere to the surface, then they will likely accrue in concentrations much greater than in the bulk, but how much greater depends upon numerous variables. Understanding the mass transport of bacteria to the surfaces and of the AMA from the surfaces can provide much needed insight when attempting to discriminate between bound and leachable agents.

Interpreting immobilized AMA data appropriately can be a challenge when trying to assign efficacy strictly to noneluting agents. It is commonplace to modify standard methods as the sample geometry or lab expertise dictates. This review describes the most commonly used efficacy test methods, highlighting the special needs that immobilized agents pose for efficacy testing and commenting on the assumptions and appropriateness of the methods for immobilized agents. Table I provides an overview of how various immobilized AMAs have been tested in the literature.

B. Zone of inhibition

Zone of inhibition (ZOI) methods involve placing an AMA loaded substrate in contact with a growth media loaded with bacteria. As the AMA elutes from the substrate into the media, a zone can be observed in which the concentration exceeds the minimum inhibitory concentration (MIC) or critical concentration for that AMA. The size of the zone is related to the diffusion constant for the AMA in the media as well as to the total amount of agent that is available to diffuse.[76–78] Drugeon and coauthors describe in some depth the physical basis of the ZOI method, and they focus on the functional dependence of the zone size on key parameters such as the agent mobility and quantity. This touches on a key point that is relevant to many immobilized AMA studies: the quantity of AMA present on the surface is severely limited, unlike with a swath of fabric or filter paper that is soaked with an antibiotic. In the case of nonporous relatively low surface area substrates modified with a densely packed monolayer of AMA, the quantity of AMA that can elute from the part is vanishingly small. This small number of available molecules can readily limit the size of the zone to microscopic distances. A brief calculation of the expected zone is recommended for anyone using this method to conclude that the agent is immobilized. It might be that even if *all* of the AMA molecules eluted from the surface, the volume corresponding to the critical concentration would correspond to an undetectably small zone. Figure 1 shows an example in which ZOI has been used with a polymer monolayer on a silicon substrate. The lack of a zone for the polymer-silicon sample and the presence of a zone for a porous scaffold soaked in the AMA were used to support a lack of leaching from the 1.2 cm square silicon substrate. Using simplistic calculations with some optimistic assumptions, we can see that if all of the agent—say, 3×10^{11} molecules, assuming a high 1 molecule/nm^2 coverage over the geometric area of the silicon—eluted from the part and penetrated uniformly into the surrounding volume, and if the minimum concentration needed in order to establish a zone in which growth was inhibited was 0.1 mM, then the zone would be on the order of 20 μm. These numbers are overly optimistic, as 0.1 mM corresponds to an aggressive AMA and the calculation assumes a uniform concentration across the 20 μm, whereas the true distribution will be a gradient with most of the agent closer to the surface. This is not to say that the polymers in Madkour's work are eluting, but that the ZOI method is insufficient to prove the lack of elution.

Furthermore, it is important to compare similar environments. The growth media used for ZOI might be inappropriate when the enumeration testing is performed in a much

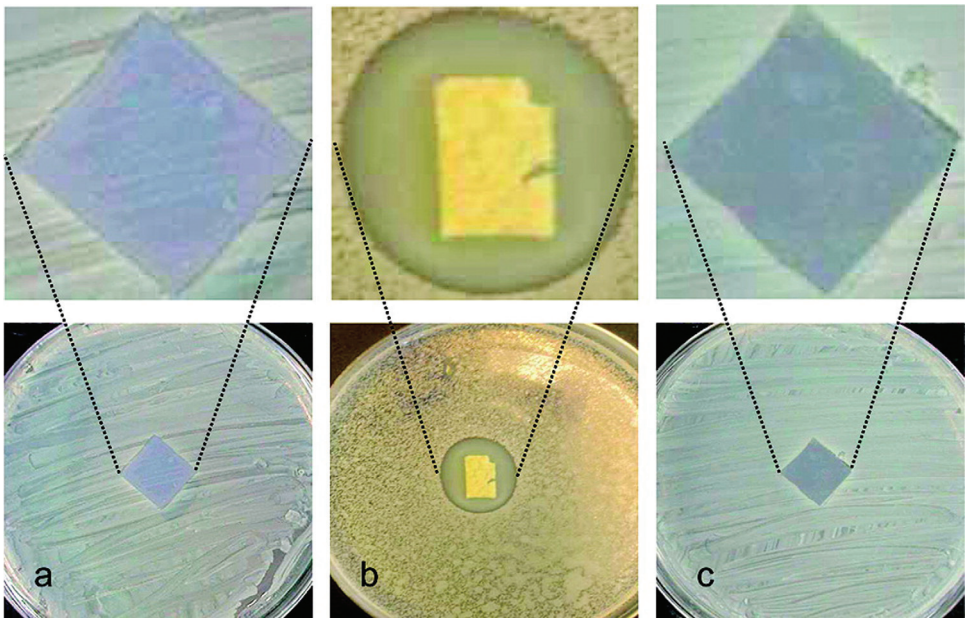

FIG. 1. (Color) Zone of inhibition assay for the antimicrobial surfaces: (a) untreated silicon wafer and (b) porous scaffold loaded with poly(butylmethacrylate)-co-poly(Boc-aminoethyl methacrylate) highlighting the typical "zone of inhibition" observed in ZOI experiments. (c) Modified silicon wafer surfaces containing 70 nm of poly(butylmethacrylate)-co-poly(Boc-aminoethyl methacrylate) shows no zone of inhibition. (Reprinted with permission from A. Madkour, J. Dabkowski, K. Nusslein, and G. Tew, Langmuir **25**, 1060 (2009). Copyright 2009, American Chemical Society.)

cleaner saline suspension that has less nutrient and which might contain fewer potential interferents. The interferences will generate a higher MIC and will reduce the dimensions of the zone by an amount that depends upon the extent of the interference. This might be especially relevant for AMAs that exploit charge-based interactions, as the nutrient rich media common to ZOI experiments contain proteins and polysaccharides that might contain ionized groups.

These two factors might lead to a spurious conclusion that the material is not leaching from the part. The ZOI tests are completely appropriate for porous pads loaded with antibiotics; however, much consideration should be given to the above-mentioned points before equating the absence of a zone to the immobilization of the agent.

C. Immersive inoculation

Many methods involve the immersion of the active sample into a media or saline solution that contains the inoculum. The American Society for Testing and Materials (ASTM) has developed a standard method for the antimicrobial efficacy assessment of immobilized antimicrobials, ASTM E2149.[79] This is one of the most widely used methods to test for the efficacy of immobilized samples. The method measures the colony forming units (CFU) that derive from an inoculum solution that was in contact with the sample. There can be confounding factors that affect the number of CFU measured from the solution; for example, agent could elute and kill cells that approach the surface of the sample, or viable cells could be preferentially removed from the solution via adhesion to the sample. Typically, a control sample is used for comparison to the active sample. Although the control and active samples are often made of the same substrate material (same size, surface area, roughness, etc.), unfortunately, due to the nature of chemical modification, the samples will likely have very different chemistries. Often the active surface is positively charged, whereas the control is not. This simple difference could have an impact on the cell-surface adhesion. In immersion methods like ASTM E2149, the removal of live cells from the solution via irreversible adhesion to the sample will produce the same effect as killing the cells.

Some researchers have immersed the sample in an inoculum-free solution and then inoculated that solution in the absence of the sample.[23] The intent is to test the efficacy of any leached compounds, and a lack of efficacy is used to rule out leaching as a factor for kill. Mass transport of the agent from a surface is key in interpreting these results,[75] and although the theory is conceptually rather simple and well known, a predictive understanding can be very complicated in a real-world system. Nonetheless, it can be instructive to consider the various possible outcomes. Firstly, if the AMA is truly immobilized, the solution will be noneffacacious. Furthermore, if the AMA molecules rapidly elute from a part to which the molecules are not strongly bound and where there is limited porosity, then the concentration in the bulk will rapidly approach a limiting value that will also approach the surface concentration. If the bulk concentration is adequate to kill, then it will be detected as efficacious, and the conclusion will be elution. If the bulk concentration is inadequate to kill (perhaps due to dilution of a limited supply of AMA), then the conclusion will be that the agent does not elute, when in fact it does but was diluted below the critical concentration in that volume.

An interesting outcome of this last condition can occur when the elution rate is comparable to or less than the rate at which the cells sample the surface. In that case, the planktonic cells approach a surface that is still eluting agent, and as they do so, they experience concentrations in excess of the bulk concentration; as a result, those cells might die even though the bulk solution remains far below the critical value (even at the end of the experiment). In this case, we would expect that the cells at the surface would be killed by eluting agent, but that the solution inoculated without the sample would not kill, perhaps leading to the incorrect conclusion that the activity was due to immobilized agents. The exact mechanism will depend upon several dynamic processes, and will likely depend upon the bacterial species and its mobility. This does not contradict previous experiments, but it suggests that a comprehensive understanding of the mechanism might benefit from a more critical tool than this test method when attempting to discriminate between the immobilized and leachable kill.

In a paper by Murata and coworkers, the authors challenged their samples with increasing inoculum and observed an interesting limit to the efficacy of their surfaces ($\sim 1 \times 10^8$ *E. coli*/cm^2). This value is approximately equal to the closest packed surface coverage of these bacteria on a surface. Testing to failure like this can be very informative, and where appropriate, it is recommended in order to provide further mechanistic insight into the test method and mode of action.

D. Direct inoculation

Several methods, such as the Japanese Industry Standard (JIS Z-2801) method, place a small droplet of inoculum directly in contact with the active surface. Although JIS Z-2801 is not explicitly designed for use with immobilized agents,[80] it has nonetheless become common practice to apply it to systems with purportedly immobilized agents. This method involves placing a small droplet of inoculum directly on the surface of the sample and then placing a coverslip or film on top of the droplet, allowing capillary forces to draw the surfaces together, thereby spreading the droplet across the surface. Following the requisite inoculation time, the entire assembly (both surfaces and the captive liquid) is agitated, and released cells are typically enumerated as CFU. This coverslip, which is not usually antimicrobial, can adhere cells from the inoculum. It is not uncommon to see JIS results for ostensibly immobilized agents with log reduction values on the order of 3 or more. Bacterial adhesion to the coverslip will likely depend upon the bacterial strain and the coverslip material; however, if even 10% of the inoculum adheres to the coverslip, and if 10% of those cells are recovered for enumeration, then the log reduction value (LRV) is expected to be limited to less than 2. Therefore, high LRVs for direct inoculation methods are seemingly in contradiction to the immobilized nature of the agent. It should be noted that the sample-coverslip separation is on the order of 5 to 25 microns, and that diffusion across this distance would be difficult to observe with ZOI, though ZOI is sometimes used in conjunction with direct methods to demonstrate efficacy and immobilization.

Some researchers have avoided this problem by excluding the coverslip, thereby providing the cells with only one solid surface, the sample surface. Our lab modified this direct inoculation method with live-dead staining, and for the strain we examined we were able to discriminate between kill at the active surface and kill at the coverslip. In summary, extra attention should be paid to these kinds of enumeration based direct inoculation methods. This is especially true if the test generates high LRVs, as this might be a good indication that there was elution.

E. Surface growth methods

A number of innovative and effective methods involve aerosol inoculation methods to apply a thin film of pathogens across the surface. The bacteria are sometimes dried in place and sometimes kept humidified. Following a specified inoculation time, the activated surfaces and controls are then used for growth based amplification via either direct contact with a slab of agar or recovery for traditional enumeration. Figure 2 shows an example of this test method, in which both glass slides were inoculated with an aerosol of bacteria. Clearly, the untreated control grew more colonies than did the sample treated with the antimicrobial agent. These test methods are excellent for emulating ambient contamination of surfaces and the corresponding antimicrobial efficacy of the surface; however, regarding discrimination between immobilized and elutable agents, there are a few points to consider.

These methods place the bacteria in very close contact with the AMA coated substrate. Even when the bacteria remain partially humidified, the volume of fluid in contact with the AMA coated substrate is extremely small. Furthermore, even if all of the fluid between the bacteria and the surface is removed, the bacterial surface is still in direct contact with the surface, and diffusion of trace nonimmobilized AMAs can still occur. In this geometry, the impact of eluted agents will be greatly amplified as compared even with the direct inoculation methods described above. By way of a very coarse example, suppose that a 1 cm^2 sample is inoculated, and suppose that the same sample elutes enough free AMA into a 10 ml solution to generate 1/1000 of the minimum bactericidal concentration (MBC). Limited by the concentration at the source and allowing sufficient time for equilibration, the concentration produced by the same amount of material released into the constrained volume of a thin film would be higher. In the case of direct inoculation methods, a 10 μm thick film would result in a concentration of 10 MBC. For aerosol based methods, the fluid layer between the cells and the substrate is much thinner (say, 100 nm), and the relative concentration can approach 1000 MBC. Of course, 100 nm is probably thicker than expected if the cell is in direct contact with the surface, and so higher concentrations are plausible, as the concentration is limited by the amount of free agent and the solubility of the agent in

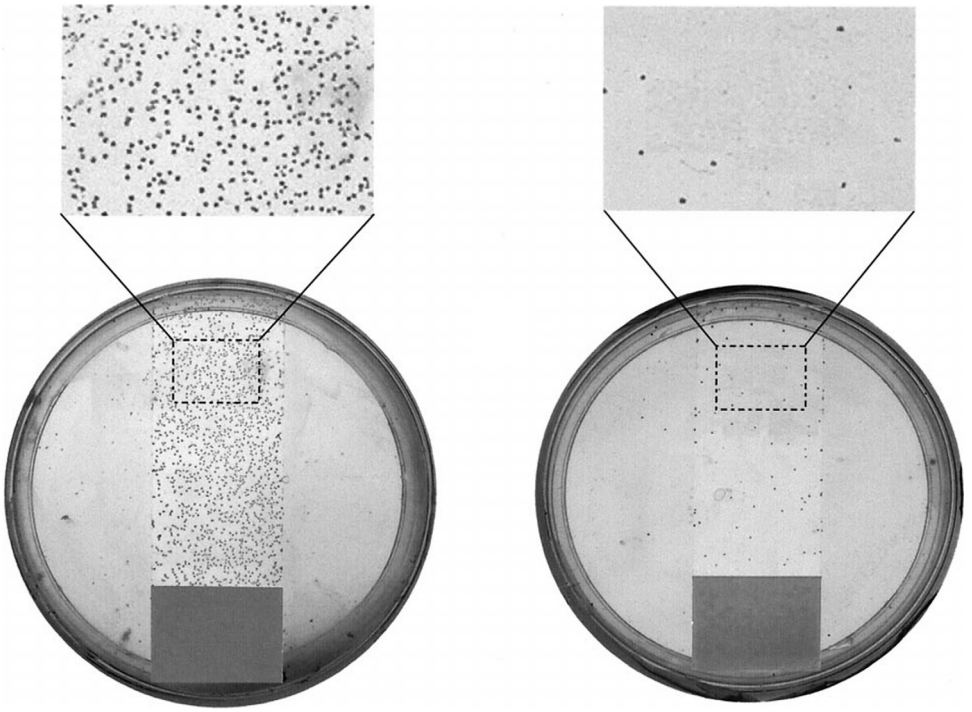

FIG. 2. Photographs of a commercial NH_2 glass slide (left) and a hexyl-PVP-modified slide (right) onto which aqueous suspensions (10^6 cells per ml of distilled water) of *S. aureus* cells were sprayed. The slides were air dried for 2 min and incubated under 0.7% agar in a bacterial growth medium at 37 °C overnight. (Reprinted with permission from J. C. Tiller, C. J. Liao, K. Lewis, and A. M. Klibanov, Proc. Natl. Acad. Sci. U.S.A. **98**, 5981 (2001). Copyright 2001, National Academy of Sciences.)

that fluid layer. This back of the envelope calculation simply highlights the potential amplification by trace elutables of the activity of the AMA modified surfaces. To the extent that the mobility of the AMA is of interest, a more detailed calculation or test would be needed in order to assess the impact that these potential elutables would have on a real system.

F. Viable but nonculturable

The above-described methods use the growth of colonies to amplify the number of bacteria for quantitation and detection. Any growth based method will have a limitation when it comes to viable but nonculturable (VBNC) microbes.[80–83] The efficacy of the agent will be convoluted with the method's ability to amplify the microbe by growth, and if the microbe has been put into a VBNC state and resists growth in the medium, then it will appear to have been killed by the agent even though it actually remains viable, awaiting an appropriate trigger or medium to reactivate. This is not a problem specific to immobilized agents, but it bears consideration in the selection of a test method for any antimicrobial efficacy testing. This is a relatively recent field of research, and as such it contains many new areas to explore. As it pertains to this review, the assessment of an immobilized AMA's ability to kill VBNCs will be strengthened by the development of alternative methods tuned to the requirements for those VBNCs. This will likely require a detailed understanding of particular species, strains, or even phenotypic properties. For example, the behavior of metabolic or membrane permeable stains is expected to be affected by the specifics of the VBNC state.

G. Luminescent signaling

Although growth based enumeration is familiar to microbiologists and produces quantitative data with a large dynamic range, that level of quantitation is not necessarily needed in order to assess the general efficacy of an AMA. There are a number of semiquantitative methods that exploit luminescence in order to detect the viability of microbes. The most common of these methods use live-dead stain kits, with which the stains probe various properties of the microbe such as its membrane permeablity, metabolic activity, etc.[84,85]

These kinds of fluorescent stains can be used with confocal or epi-fluorescent microscopy, and recently these have been exploited for enumeration based methods when combined with flow cytometry.[86,87] Our group has devised a conceptually simple live-dead staining technique that can determine whether the antimicrobial agent kills cells at the surface or at a distance.[88] The method uses a direct inoculation method with spacers to separate an iAMA surface from a control coverslip surface. The method generates three populations of bacteria that can be compared: (1) those at the control surface, (2) those at the test iAMA surface, and (3) those freely floating in the solution. A comparison of the bacterial fluorescence in the three populations can provide insight as to whether the agent acts only at the substrate or is able to affect the control bacteria. The process could be

extended to include alternate fluorophores, thereby probing the metabolic activity or other properties of the microbes. Live-dead staining has many potential limitations. For instance, it has a limited dynamic range (usually from 5% to 95%, compared to enumeration methods that can vary over several orders of magnitude). The live-dead staining can depend upon the bacterial species, the strain, or the medium. In the case of immobilized surfaces, the stains can sometimes interact with the substrate, producing high fluorescent backgrounds.

As with the methods mentioned above, bacterial adhesion to the surfaces is important when interpreting results for surface tethered AMAs. The iAMA and control surfaces are chemically different, and the relative adhesion of live and dead cells to these two surfaces is typically unknown. Rinsing of the surface prior to imaging could potentially bias the results by preferentially removing live or dead cells from either of the two surfaces. In some cases when bacterial adhesion is the goal, some researchers have exploited this by combining rinsing with fluorescent imaging in order to probe the extent of bacterial adhesion in antifouling experiments.

Some methods take advantage of bioluminescence generated within the cell to measure the metabolic activity of the cell.[60,89] These use genetically engineered lux-reporter strains, and the luminescence is usually a measure of the respiration. Lux-reporters require some effort in order to create each new bacterial strain, and therefore they are used for only a limited number of species. Furthermore, the lux-reporter cells are now different from the original strains, because some of the lux-reporter cellular energy is diverted in order to maintain the luminescence.

III. IMMOBILIZATION STRATEGIES

When the agent is identified as potentially attractive as an immobilized AMA, the next step is to formulate a strategy to immobilize the agent to a surface. Figure 3 illustrates a few of the different strategies for the immobilization of AMA to substrates: (1) "graft-to" strategies involve the covalent coupling of the intact AMA to a surface via covalent linker chemistries; (2) "physical adsorption" methods involve physisorption of the AMA through noncovalent but strong or multidentate interactions at the surface; (3) "surface initiated" strategies involve the synthesis of the AMA from initiators covalently immobilized to the surface; and (4) "as-formed" methods involve creating a substrate that contains the AMA when the substrate is formed.

A. Graft-to

Many of the iAMAs are formed by means of graft-to strategies and begin with the synthesis of a potentially surface reactive AMA. Frequently, the surface requires an activation process that generates amine, carboxylic acid, aldehyde, or thiol functionalities. When activated, the substrate is ready for additional reaction with heterobifunctional linking chemistries that contain reactive groups such as succinimide, carbodiimide, maleimide, or aldehyde.[90] Sometimes

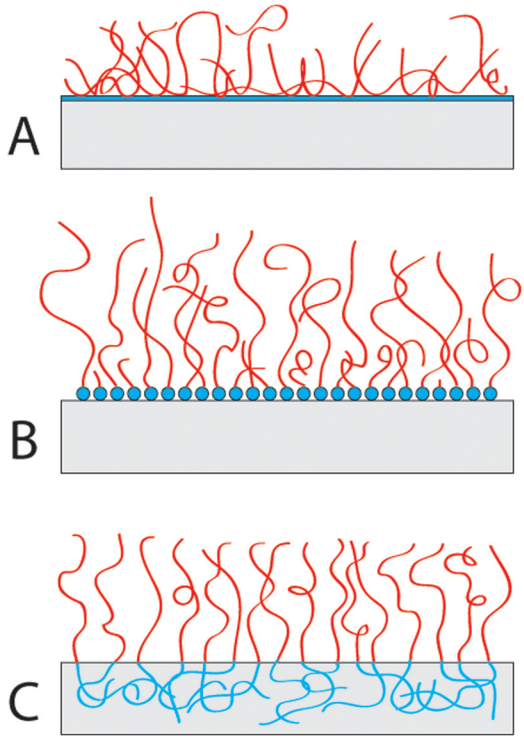

FIG. 3. (Color online) Various methods for immobilization: (a) graft-to or physical adsorption, (b) surface-initiated synthesis, and (c) as-formed.

these linkers contain spacers such as polyethyleneglycol, which serve to enhance the degrees of freedom for the AMA, thereby enabling more modes of action and increasing the efficacy. Alternatively, the AMA could have been modified to exploit click-chemistry for rapid clean immobilization.[91] Often, as in the case of polyethyleneimine, the immobilization of the AMA is followed by further surface-based reactions such as quaternization in order to produce the final immobilized AMA.

B. Physical adsorption

The methods mentioned above involve the formation of a chemical bond to the AMA. However, a single covalent bond can be weaker than numerous noncovalent bonds. This is the strength of many self-assembled structures such as the deoxyribose nucleic acid double helix. This physical adsorption can take the form of hydrogen bonding, ionic bonding, or even steric interactions caused by entanglement during the solvent swelling of polymer films. One of the best examples of robust physically adsorbed films is the layer-by-layer (LbL) film that uses multidentate polymer interactions to bind the polymers to the surface.[92] These are often combined with eluting strategies, but in some cases quaternary ammonium or another AMA is used as the outer layer of the LbL system.[93] Other physical absorption methods involve exploiting the charge pairing or strong ionic bonding in order to hold a smaller AMA to a substrate with an opposite charge. When the physical interaction involves ionizable groups, the *p*H of the environment is of key importance to the stability of the film and the robust nature of the iAMA.

C. Surface initiated or graft-from

In the surface initiated or graft-from strategy, the AMA is essentially synthesized from surface bound initiators. Well-defined polymeric structures have been created through "living" or controlled polymerization techniques,[94] such as reversible addition fragmentation chain transfer polymerization,[95,96] nitroxide mediated polymerization,[97] and metal catalyzed living radical polymerizations such as atom transfer radical polymerization (ATRP).[98] In a similar but substantially more controlled manner, researchers can use solid phase synthesis methods to graft specific sequences of peptides and peptoids directly from a substrate. Cellulose-amino-hydroxypropyl ether has been used to synthesize antimicrobial peptides (AMPs) directly on cellulose substrates.

In a combination of surface-initiated and graft-to methods, researchers have used "dry" chemistries such as plasma and chemical vapor deposition (CVD) to modify substrates with complex polymer films.[38] CVD was used to deposit a polymer film from dimethylaminomethylstyrene and a ditert amylperoxide initiator. The formed films were not structurally characterized, but the researchers recorded rapid kill with ASTM E2149 and observed no zone of inhibition around the high surface area fabric.

D. As-formed

The as-formed strategy involves the inclusion of the AMA within the polymer used to create the device substrate. By mixing before forming, one is able to immobilize the AMA via the nature of the substrate formation process. Either crosslinking or entanglement with the substrate polymers results in the AMA's being immobilized. Nagel and coworkers have demonstrated that surface reactive injection molding can generate permanently modified parts, in their case polycarbonate with polyethyleneimine (PEI) presented at the surface.[99] Namba *et al.* included an AMA within the ingredients for methacrylic polymerization, thereby encapsulating the AMA as an integral component of the matrix.[12]

IV. IMMOBILIZED AGENTS

A wide range of molecules have been immobilized and tested as antimicrobial agents, including amine containing polymers, quaternary ammonium polymers, guanides, enzymes, chitosan, peptides, peptoids, and other peptide mimetics. The following sections highlight key publications that have explored each of these different classes of agents.

A. Quaternary ammonium silanes and other small molecules

Historically, the first immobilized antimicrobial agent was the silane (3-trimethoxysilyl) propyldimethyloctadecyl ammonium chloride[10] (Si-QAC). This molecule, and its related silane analogues, can form direct covalent linkages to silicates, oxides, and many plasma activated polymers. Furthermore, as with any tri-alkoxy silane, this molecule can autopolymerize to form long branched polymer chains with an (-Si-O-) backbone. The quaternary ammonium side chains on this siloxane polymer form a motif similar to that of the quaternary ammonium polymers that followed.

In a seminal paper by Isqueth, Abbott, and Walters,[11] a quaternary ammonium silane was immobilized and found to retain its antimicrobial efficacy. The silane was bound to a wide range of different substrates, including siliceous surfaces, man-made fibers, natural fibers, metals, and assorted industrial materials. These modified surfaces were tested against bacteria (both Gram positive and Gram negative), yeast, algae, and fungi. A modified version of ASTM 2149E and an aerosol method were used for antimicrobial efficacy, and elution of the agent was tested using radiolabeled agents.

In a paper by Gottenbos *et al.*,[16] this silane was reacted with argon plasma activated silicone rubber, and a suite of surface analytical tools supported the presence of the silane on the surface. Interestingly, this study used a flow cell combined with rinsing and staining with a live-dead kit. Although the live-dead stain does not provide a large dynamic range, it clearly demonstrated that the modified surfaces supported more dead bacteria than did the unmodified controls. Whether this was due to a causal based surface killing of the bacteria or an enhanced adhesion of membrane compromised cells to the surface is unclear. Nonetheless, the effect was observed even after the surfaces were exposed to human plasma, as shown in Fig. 4. The authors also performed a series of *in vivo* experiments. In one case, the samples were inoculated and rinsed *ex vivo* prior to being implanted, and in another study samples were implanted and inoculated *in vivo*. The authors observed that the samples inoculated *ex vivo* were efficacious, whereas those inoculated *in vivo* were not.

Still others have used this Si-QAC as a nonleaching treatment for cellulose.[14] Although FTIR and XPS demonstrated that the molecule was associated with the substrate, the data do not definitively prove that the molecules are necessarily bound to the substrate. The authors used ZOI along with JIS-Z2801 to demonstrate nonleachability.

In addition to these quaternary ammonium silanes, other small molecules such as aminoglycoside antibiotics have been immobilized. Osinska-Jaroszuk *et al.* reacted gentimicin and amikacin with vascular stents via aldehyde coupling chemistry.[12] Although some of the molecules might have been covalently reacted with the stents, the parts clearly developed a substantial zone with ZOI, seemingly in direct contradiction to the covalent nature of the agent. Nonetheless, the stents are efficacious and can function as intended, though most likely via elution of weakly associated antibiotic molecules.

In a similar study, Namba and coworkers immobilized cetylpyridinium chloride (CPC) via physical incorporation within a polymethacrylate matrix using an as-formed method.[13] They demonstrated that these surfaces function to inhibit biofilm formation, and they used ZOI to support the assessment that the CPC was not eluting. Based on the discussions above and the lack of any clear force holding the

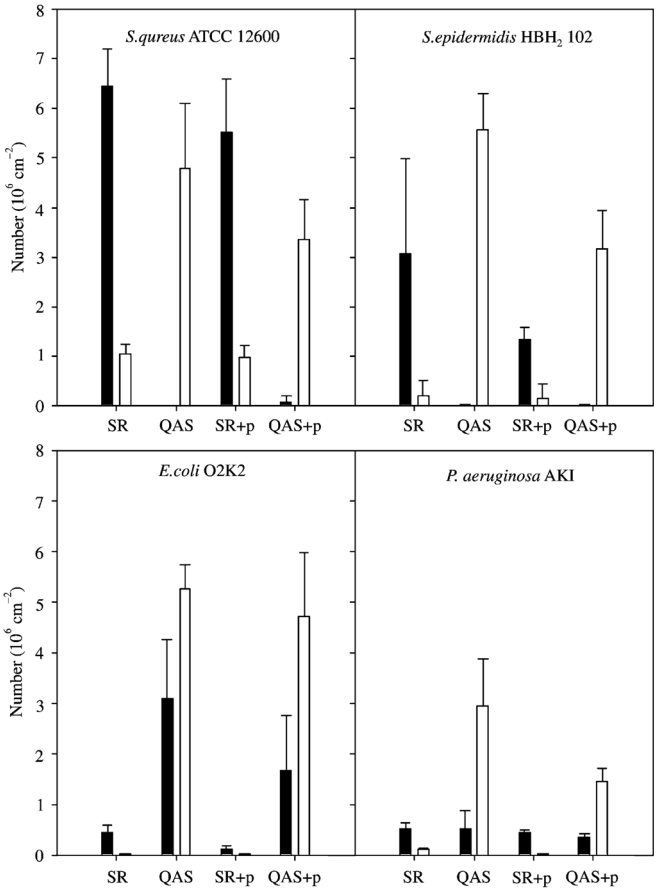

FIG. 4. Numbers of adhering viable (black bars) and nonviable (white bars) bacteria on silicone rubber (SR) and quaternary ammonium silanized silicone rubber (QAS) with and without adsorbed plasma proteins (SR+p and QAS+p). Error bars represent the SD over six images collected in two experiments, with separately cultured bacteria and differently prepared coatings. (Reprinted with permission from B. Gottenbos, H. C. van der Mei, F. Klatter, P. Nieuwenhuis, and H. J. Busscher, Biomaterials **23**, 1417 (2002). Copyright 2002, Elsevier.)

CPC to the substrate, it seems plausible that the agent is eluting to kill bacteria at or near the surface.

B. Quaternary ammonium polymers

The most thoroughly studied class of AMAs is the quaternary ammonium polymers,[18–21] and these have included polymer backbones such as PEI, polyvinylpyridinium (PVP), chitosan, and assorted acrylates. These polymers have been immobilized using virtually every method listed above, and they are often modified by post-immobilization reactions. The Klibanov group has pioneered much of the graft-to work with PVP and PEI. Immobilization of the amine containing polymer usually involves control over the surface density and is usually followed by on surface quaternization with different sidechains and counterions.[17,22,30,31] Other groups, most notably Russell and Matyjaszewski, have led the surface initiated efforts using controlled ATRP reactions.[24,26–29] The works from the Klibanov and Russell groups have systematically probed the antimicrobial impact of key properties such as the surface charge density, polymer chain length, polymer chain density, counter ion identity, and quaternary ammonium sidechain length.

1. Aliphatic quaternary ammonium polymers

Lin and coworkers performed a comprehensive study of immobilized PEI using graft-to methods that examined the length of the sidechain and the charge of functional groups.[35] The immobilization was followed by alkylation, acylation, or carboxyalkylation to generate cationic (quaternary ammonium), neutral (amide), and zwitter-ionic (quaternary ammonium carboxylic acid) functional groups, respectively. Alkylation was performed with a range of chainlengths (ethyl, Butyl, hexyl, dodecyl, and octadecyl) followed by subsequent methylation to quaternize the amine. These reactions were performed on glass slides as well as iron oxide nanoparticles. The efficacy testing for the glass slides involved the aerosol inoculation of slides, followed by drying. After a time, the slides were covered with agar and incubated, and the subsequently grown colonies were counted. The bactericidal efficiency was determined by taking the ratio of the colonies formed on the sample to the number formed on the aminosilane control. Their results showed that a positive charge was necessary, because the neutral and zwitter-ionic surfaces were not efficacious. The authors also concluded that when it is terminally methylated, the alkylating group's chainlength should be greater than that of n-Butyl for a high efficacy. They found similar results for both Gram positive (*S. aureus* and *S. epidermidis*) and Gram negative (*E. coli* and *P. aeruginosa*) bacteria.

Haldar and coworkers also examined the charge and the sidechain length, but with an immobilization scheme that is like a combination of an as-formed and a physical adsorption approach. They painted hydrophobic derivatives of branched and linear PEI onto glass slides.[23] The derivatives covered a range of molecular weights, were formed prior to painting, and were modified to have net cationic (N,N-dodecylmethyl-PEI, N,N-docosylmethyl-PEI), zwitter-ionic (N-(15-carboxypentadecyl)-PEI HCl salt), anionic (N-(11-carboxyundecanoyl)-PEI), and neutral (N-undecanoyl-PEI) charges. The painting process did not immobilize the molecules covalently; instead the authors exploited the inherently poor solubility of these polymers as a barrier to dissolution in the inoculum. The study demonstrated the efficacy of the cationic polymers against aerosol based microbes (*E. coli*, *S. aureus*, and influenza virus) with a greater than 4 log reduction in CFU or plaque forming units within minutes of exposure. In an effort to demonstrate that leaching was not impactful, the authors performed two tests: (1) an extraction from a painted sample, and (2) an extraction from 200 mg/ml of the pure agents. In each case the bulk "extraction" buffer solution was then inoculated with microbes and tested for efficacy. As with many elution tests, this probes the amount of material that can dissolve into the aqueous phase and asks whether this bulk solution concentration is adequate to impact the efficacy test. The second method is much

stronger, as it involves what is essentially a saturated solution of the agent, whereas the first method might still have a locally high concentration at the surface due to the diffusion gradient's slowly increasing the bulk solution concentration. However, both methods are expected to yield concentrations that are much less than the apparent concentration at the interface.

Compared with living polymerization methods, conventional radical polymerization tends to offer poor control over the molecular weight, polydispersity, and chain branching structure.

In order to address the lack of control, Huang and coworkers performed graft-to immobilization of block polymers created by ATRP to contain surface grafting regions and dimethyl amine regions.[26] The surface density of the immobilized quaternary ammonium (QA) groups was controlled via the polymer solution concentration, immersion time, and molecular architecture. Fluorescence and atomic force microscopy (AFM) measurements were used to quantify the density of chains on the surface, and the results were correlated with the antimicrobial efficacy, as shown in Fig. 5. Interestingly, when comparing graft-to and graft-from surfaces with comparable densities of QA groups, the graft-to surfaces were more efficacious. The authors hypothesize that this is due to the observed heterogeneity in the graft-to films, which results in local regions of higher relative QA densities. By using micropatterning they were able to generate areas of the substrate where the agent was immobilized directly next to areas free of the AMA, as seen in Fig. 6. This enabled their live-dead stain images to spatially differentiate between kill over the immobilized agent and kill in neighboring, unmodified areas (less than a few microns away). This provides a highly credible method for stating that the agent does not kill by elution, and it is essentially a microscopic version of the ZOI test, but instead of taking place under growth conditions, it occurs under the more relevant test conditions. The paper makes frequent connections between interesting mechanistically relevant molecular properties of the film and

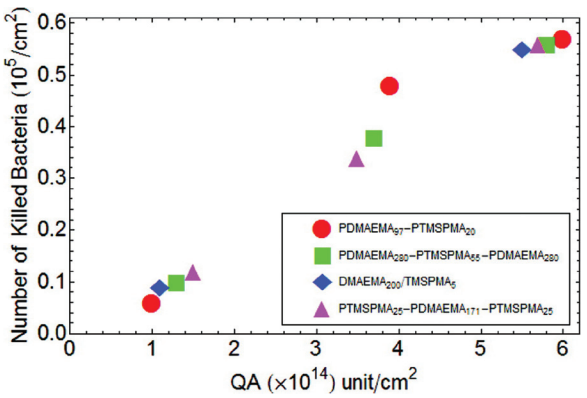

FIG. 5. (Color) Biocidal activity of the surfaces vs the density of QA units on surfaces (2.9×10^5 bacteria in control; surface area = 5 cm^2). (Reprinted with permission from J. Huang, R. R. Koepsel, H. Murata, W. Wu, S. B. Lee, T. Kowalewski, A. J. Russell, and K. Matyjaszewski, Langmuir **24**, 6785 (2008). Copyright 2008, American Chemical Society.)

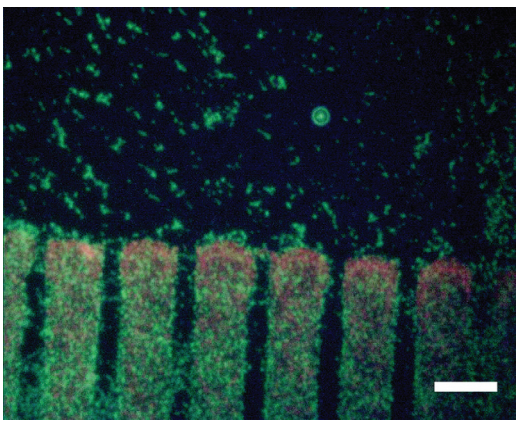

FIG. 6. (Color) Fluorescence microscopy image of *E. coli* on a PQA-patterned glass slide. The image is a result of the superposition of an image with a green band-pass filter showing intact bacteria and an image taken using a red band-pass filter showing bacteria with damaged cell membranes. Bar size is 50 μm. (Reprinted with permission from J. Huang, R. R. Koepsel, H. Murata, W. Wu, S. B. Lee, T. Kowalewski, A. J. Russell, and K. Matyjaszewski, Langmuir **24**, 6785 (2008). Copyright 2008, American Chemical Society.)

the efficacy—for example, the number of QA groups needed to kill a bacterium, whic in this case is 10^{10} QA/bacterium.

In order to take the level of control further, quaternary ammonium polymers (PQAs) have also been formed via surface initiated living polymerization methods. In a paper by Murata *et al.*, glass surfaces coated with initiators were used to polymerize dimethylaminoethylmethacrylate via ATRP.[24] These polymers were then quaternized via reaction with alkylhalides to form immobilized PQAs. By controlling the surface density of the initiators and the reaction time for the polymerzation, they were able to independently control the polymer density and polymer length, respectively. By using fluorescence to quantify the surface charge density, as well as live-dead staining to determine the local efficacy, the functional dependence of the efficacy on the surface charge density was demonstrated. The authors concluded that the key operational parameter in the efficacy was the surface charge density, and not necessarily the polymer length. This has mechanistic implications, which they explored and compared to other examples in the literature. They determined that a threshold charge density of 5×10^{15} charges/cm^2 was needed for efficacy against *E. coli*. They also noted that the surface charge for an *E. coli* is in the range of 10^{14}–10^{15} charges/cm^2, depending on the growth stage of the cell.

Milovic and coworkers reported on the apparent lack of resistance that the bacteria develop upon repeated exposure to fresh surfaces of graft-to N-alkylated quaternary PEI.[30] In this insightful study, the samples of aminopropylsilane modified glass slides were coupled to PEI that was subsequently N-hexyl and N-methyl quaternized. Based on the live-dead staining data, both *E. coli* and *S. aureus* were effectively killed within 1 h of exposure to the surface. Only a small fraction of the aerosolized bacteria that contacted the surface developed colonies via a surface growth amplification method. By repeatedly sampling bacteria from the

surviving colonies and re-challenging each new culture with fresh surfaces, the authors demonstrated that the bacteria did not develop resistance over the course of 11 exposures.

2. Aromatic quaternary ammonium polymers

In addition to the commonly observed aliphatic quaternary ammonium AMA, some researchers have been exploring the impact that pyridinium based quaternary ammonium ions have on antimicrobial efficacy.[32] Tiller and coworkers not only detail the dependence of the side-chain chainlength with pyridinium polymers, but they also present a spray-growth amplification method for qualitatively assessing the efficacy.[21] Figure 7 shows that these authors found that the chainlength of the N-alkylated group impacted the efficacy of the agent, with hexyl ammonium quats having the greatest efficacy relative to the longer or shorter chainlengths. Substrates were challenged by airborne and waterborne *E. coli* experiments to test for antimicrobial activity. The airborne assay was performed by spraying *E. coli* onto surfaces and allowing it to dry. The substrates were placed in a sealed Petri dish with growth agar and incubated in order to allow the viable bacteria to grow into colonies for counting. The waterborne test was performed by immersing the substrate in a suspension of microbes and then rinsing it before it was sealed and incubated. Colony growth was tracked with scanning electron microscopy and through colony counting in the agar with optical microscopy.

A careful reading of the literature will show that the most efficacious chainlength for the side chain is a point of contention and varies from Butyl to hexyl (as above) to decyl and dodecyl. Although the mode of action is still unclear, it

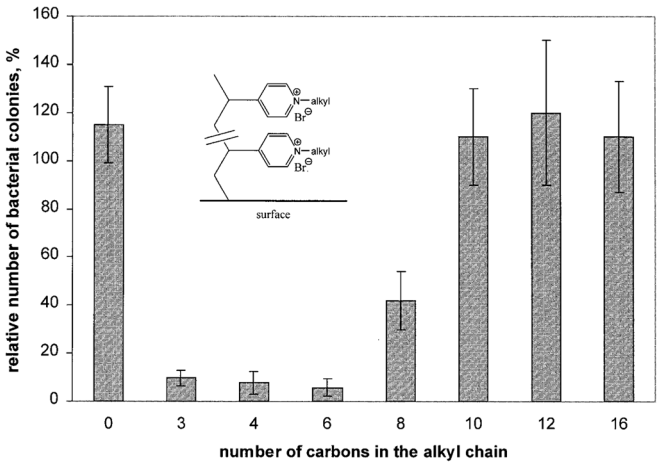

FIG. 7. Percentage of *S. aureus* colonies grown on the infected surfaces of glass slides modified with PVP that was N-alkylated with different linear alkyl bromides relative to the number of colonies grown on a commercial NH_2-glass slide (used as a standard). The bacterial cells were sprayed from an aqueous suspension (10^6 cells per ml) onto the surfaces. All experiments were performed at least in quadruplicate, and the error bars indicate the standard deviations from the mean values obtained. (Reprinted with permission from J. C. Tiller, C. J. Liao, K. Lewis, and A. M. Klibanov, Proc. Natl. Acad. Sci. U.S.A. **98**, 5981 (2001). Copyright 2001, National Academy of Sciences.)

is the authors' opinion that the charge density mechanism, which has been postulated by several labs, is likely the dominant factor. This mode has been hypothesized to be rooted in ion exchange with membrane and cell wall components. It seems plausible that the ion exchange will be affected by the polarizability and local environment of the immobilized AMA cations. In the case of the aromatic groups for which no methylation is needed, the environment is markedly different from that of the aliphatic tetrahedral quaternary ammonium group. With differences such as these, it is perhaps not surprising that the optimum chainlength would be different. The authors do not subscribe to the notion that the optimum chainlength is related to the hydrophobic side chain penetrating the membrane. The steric limitations of the polymer and surface structure caused by immobilization combined with the dimensions of the bacterial envelope seem to be at odds with this kind of intercalation. Of course, if trace amounts of the agent are free to elute from the surface, then this kind of molecular intercalation (and corresponding chainlength dependence) could certainly be more plausible.

Kugler *et al.*[25] performed charge measurements on quaternized PVP films and reported rapid kill in less than 10 min with live-dead staining. They also performed some interesting analyses of the film thickness and bacterial state. The film thickness was measured using ellipsometry. They found that the different cellular states (low or high cell division conditions) required different surface charge densities for *E. coli* and *S. epidermidis* of 10^{14} N^+/cm^2 in the low cell division state and 10^{13} and 10^{12} N^+/cm^2 in the high division conditions, respectively. Given that they observed efficacy for films with a thickness of 2 nm, they proposed that their data support an ion exchange mechanism of efficacy that has been previously discussed.[21,100,101]

Poly(vinylpyridine) AMAs have been synthesized from the surfaces of cellulose, polyethyleneterephthalate, and electrospun polyurethanes.[33,34] In each of these experiments, the authors initiated the polymerization of PVP from plasma activated surface sites. These PVP modified surfaces were then quaternized with hexylbromide. Yao *et al.* challenged the electrospun membranes with *S. aureus* and *E. coli* via an immersion method. The modified membranes showed a higher propensity for cell death for the *S. aureus* than the *E. coli*, with the former having a 5 log reduction in viable cell count after 4 h and the latter having an LRV of only 3. SEM images of the membranes showed the absence of intact cellular material on the treated samples; therefore, the reduction was not due simply to selective adhesion to the sample.

3. Fluorinated quaternary ammonium compounds

The nature of the side-chains on the quaternary amine has also been explored. The most notable success has involved the use of perfluorinated side groups. Krishnan *et al.* quaternized pyridinium polymers with a perfluorinated side chain,[92] although this work was not a direct comparison of fluorination because the fluorinated ($F(CF_2)_8(CH_2)_6Br$) and nonfluorinated ($H(CH_2)_6Br$) molecules were of different

lengths. The samples were glass slides that were coated with styrene ethylene butylene styrene (SEBS). This polymer surface was then sprayed with PVP and annealed. In this way the PVP was physically adsorbed to the SEBS film. The resulting surface was then quaternized with the aforementioned alkylbromides. As an aside, they also noted that complete quaternization led to reduced efficacy, and that better antimicrobial properties were observed with a lower quaternization fraction.

C. Amine containing polymers

The quaternary ammonium compounds are known to be antimicrobial, but some work has also examined the efficacy of the less substituted tertiary, secondary, and primary amines. Lichter and Rubner studied the efficacy of LbL films composed of the primary amines polyallylamine hydrochloride (PAH) and polystyrene sulfonate (PSS).[39] Spray and immersion based methods were used to challenge the surface, followed by growth based amplification methods to quantify the viability. Their conclusion was that the efficacy depended primarily upon the generation of a surface with a sufficient positive charge density, a hypothesis further supported by work done by Russell's group.[26] In that work, they affected the charge via the choice of the last layer (positive for PAH or negative for PSS) and the *p*H of the final rinse solution relative to the pKa of the PAH.

Martin *et al*. used plasma polymerization methods to polymerize the tertiary amine dimethylaminomethylstyrene to create polymer coated fabric surfaces.[38] They characterized the coating process via added mass and FTIR spectroscopy. A correlation between the reaction time and the amount of polymer added was established, and the FTIR was consistent with the presence of tertiary amine. The efficacies of these fabric swatches were tested via ASTM E2149, and the authors observed that a critical added mass was needed in order to attain substantial antimicrobial efficacy, a finding that is consistent with previous work that observed a critical charge density. A ~6 to 7 log reduction in microbe activity relative to unmodified controls was observed, and the authors confirmed that their fabric swath generated no visible "zone" with a ZOI test. Finally, the extracted solution in contact with the sample also generated no zone.

Madkour and coworkers created ATRP polymethacrylates with Butyl and ethylamine groups.[40] The film thicknesses ranged from 3 to 70 nm. All gave rapid and complete kill, and dilution of the surface initiators from 100% to 1% had virtually no impact on the performance of the film. Furthermore, the films lost antimicrobial activity with repeated exposure/rinsing cycles. The paper reported massive kill via the aerosolized inoculation method as determined by live-dead stain within 5 min of aerosolization. A modified JIS style test avoided the use of a coverslip, but spray deposition on the substrate enhanced any impact that potentially trace elutable agents would have by effectively concentrating them in a miniscule liquid between the cells and the substrate. The evidence against elution was the ZOI test, based on the fact that these polymers have been shown to be efficacious in solution.[41]

D. Guanidinium-based molecules and polymers

Guanides, biguanides, and their polymers have long been recognized for their antimicrobial activity and low human toxicity. Chlorhexidine (CHX), polyhexamethylenebiguanide, polyhexamethyleneguanide (PHMG), various oligoguanides, and other biguanides are known to be efficacious in solution.[4,76] Furthermore, their mode of action has long been linked to the disruption of the bacterial membranes. Asadinezhad and coworkers reacted chlorhexidine with surface initiated polyacrylic acid via ethyl(dimethylaminopropyl) carbodiimide (EDC) coupling to the CHX secondary amine groups and then used glutaraldehyde to crosslink neighboring CHX molecules. The reactions on the polyvinyl chloride (PVC) substrate were followed by FTIR, XPS, SEM, and finally bacterial adhesion.[102] The authors concluded that medical grade PVC modified in this way was more resiatant to the bacterial adhesion of *S. aureus* and *E. coli*.

In a paper by Chen *et al*.,[103] the authors reported combining electrospun cellulose acetate with chlorhexidine, polyethyleneglycol (PEG), and a triethanolamine titanium complex (Tyzor® TE) to generate composite polymer fiber meshes. The fibers were cured and the CHX composition was characterized via FTIR, Raman spectroscopy, and XPS. ZOI tests were used to assess the lack of elution and provide support for the immobilization of the CHX. The authors offer some detailed calculations for the ZOI beyond the normal treatment, and they used ASTM E2149 to verify that the agent modified parts were efficacious. They observed that the chlorhexidine modified parts killed at a ~2 to 3 log reduction at the highest weight percents of ~8% CHX.

Guan and coworkers[104] reacted the primary amine end groups of PHMG with glycidyl methacrylate to make a unique polyguanidylmethacrylate monomer. They then copolymerized this monomer from sulfite pulp (cellulose fibers) using ceric ammonium nitrate as an initiator. This modified cellulose was then characterized via energy dispersive x-ray, FTIR, charge density, AFM (topography and adhesion), and gravimetry (grafting efficiency). The authors measured the antimicrobial efficacy via a shake flask method very similar to that described in ASTM E2149. The results showed a substantial reduction (LRV > 4) of the bacterial viability within a 48 h inoculation.

E. Chitosan

Like guanide compounds, chitosan has long been reported as a naturally occurring antimicrobial polymer. Numerous researchers have immobilized chitosan to fibrous substrates such as wool, cotton, pulp, etc. These investigations have typically combined immersion inoculation methods with ZOI in order to demonstrate efficacy and support a lack of elution; because neither method reveals much about the immobilization of the molecules, the immobilized status of

the molecules is suspect. In a study by Vartiainen and coworkers, polypropylene films were plasma activated and coupled to medium molecular weight chitosan.[45] The coupling involved plasma treatment with an NH_3 or CO_2 rich atmosphere, with the intent of creating amine and carboxylic acid groups on the polymer surface. Chitosan was coupled to the substrate by using either glutaraldehyde or EDC coupling chemistries, and the samples were characterized via FTIR, SEM, and contact angles. The antimicrobial activity was assessed by placing 0.1 ml of inoculum in direct contact with the surface. The samples were inoculated for 24 h at 25 °C under humidified conditions. The cells were then diluted and displaced from the sample by gentle shaking, and the viability of the cells in the supernate was enumerated via standard plating methods. The authors observed a 3 to 5 log reduction in the viable bacteria, depending on the bacterium (*E. coli* or *B. subtilus*). Carlson and coworkers[49] coated PMMA substrates with chitosan and observed that the surfaces were antimicrobial. By exploiting live-dead staining and time-lapse confocal fluorescence microscopy, they were able to observe that as the cells approached and interacted with the surface, the cell membranes became permeable to the fluorescent dye.

F. Peptides

Bacteria, protozoa, fungi, plants, and animals produce a host of antimicrobial peptides,[105–107] many of which are documented in an online antimicrobial peptide database.[108] The database can be searched and sorted according to the mode of action on record. By restricting immobilization candidates to those AMPs that are known for membrane disruption, the list is reduced to a manageable size, such as magainin I, polymyxin B, defensins, apoprotinin, nisin, etc. Hilpert and coworkers put together an excellent piece of work that correlates the peptide structure (charge, hydrophobicity, and spatial structure) with the antimicrobial function,[60] and through systematic variations around known peptide sequences they identified a number of efficacious agents. They characterized the peptide efficacy by both solution (MIC) methods and luminescence from lux-reporter strains in wells with surfaces modified with the agent. Their review demonstrated that many of the efficacious AMPs were able to form some sort of amphipathic and cationic structures.

Humblot and coworkers immobilized magainin I (MAG) to a self-assembled monolayer of carboxylic acids on gold by using standard EDC/N-hydroxysuccinimide coupling to react with free amine groups on the MAG.[56] The surfaces were fully characterized via polarization modulation infrared reflection absorption spectroscopy, XPS, and AFM. The gold samples were inoculated by immersion and then rinsed. The adhered bacteria were live-dead stained, and fewer than half of the cells were stained alive. The authors concluded that although the MAG was effective at killing the cells that came into direct contact with the surface, the remnant dead cells might offer support for further cell attachment and growth that would be protected from the immobilized agent.

Glinel and coworkers grew a polymer film via ATRP composed of hydroxyl-terminated PEG groups. This largely antifouling surface was augmented by graft-to reactions of C-terminal cysteine modified MAG with the hydroxyl groups using p-maleimidophenyl isocyanate (a heterobifunctional reagent used to couple hydroxyl groups to thiols). In this way, the MAG was bound at a specific site and on the end of PEG tethers hanging off of the brush of a polymethacrylate backbone. The authors were able to vary the surface density of the peptide, and they found that even low immobilization densities were efficacious. The samples were inoculated via immersion in suspensions of two Gram-positive bacteria (*Listeria ivanovii* and *Bacillus cereus*), lightly rinsed,and stained with a live-dead stain. The efficacy was assessed via confocal laser scanning microscopy of the stained cells. The images demonstrated that some of the filamentous *B. cereus* and all of the *L. ivanovii* cells that remained following rinsing were dead.

Polymyxin B (PMB) has been immobilized to adhere and subsequently detect *Salmonella typhimurium* and *E. coli*.[57] Tzoris and coworkers were able to immobilize PMB to a radical polymerized copolymer of ethylacrylate and hydroxyethylacrylate. The hydroxyl groups were reacted with the amine groups on the PMB via standard coupling reactions using tresyl chloride. The authors determined that the PMB was not leaching via a novel ion selective FET method as well as a fluorescent labeling experiment. This led them to conclude that the elution in solution would be much less than the MIC for PMB.[58] They then determined the antimicrobial efficacy using an optical density measured growth curve lag between inoculated media that was or was not exposed to PMB immobilized glass. The bacteria in the media exposed to PMB glass had a delayed growth curve. From this, the authors concluded that the PMB was immobilized and efficacious while immobilized. Elastomers such as polydimethylsiloxane have also been modified with AMPs in an effort to attain antibiofilm properties.[59]

When considering AMPs, it is instructive to consider the mode of action. Although the modes of action of AMPs are still relatively unknown, there is substantial literature hypothesizing concerted multipeptide arrangements into complex quaternary structures (barrels, staves, and carpets) at the bacterial membrane. As a result, the antimicrobial performance of AMPs might be even more sensitive to immobilization than that of the simple polycationic systems mentioned above.

Patch and Barron give an excellent review of non-natural peptidomimetic oligomers.[63] As mimics can be found for virtually any peptide function, it is not surprising that some mimics have been identified for antimicrobial functionality. Statz and coworkers[65] examined the impact that surface bound peptide mimetics have on *E. coli* adhesion. In that study, the authors immobilized three different peptoid sequences to titania substrates: an antimicrobial peptoid, an antihemolysis/antifouling peptoid, and a filler peptoid.

Immobilization was confirmed with an assortment of surface analytical tools, and the antimicrobial efficacy of the surfaces was determined via fluorescence microscopy. The fluorescence data agreed with the solution phase MIC data for the free peptoids. One of the key advantages of these artificial peptoid motifs is their resistance to natural enzymes. This area is still very new, and relatively few mimetics have been immobilized and tested for antimicrobial efficacy.

G. Enzymes

A number of enzymes have evolved as antimicrobial agents in natural settings, and several of these naturally occurring enzymes have been used as bactericidal and antibiofilm agents. Chitinases have been mobilized against fungi, and proteases have been applied against prions. Autolysins are a group of enzymes generated by bacteria for regulation of their own cell wall, and they are usually highly specific to the originating bacteria. Common antimicrobial enzymes include proteinase K, trypsin, subtilisin, protease A, papain, umamizyme, dispersin B, neutrophil elastase, phospholipase A2, and of course lysozyme. Lysozyme has been immobilized to fabrics such as cotton[51] and wool,[50] as well as to polymer substrates such as polymethylmethacrylate, polyethylene, polypropylene, and polystyrene.[52,53] Conte *et al.* reacted crosslinked polyvinylalcohol (PVA) with lysozyme via gluteraldehyde, and the crosslinked solution was cast onto PMMA substrates. The elution of the lysozyme was tracked using high-performance liquid chromatography and, following extensive rinsing, was determined to be negligible. The antimicrobial efficacy of the immobilized enzyme was monitored in the same way that the activity of the enzyme would be determined, via a UV absorbance assay for the lysis of *Micrococcus lysodeikticus*. The authors found that the efficacy increased with the quantity of lysozyme immobilized in their PVA matrix.

Efforts by Vartiainen and co-workers have led to the immobilization of glucose-oxidase onto amine and carboxy activated polypropylene using standard coupling chemistry of glutaraldehyde and carbodiimide linkers, respectively.[54,55] These films were found to be antimicrobial using a "drop test" method[109] similar to that in an uncovered JIS-Z2801. In these cases, it should be noted that while the glucose oxidase is immobilized, the efficacious agent (hydrogen peroxide) is clearly free to diffuse from the surface, similar to chlorine and N-halamines. Therefore, this is not actually an immobilized enzymatic AMA. Enzymes have also been used to provide antifouling capabilities, as in the work by Asuri and coworkers[110] in which enzyme-nanotube composites were created and the antifouling nature was verified.

V. CONCLUSIONS AND FUTURE DIRECTIONS

In summary, there is a significant need for increased understanding of the modes of action of these surface bound antimicrobial agents. The immobilization places restrictions on the traditionally discussed modes of action. Some new modes have been suggested based on the overwhelming correlation with charge density, but these modes remain hypothetical, with limited experimental evidence. To the extent that the elution needs to be prevented and that the mode of action should work while the agent is immobilized, additional testing methods should be employed. In the least, a more critical use of the traditional testing methods should be adopted. Zone of inhibition should be abandoned as a proof of immobilization in all but extreme cases, and where used it should be accompanied by some simple calculations that demonstrate that its use is appropriate.

Of the various immobilization strategies, the surface-initiated or graft-from approaches offer the most confidence in the generation of samples that do not elute agent (truly immobilized AMAs). The graft-to methods can be adequate, as demonstrated in some cases described above, but in many cases the surface associated AMAs are a mixture of physically and covalently adhered molecules, allowing for trace elution of the physically adhered molecules over time. Furthermore, some of the common linking chemistries employed are labile (hydrolysis) and might degrade over time or in some conditions of use. Physical adsorption is generally a weaker immobilization method, but in some cases, such as LbL films, the multidentate charge interactions can lead to strong, essentially irreversible adhesion comparable to or greater than that found in covalent systems. However, depending upon the molecules, attention needs to be given to the potential *p*H or ionic strength sensitivity of the interactions, and those molecules not physically adsorbed by these multiple interactions are at much greater risk of elution. Perhaps the weakest systems, with respect to immobilization, are the as-formed systems. These systems often rely upon polymer intercalation or hydrophobicity to keep the molecules associated with the sample. However, time, *p*H, ionic strength, and the local environment of a cell in contact with the surface can enable increased elution of the molecule from the surface. However, the larger issue with these methods comes from the nature of the agent. Frequently, the agent is a high molecular weight polymer, but these are not monodisperse, and shorter molecular weight analogues will be more mobile and can elute while the larger molecules remain at the interface. In each of these cases, spectral data that associate a molecule with the surface do not equate to immobilization. In addition to surface association, some evidence for the molecules' irreversible state should also be presented. The difficulty with the analysis is that depending upon the efficacy test, trace amounts of elution can have a biological impact, and so the detection limits needed for the analysis can be quite challenging.

When evaluating the appropriateness of various immobilized agents for a specific application, there are several factors to consider, including the cost of materials and processing, the efficacy, sensitivity to and methods of inactivation, ease of use, the lifetime of the agent, and manufacturability and stability to sterilization procedures. The surface-initiated polymerization of highly positively charged surfaces (quaternary ammonium polymers) has demonstrated

efficacy and lack of elution at reasonable costs. Although manufacturability requires more planning, control, and cost than graft-to methods, groups such as the Matyjaszewski group have demonstrated that even controlled processes such as surface ATRP can be implemented in manufacturing settings. As with all immobilized AMAs, surface contamination can lead to inactivation of these surfaces, and, in fact, because these surfaces by their nature are highly positively charged, they are expected to strongly interact with anionic polymer cellular material. This inactivation simply limits the application space to certain environmental conditions. Graft-to coupling reactions of polyamines such as chitosan, biguanides, and quaternary ammonium polymers are easy to perform with standard chemistries and are readily amenable to manufacturing at relatively low costs. These are typically also highly charged and thus are subject to similar contamination limitations. Peptides and enzymes need to be coupled via graft-to, and whereas the cost for grafting is low, the cost for the agents can sometimes be very high, especially in the case of the peptides. In the case of biologically created enzymes, the source of the molecules can sometimes create additional regulatory hurdles and costs. In addition to the increased material costs, these might also pose greater manufacturing difficulties associated with the sensitivity of the molecules to processing solvents, sterilization, or other manufacturing conditions. Further limitations of these will result from their restricted mobility when immobilized, as their modes of action might require access to parts of the cell that are difficult for them to reach while immobilized. Lastly, and in addition to typical contamination based inactivation, these agents will have a reduced lifetime as a result of proteolytic degradation due to enzymes originating from host, the pathogen, or the environment.

Emerging directions for the agents and the agent properties include a focus on combining antifouling with antimicrobial properties. Alternate, promising directions include responsive or smart materials capable of switching from antifouling to antimicrobial when stimulated by the presence of microbes. In a closely related direction, some researchers have focused on microstructured surfaces used to minimize biofilm formation. Future developments of this textured aspect might generate added functionality, assuming that the data are truly representative. As new materials are added to surfaces for biomedical devices, the cytotoxicity will be of interest; however, given that the agents are immobilized, the toxicity is expected to be less important than for the corresponding biomedical devices with leaching antimicrobial agents.

Future developments will likely witness the application of additional test methods for immobilized AMA devices. Emerging areas for method development include VBNC test methods and measurement capabilities with larger dynamic ranges that properly measure and identify the kill as immobilized or eluted. Rapid microbiology methods have traditionally been utilized for the diagnosis of infectious diseases and, more recently, are being implemented to provide better control over manufacturing processes, as well as the earlier release of products. These methods typically use fluorescent probes or molecular biology methods such as reverse transcription polymerase chain reaction and mass spectroscopy to probe genomic, proteomic, or phenotypic differences in order to detect and identify viable organisms. These methods have not found their way into the efficacy testing of immobilized agents in the literature; however, they have strengths with respect to VBNCs and complicated realistic colonies made up of multiple species or strains. Perhaps as improved methods become available, some previously examined systems could be reexamined, with added attention paid to their mechanistic implications.

Depending upon the stringency of the immobilized criteria, there might be many examples of immobilized agents or very few. This pursuit is complicated by the testing method appropriateness, bacterial species/strains, resistance, and simple microbe surface interactions. This review highlights the efforts to date with regard to the immobilization of antimicrobial agents, and it is intended to cast some critical light on the appropriateness of the efficacy testing as it pertains to truly immobilized agents.

Nomenclature

AFM = atomic force microscopy
AMA = antimicrobial agent
AMP = antimicrobial peptide
ASTM = American Society for Testing and Materials
ATRP = atom transfer radical polymerization
B. subtilus = *Bacillus subtilis*
CFU = colony forming units
CHX = chlorhexidine
CPC = cetylpyridinium chloride
CVD = chemical vapor deposition
E. coli = *Escherichia coli*
EDC = ethyl(dimethylaminopropyl) carbodiimide
FET = field effect transistor
FTIR = Fourier transform infrared
HAI = hospital associated infection
iAMA = immobilized antimicrobial agent(s)
JIS = Japanese Industry Standard
LbL = layer by layer
LRV = log reduction value
MAG = magainin I
MBC = minimum bactericidal concentration
MIC = minimum inhibitory concentration
PAH = polyallylamine hydrochloride
PEG = polyethyleneglycol
PEI = polyethyleneimine
PHMG = polyhexamethyleneguanide
PMB = polymyxin B
PSS = polystyrene sulfonate
PVA = polyvinylalcohol
PVC = polyvinyl chloride
PVP = polyvinylpyridinium
QA = quaternary ammonium

S. aureus = *Staphylococcus aureus*
SEBS = styrene ethylene butylene styrene
SEM = scanning electron microscopy
Si-QAC = (3-trimethoxysilyl) propyldimethyloctadecyl ammonium chloride
VBNC = viable but nonculturable
XPS = x-ray photoelectron spectroscopy
ZOI = zone of inhibition

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