Immobilized Antimicrobial Agents: A Critical Perspective

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Traditionally antimicrobial devices and applications have leveraged the elution of biocides from devices which were infused with these active molecules. The elution of these agents can, in some cases, limit the application of the device and can potentially lead to unwanted side effects. Following the ground breaking works of Isquith, Abbott and Walters, surface immobilized antimicrobial agents (iAMA) have been explored. Devices sporting immobilized agents may remain active longer and minimize side effects. Researchers have examined the structure-activity of antimicrobial agents appropriate for immobilized applications. A great deal of effort goes into the design, synthesis, immobilization and characterization of the AMA’s, and yet it is common for the activity measurement to be off-the-shelf methods that are poorly aligned with the needs of immobilized agents. As a result, much time and effort is wasted developing structure-activity relationships based on questionable interpretations. The purpose of this review is to educate and guide researchers planning to develop immobilized antimicrobial agents. As such, the focus is not only to review the agents which have been immobilized, but also to highlight the key means of immobilization and importantly the activity testing methods.

Keywords immobilized antimicrobial agent; efficacy testing, immobilization strategies,

Introduction

Microbial contamination and infection pose a serious concern for food, military and medical industries. The impact to healthcare is undisputed, with hospital associated infections (HAIs) from bacterial, fungal and viral agents contributing to ~100,000 deaths annually in the USA alone[1,2]. The first line of defense against food spoilage and medical infections is the avoidance of microbial load, followed by the appropriate conditions that minimize microbial growth. Additional protection can be added through the use of containers and devices that possess antimicrobial or antifouling properties.

Most of the antimicrobial applications have exploited eluting agents[3-7] such as benzalkonium chlorides, cetylpyridinium chloride, aldehydes, anilides, diamidines, silver, chlorhexidine, triclosan, N-halamines, and povidone-iodine. While such agents are known to be efficacious and appropriate for specific applications, their extension to some medical device applications may be hindered by the elution of the agent, due to a limited reservoir capacity or potential side-effects caused by these extractables. Immobilized antimicrobial agents offer an alternative motif which 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, as well as natural and man-made fabrics[66].

This chapter will guide the reader through the various antimicrobial agents that have been immobilized, and then touch on some key points such as mode of action and the testing methods. These last points are especially relevant for researchers who are developing new materials or are evaluating established materials. The antimicrobial mode of action[2,67-75] for an agent is expected to be affected by the process of immobilization, since the chemical composition and dimensions of the extra-cytoplasmic bacterial components (membranes, peptidoglycan wall, capsule, fimbiae and flagella if present), are relevant to the performance of surface tethered AMAs. For example, given that the mode of action for tetracycline involves disruption of the binding between 16S rRNA and tRNA[76], surface immobilization via a short tether severely restrict it’s access from the cell interior, thus dramatically reducing if not eliminating the AMA’s efficacy. Once immobilized, the appropriate choice of test method and the interpretation of the results can be complicated by numerous variables. This chapter will attempt to provide insight into these factors and assist researchers in the appropriate choice of test methods for their immobilized AMA samples.

Immobilization Strategies

There are a number of different strategies for immobilization of AMA to substrates: (1) “graft-to” strategies involve the covalent coupling of the intact AMA to a surface; (2) “physical adsorption” methods involve physisorption of the AMA through non-covalent 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 which contains the AMA at the time the substrate is formed. Frequently researchers employ a multi-step process that involves two or more strategies, for example graft-from synthesis of polymer brushes which are then reacted with AMA’s via graft-to pathways. Graft-to strategies begin with the synthesis or purchase of a potentially
surface reactive AMA. Frequently, the surface requires an activation process that generates amine, carboxylic acid, aldehyde or thiol functionalities. The AMA can be coupled to the activated substrate by use of heterobifunctional linkers such as succinimide, carbodiimide, maleimide, or aldehyde[77]; though sometimes the linking uses click-chemistry[78]. Sometimes these linkers contain polymer 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. While the graft-to methods appear strong and irreversible by the nature of the covalent bonds, sometimes these bonds can be hydrolyzed or broken by mechanical forces. Physisorption can sometimes be stronger and more irreversible than covalent bonding because of multi-dentate interactions such as hydrogen bonding, ionic bonding, or even steric interactions caused by entanglement during solvent swelling of polymer films. The best examples of robust physisorbed films are the layer-by-layer or LbL films[79], which are often combined with eluting strategies, but some cases use quaternary ammonium or other AMA as the outer layer of the LbL system[80]. Other physical absorption methods involve exploiting the charge pairing or strong ionic bonding to hold smaller AMA to a substrate with opposite charge. When the physical interaction involves ionizable groups, the pH of the environment is of key importance to the stability of the film. In as-formed strategies the molecules are incorporated during the substrate formation process, and become crosslinked or entangled within the substrate polymers. Nagel and coworkers demonstrate that surface reactive injection molding can generate permanently modified parts, in this case polycarbonate with PEI presented at the surface[81]. Namba et al., included an AMA within the ingredients for methacrylic polymerization, thus entangling the AMA within the substrate matrix[12]. Unlike the above methods, surface initiated or graft-from strategies provide a higher degree of control over the immobilization process. In these methods, molecules are synthesized directly from the surface, as in the case of solid phase synthesis of peptides or oligonucleotides. Well defined polymeric structures have been created through “living” or controlled polymerization techniques[82], such as reversible addition fragmentation chain transfer polymerization (RAFT) [83,84], nitroxide mediated polymerization (NMP)[85] and metal catalyzed living radical polymerizations like atom transfer radical polymerization (ATRP).[86]. 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 (CAPE) has been used to synthesize antimicrobial peptides (AMP) directly on cellulose substrates. Sometimes researchers use multi-step methods by combining strategies. In one example, researchers combined graft-to and surface-initiated methods to modify substrates with complex polymer films[38].

![Immobilization schemes](image)

**Figure 1 Some immobilization schemes.** (A) Physical adsorption methods by noncovalent perhaps multidentate interactions e.g., LbL films, block polymers, etc. (B) Graft-to methods by creating covalent bonds with the surface e.g., PEIs, AMPs, enzymes. (C) Graft-from or surface-initiated via synthesis of AMA from initiator at surface perhaps by ATRP e.g., PVP, PDMAEMA, methacrylates. From Barbey et al. Chemical Reviews. 2009;109(11):5437-5527.

**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 these different classes of agents. The literature is full of potential explanations for the mechanism or mode of action for each of these agents, and much of these hypotheses are based upon the results from standard efficacy tests. These AMA specific sections will briefly touch on the test methods, but they will be covered in more depth and with more critical assessment later. The field of immobilized AMAs has become quite expansive. This review is not able to cite everything, but instead aims to highlight those key publications.
Quaternary ammonium silanes and other small molecules

Perhaps the most well-known immobilized antimicrobial agent is the silane (3-trimethoxysilyl) propyldimethylolctadecyl ammonium chloride[10], Si-QAC. This molecule, as with any tri-alkoxy silane, can autopolymerize to form long branched polymer chains with an (-Si-O-) backbone. The quaternary ammonium side chains on this siloxane polymer form a similar motif to the quaternary ammonium polymers discussed below. In the initial work on this molecule, the silane was bound to a wide range of different substrates, including siliceous surfaces, man-made fibers, natural fibers, metals and assorted industrial materials[11]. These modified surfaces were tested against a host of microbes; including bacteria (both gram positive and negative), yeast, algae and fungi. A modified ASTM 2149E and an aerosol method were used to probe antimicrobial efficacy, and radiolabeling was used to prove that the agent did not elute from the surface. This initial work was followed by more detailed publications of the silane immobilized to different substrates and under different application conditions[14]. Each treatment was slightly different, and the resulting films may be different from the films created by the initial researchers. In one example of note, Si-QAC was reacted silicone rubber, and using a flow cell combined with rinsing and live-dead staining, they clearly demonstrated that the modified surfaces supported more dead bacteria than the unmodified controls[16]. 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. The effect was even observed after the surfaces were exposed to human plasma. In even more extreme applications, the authors performed a series of in vivo experiments, and they observed that the samples inoculated ex vivo were efficacious while those inoculated in vivo were not.

In addition to these quaternary ammonium silanes, other small molecules such as aminoglycoside antibiotics and cetlypyridinium chloride (CPC) have been immobilized[12][13]. However, in each case whether immobilized by covalent coupling or incorporated in as-formed films, these examples usually show tell-tale signs of elution of the agents. As such, even though some of the agents may have been immobilized and may remain associated with the part throughout the experiment, these surfaces are likely efficacious via the elution of AMAs. In some cases this was evidenced by ZOI data, and in some by the basic absence of any physical mechanism to immobilize the small soluble efficacious molecules.

Quaternary ammonium polymers

The most thoroughly studied class of AMAs are the quaternary ammonium polymers[18-21], PQAs, with polymer backbones such as polyethyleneimine (PEI), polyvinylpyridinium (PVP), chitosan, and assorted acrylates. These polymers have been immobilized by virtually every method listed above, and are often modified by post immobilization reactions. Much of the graft-to work has involved PVP and PEI using the immobilization process to control the polymer surface density and subsequent on-surface quaternization with different sidechains and counterions[17,22,30,31]. Other groups have focused efforts on surface initiated polymerization 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 surface charge density, polymer chain length, polymer chain density, counter ion identity, and quaternary ammonium sidechain length.

Aliphatic quaternary ammonium polymers

The most common aliphatic polymer systems studied have been polyethyleneimine (PEI) and its derivatives. It is common to immobilize the PEI by graft-to methods, although some groups have used as-formed methods akin to painting to create a thick film of the PEI. The painting process did not immobilize the molecules covalently, but instead they exploited the inherently poor solubility of these polymers as a barrier to dissolution in the inoculum. Two specific studies focused on the length of the sidechain and the charge of functional groups[23][35]. The PEI 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. Their results showed that positive charge was necessary, and that neither the neutral nor the zwitter-ionic surfaces were efficacious. They also concluded that when terminally methylated, the chainlength of the alkylating group should be greater than n-butyl for a high efficacy. The covalent strategy found similar results for both Gram positive (S. aureus and S. epidermidis) and negative (E. coli and P. aeruginosa) bacteria. The painted study demonstrated 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 PFU 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 200mg/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 is essentially a saturated solution of the agent, while the first method may still have locally high concentration at the surface due to the diffusion gradient slowly increasing the bulk solution concentration.
However both methods are expected to have concentrations much less than the apparent concentration at the interface. Interestingly, one group examined the bacterial resistance (or lack thereof) with respect to these quaternized PEI surfaces[30]. Surfaces with PEI that was hexyl,methyl quaternized, showed efficacy against both \textit{E. coli} and \textit{S. aureus}. 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.

![Figure 2](image)

\textbf{Figure 2} No resistance developed for quaternary ammonium surfaces. A series of bactericidal activity assays for amino-glass slides covalently coupled with N-hexyl,methyl-PEI against airborne \textit{S.aureus} (A) and \textit{E. coli} (B). In each assay, the bacterial population originated from a single colony of surviving bacteria from the preceding assay. From Milovic et al. \textit{Biotechnology and Bioengineering}. 2005;90(6):715-722.

Like many of the PQAs, polyethyleneimine is formed via relatively uncontrolled methods, that generate a wide range of different molecular weights as well as branching topologies. A number of groups have used controlled radical polymerization to produce PQAs that are monodisperse and with uniform molecular architectures. Some researchers have used graft-to immobilization of these uniform and controlled block polymers created by ATRP[26]. The polymers contained surface grafting regions as well as dimethyl amine regions. The surface density of the immobilized PQA was controlled via the polymer solution concentration, immersion time and molecular architecture. They were able to correlate the surface charge density with their observed efficacy. In the previous work, the initiator for ATRP was free in solution when the polymer was synthesized; however, by immobilizing the initiator to the substrate the same controlled polymer morphology could be achieved, and with some added control over the polymer chain density. The same group coated glass substrates with ATRP initiators for the controlled polymerization of dimethylaminoethylmethacrylate[24]. Subsequent quaternization with alkylhalides formed the immobilized PQAs. By controlling the surface density of initiators and the polymerization reaction time, they were able to independently control the polymer density and polymer chainlength respectively. This enabled systematic evaluation of key parameters without concern for elution of polymers. They concluded that the key operational parameter in efficacy was the surface charge density, and not necessarily the polymer length. This has mechanistic implications, which they explored and compared to other literature. According to this work, as long as the PQA surface charge density is greater than $5 \times 10^{15}$ charges/cm$^2$ the film will be efficacious against \textit{E. coli}, which coincidentally also has a surface charge density of $10^{14}-10^{15}$ charges/cm$^2$ depending on the growth stage of the cell. Of course both of these studies fully characterized their substrates via surface analytical tools, and an interesting observation came when comparing the efficacies of 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, resulting in local regions of higher relative QA densities. By using micropatterning they were able to generate areas of the substrate where agent was immobilized directly next to areas free of the AMA[26]. This enabled their live-dead stain images to spatially differentiate between kill over the immobilized agent from 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 is essentially a microscopic version of the ZOI test, but instead of being under growth conditions, it is under the more relevant test conditions. This paper makes frequent connection between interesting mechanistically relevant molecular properties of the film and the efficacy; for example, the number of QA groups needed to kill a bacterium, in this case $10^{10}$QA/bacterium.
Aromatic quaternary ammonium polymers

Of the aromatic PQAs, none is more studied than polyvinylpyridinium and the quaternized derivatives[32]. Given the nature of the pyridinium nitrogen, alkylation with a single group renders it quaternized. Researchers have focused on the nature of the sidechain, its length and chemistry[21]. The authors found an interesting relationship between chain length and efficacy, in that there was an optimal length of the N-alkylated group, with hexyl ammonium quats having the greatest efficacy relative to the longer or shorter chainlengths. Other groups performed charge measurements on quaternized PVP films and reported rapid kill in less than 10 minutes with live-dead staining[25]. They also performed some interesting analysis of the bacterial state. They found that the different cellular states (low or high cell division conditions) required a different surface charge density \((10^{14} \text{ N}^+/\text{cm}^2)\) for \(E. \text{coli}\) and \(S. \text{epidermidis}\) in the low cell division state, but \(10^{13}\) and \(10^{12}\) in the high division conditions respectively. Given that they observed efficacy for films with thicknesses of 2nm, they proposed that their data supports an ion exchange mechanism of efficacy[21,87,88]. PVP AMAs have also been synthesized from a host of surfaces e.g., cellulose, polyethyleneterephthalate (PET) and electrospun polyurethanes[33,34]. In each case, the polymerization was initiated from plasma activated surfaces. These PVP modified surfaces were then quaternized with hexylbromide, the optimum length reported above. Yao et al. challenged the electrospun membranes with \(S. \text{aureus}\) and \(E. \text{coli}\) by an immersion method. The modified membranes showed a higher propensity for cell death for the \(S. \text{aureus}\) than the \(E. \text{coli}\), with the former having a 5 log reduction in viable cell count after 4 hours, 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 simply due to selective adhesion to the sample.

The common form for these quaternary ammonium compounds include not only the quaternary ammonium group, but also a hydrophobic side chain. The historical hypothesis is that the length and hydrophobicity of the side chain functions to interact with the bacterial membrane, thereby assisting with the disruption of the membrane. As such most of the sidechains have been aliphatic hydrocarbons; however, some work has explored alternative hydrophobic groups, notably perfluorinated side groups[79]. This work compared the use of fluorinated \((\text{F(CF}_2)_n(\text{CH}_2)_n\text{Br})\) and nonfluorinated \((\text{H(CH}_2)_n\text{Br})\) molecules for the quaternization of PVP coated styrene ethylene butylene styrene (SEBS). Their primary observation was that fluorinated side chains improved efficacy, but they also observed high levels of quaternization led to a reduced efficacy.

Figure 3 Biocidal dependence on surface charge density.
The biocidal efficacy is related to the surface charge density of QA groups, as probed by charge coupled fluorescence assays. The grafting-to surfaces gave higher efficacy at apparently lower densities, and this was hypothesized to result from film heterogeneity, resulting in small areas with higher charge densities. From Huang et al. Langmuir. 2008;24(13):6785-6795.

Figure 4 Optimum chainlength.
A series of different n-alkylhalides were used to quaternize surface bound pyridinium polymers. The efficacy depended on the length of the alkylhalide, with the optimum length corresponding to hexyl bromide. From Tiller et al. Proc. Natl. Acad. Sci. U.S.A. 2001;98(11):5981-5985.
Amine containing molecules and polymers

Based on modes of action that involve molecular charge, the less substituted amines should be efficacious as well[41]. Under normal pH conditions primary, secondary and tertiary amines will be protonated, and therefore positively charged. In an interesting series of experiments, Lichter and Rubner studied the efficacy of LbL films composed of alternating layers of the positively charged primary amine polyallylaminehydrochloride (PAH) and the negatively charged polystyrenesulfonate (PSS)[39]. As with the alternate work described above[26], they observed that the efficacy depended primarily upon generating a surface with sufficient positive charge density. Using plasma methods, dimethylaminomethylstyrene was polymerized on textiles[38]. They found that a critical added mass was needed to attain substantial antimicrobial efficacy, consistent with previous work that observed critical charge density. Although based on the method of formation, these films are expected to be composed of many loosely bound polymers. Meaning that thicker films might release more free molecules thereby leading to better efficacy via elution mechanisms. A 6-7 log reduction in microbe activity relative to unmodified controls was observed, and they used zone of inhibition tests to confirm that their fabric swatch was not eluting. Still other groups created surface initiated ATRP growth of polymethacrylates with butyl and ethylamine groups[40]. Film thicknesses ranging from 3nm to 70nm all gave rapid and complete kill; furthermore, dilution of the surface initiators from 100 to 1% had virtually no impact on the performance of the film. Unfortunately the films lost antimicrobial activity with repeated exposure/rinsing cycles. They observed massive kill within five minutes inoculation contact time. As with many researchers they used ZOI as a method to prove that the agent was not leaching.

Chlorhexidine (CHX), polyhexamethylenebiguanide (PHMB), polyhexamethyleneguanide (PHMG), various oligoguanides and other biguanides have long been recognized for their antimicrobial activity and low human toxicity[4][75]. As membrane disruptive agents, they may retain some efficacy when immobilized. Researchers have immobilized biguanides by crosslinking to polyacrylic acid[89], electrospinning[90] as a polymer composite and by polymerization of synthetic biguanide-acrylates[91]. In each case, analytical methods associated the biguanides with the substrate, but the arguments against elution based efficacy were supported by at best ZOI testing.

Chitosan is an inexpensive, naturally occurring polymer that has shown efficacy in solution as an AMA and on surfaces as an antifouling agent. It has been immobilized to fibrous substrates such as wool, cotton, pulp, etc, and these investigations have almost exclusively used shake-flask and ZOI testing to demonstrate efficacy and immobilization. Typically combined immersion inoculation methods with ZOI to demonstrate efficacy and support the lack of elution. In one study, polypropylene films were modified with medium molecular weight chitosan via standard coupling chemistries[45]. The authors observed a 3-5 log reduction in the viable bacteria depending on the bacterium (E.coli or B. subtilis). Another group 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 visualize the real-time permeabilization (death) of the microbes as they approached and interacted with the surface[49].

Peptides

All organisms produce antimicrobial peptides[92][93][94], and the online antimicrobial peptide database has an outstanding coverage of the known peptides that have been studied in the literature[95]. The database is an excellent
resource for anyone interested in AMPs. While the cost of AMPs will be prohibitive for many applications, there may be some device applications where the quantity needed may be appropriate. The fraction of peptides in the database that are known or suspected to be membrane disruptive is relatively short, including molecules such as, magainin I, polymyxin B, defensins, apoprotinin, nisin, etc. The mode of action for membrane disruptive peptides suggests some key structural properties, and some authors have correlated structure (charge, hydrophobicity and spatial structure) with the antimicrobial performance[60]. In this work, they identified target peptides and verified that they were efficacious. The key structure of the efficacious peptides was that they possessed amphipathic and cationic structures. One well-known AMP was immobilized to gold alkylthiolate monolayers, and these surfaces exhibited contact kill[56]. Still other groups tethered MAG to the end of surface bound antifouling PEG chains. Unlike much of the AMP-surface crosslinking work which reacts with amine groups on the peptide, these authors exploited a thiol group on the MAG to orient peptide. Interestingly, they observed 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 by 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 which remained following rinsing were dead. In addition to MAG, another popular AMP is Polymyxin B (PMB), which has been immobilized for biowarfare sensors[57]. Like MAG, immobilized PMB has demonstrated some efficacy, though by rather non-standard testing method. In these cases, the authors examined a shift in the growth curve for bacteria in the presence of the modified surface[58]. Unlike most leaching tests, these authors also used a novel field effect transistor method to confirm a lack of leached PMB. Elastomers such as PDMS have also been modified with AMPs, in an effort to attain antibiofilm properties[59].

![Image](image.png)

**Figure 6 Immobilized peptides show kill.** The antimicrobial peptide magainin I was immobilized to gold thiolate monolayers with standard coupling chemistry. By using live-dead staining, the control thiol surface without peptide (a) was not efficacious, while the magainin I modified surface (b) was efficacious. From Humblot et al. Biomaterials. 2009;30(21):3503-3512.

In addition to the naturally occurring and synthetic peptides, some groups have been working on synthetic peptoids (molecules that will not be vulnerable to proteolytic degradation. Patch and Barron give an excellent review of non-natural peptidomimetic oligomers[63], and Statz and coworkers[65] examined the impact that surface bound peptide mimetics have on *E. coli* adhesion. In this 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 were determined with fluorescence microscopy. Their fluorescence data agreed with the solution phase minimum inhibitory concentration data for the free peptoids.

**Enzymes**

As with peptides, most organisms generate enzymes as a part of their antimicrobial strategy. Several of these naturally occurring enzymes have been used as bacteriocidal and antibiofilm agents. Also like the peptides, these will likely have limited applicability, due both the cost and the sensitivity to thermal and proteolytic degradation. Chitinases have been immobilized against fungi, and proteases have also been applied against prions. Autolysins, are a group of enzymes generated by bacteria for regulation of their own cell wall, and these are usually highly specific to the originating bacteria. Some common antimicrobial enzymes (AMEs) 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 polymer substrates such as polymethylmethacrylate, polyethylene, polypropylene, polystyrene[52][53]. The elution of the lysozyme has sometimes been tracked with
HPLC, and following extensive rinsing was determined to be negligible. There are sensitive assays that can detect trace quantities of free enzyme. 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. Enzymes have also been used to provide antifouling capabilities[96].

**Efficacy testing**

One of the most challenging components to the assessment of immobilized antimicrobial agents is the efficacy test. The methods are not inherently difficult to perform, in fact many of the test methods are relatively simple and readily implemented. The difficulty centers on the interpretation of the results, which are directly related to the choice of method. Method selection and implementation requires the synthesis of not only microbiology but also surface science, fluid dynamics and assorted chemical engineering fields. The test methods that have been used to date can be grouped into two different categories: (1) growth-based amplification/inhibition; and (2) live-dead staining. Given that these studies are for immobilized agents, the methods are necessarily heterogeneous, with a solid phase on which the agent is immobilized and a liquid phase. The immobilized substrate can be inoculated with the microbes in a range of different manners, including immersion of the sample within a large volume of inoculum, direct contact of a small droplet onto the surface of the sample, aerosolization of microbes onto the surface, as well as other interesting variants. When choosing the method, and in anticipation of results and interpretation, it is useful to consider mass transport. Mass transport of the microbe to and from the substrate as well as transport of potentially eluting agents from the substrate. These processes will depend upon the method chosen, and open a range of questions that directly impact the interpretation of the results. For example, does the eluted agent develop a concentration gradient at the surface, perhaps within 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 experience concentrations much greater than the bulk, but how much more will depend upon numerous variables.

**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 may be observed where the concentration exceeds the 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 the total amount of agent that is available to diffuse[98-100]. For truly immobilized AMAs the quantity of material is likely to be severely limited, and can be estimated by knowledge of the approximate surface area of the part. A brief calculation of the expected zone is recommended for anyone using this method to conclude that the agent is immobilized. It may be that even if *all* of the AMA molecules eluted from the surface, the volume corresponding to the critical concentration may correspond to an undetectably small zone. It is also important to compare similar environments. The growth media used for ZOI may be inappropriate when the enumeration testing is performed in a much cleaner saline suspension, which may 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 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 may contain ionized groups. Together these factors can negate the value of ZOI testing for samples where the agent was intended to be immobilized.

**Figure 7 Zone of inhibition.** The zone of inhibition test is a visual test where bacterial growth is inhibited from a region around a sample. The sample is in or on growth media, and the zone represents the diffusion volume wherein the agent has eluted with concentrations adequate to inhibit growth (MIC) in the growth medium. From Lee et al. Langmuir. 2005;21(21):9651-9659.
Immersion inoculation

The most common tests used for immobilized AMAs are the immersive inoculation methods, especially the shake flask method ASTM E2149 which has been specifically called out for use with immobilized agents[101]. These methods involve the complete immersion of a sample into an inoculum solution which is agitated for a specified time. After the inoculation time, an aliquot of the microbial suspension is sampled and the viability is tested, usually via growth based amplification. Factors such as cell adhesion and elution of the agent can dramatically impact the interpretation of the results, as will be discussed below. Since the method actually measures the viability of the cells that remain suspended, as viable cells are removed from the suspension by adhesion to the sample, they are counted as dead cells. A control sample is used for comparison to the active sample, and while the control and active samples are often made of the same substrate material (size, surface area, roughness, etc.), the nature of chemical modification, necessitates that the sample and control will have very different chemistries. As shown above, most of the antimicrobial agents are positive, and frequently there is a correlation between the efficacy and the positive charge density on the surface. This charge difference could have a dramatic impact on the cell-surface adhesion, since many of the bacterial cell surfaces contain negatively charged polymers.

In an effort to prove that the leaching of the AMA is irrelevant to the test method, some researchers have immersed the sample in an inoculum-free solution, and then inoculated this solution in absence of the sample[23]. The lack of efficacy of this post-sample-contact solution was used to rule out leaching as a factor for kill. Mass transport of the agent from a surface is key to interpreting these results[97], and while the theory is conceptually rather simple and well-known, a predictive understanding can be very complicated in a real-world system. Nonetheless, a thought experiment that examines the various possible outcomes may be instructive. Firstly, and as expected, if the AMA is truly immobilized, then the solution will be non-efficacious. Furthermore, if the AMA molecules rapidly elute from a part, then the concentration in the bulk will rapidly approach a limiting value which may exceed the MBC for this solution. If it does then it will be detected as efficacious, and the conclusion will be elution. However, 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 at a level needed to kill the microbes, when in fact it did elute, but it was diluted below the critical concentration in that volume. These are all reasonable conclusions; however, an interesting outcome of this last condition may occur when the elution rate is comparable to or less than the rate at which the cells sample the surface. In this case, the planktonic cells approach a surface which is still eluting agent, and as the microbes do so, they will experience concentrations in excess of the bulk concentration, and the microbes may die as a result, even though the bulk solution remains far below the critical value (even at the end of the experiment). The conclusion of this would be that the surface killed but not by elution, since the bulk solution concentration remains non-biocidal. This oversimplified thought experiment can be used as a guide, but the reality is expected to be significantly more complicated. This thought experiment does not contradict previous real experiments, but it does suggest that a comprehensive understanding of the mechanism may benefit from a more critical tool capable of discriminating between the immobilized and leachable kill. Perhaps some microscopic imaging methods near the surface under test conditions comparable those of the shake flask method would provide insight.

Direct inoculation

Several groups have been using direct inoculation methods which place a small droplet of inoculum directly in contact with the active surface. Two commonly cited methods have been developed by the Japanese Standards Association and the International Standards Organization, JIS-Z-2801 and ISO 22196 respectively. Although these standard methods are not explicitly designed for use with immobilized agents[102], it has nonetheless become common practice to apply these direct methods to systems with purportedly immobilized agents. This method involves placing a small droplet (10-100µL) of inoculum directly on the surface of the sample, followed by placing a 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 the released cells are typically enumerated as CFUs. This coverslip, which is not usually antimicrobial, can adhere cells from the inoculum. It is not uncommon to see direct inoculation results for ostensibly immobilized agents with log reduction values on the order of 3 to 8. While bacterial adhesion to the coverslip will depend upon the bacterial strain, some groups have observed 10-25% of some strains of S. aureus and E. coli strongly adhere to the coverslip. If even 10% of the inoculum adhere to the coverslip, and if 10% of these 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 5-25 microns, and that diffusion across this distance would be difficult to observe with ZOI, none the less ZOI is commonly used in conjunction with direct methods to demonstrate that the agent is immobilized. The coverslip adhesion can be avoided by excluding the coverslip, thereby providing the cells with only the sample surface. 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.
Surface growth methods

Whatever the means of inoculating the sample, the viability of microbes on the sample surface may be tested via taking the surface and placing it in direct contact with a semi-solid nutrient rich media. Under appropriate conditions the viable cells on the sample will grow into colonies that can then be enumerated. These methods amplify the viable cells to visible colonies; however, they cannot be diluted, as they are attached to the surface, and so neighboring microbes that are positioned within a set distance may lead to a single visible colony. Nonetheless, this semi-quantitative method can be used to screen surfaces that are efficacious from those that are not. While any method of inoculation may be used, it is most common to use this test method for aerosolization based inoculation experiments, wherein a thin film of pathogens is applied across a 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.

These test methods are excellent methods to emulate ambient contamination of surfaces and the corresponding antimicrobial efficacy of the surface; however, as it pertains to 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, if all of the fluid between the bacteria and the surface was removed, the bacterial surface is still in direct contact with the surface, and diffusion of trace non-immobilized AMAs can still occur. In this geometry, the impact of eluted agents is likely to be greatly amplified, as compared even with the direct inoculation methods above. By way of a very coarse example, suppose that a 1cm² sample is inoculated, and suppose that the same sample elutes enough free AMA into a 10mL 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 the direct inoculation methods, a 10µm 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 100nm) the relative concentration may approach 1000MBC. Of course, 100nm is probably thicker than expected if the cell is in direct contact with the surface, and so higher concentrations are plausible, with a real limit being the amount of free agent and the solubility of the agent in that fluid/extracellular layer. This simplistic back-of-the-envelope calculation simply highlights the potential amplification of trace elutables on 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 to assess the impact that these potential elutables would have on a real system.

Fluorescent and luminescent methods

There are a number of semiquantitative methods that exploit luminiscence to detect the viability of microbes. The most common of these methods use commercially available live-dead stain kits, where the stains probe various properties of the microbe such as membrane permeability, metabolic activity, etc.[106,107] These kinds of fluorescent stains can be used with fluorescent microscopy, as well as flow cytometry.[108,109] One conceptually simple live-dead staining technique has demonstrated the ability to determine whether the antimicrobial agent kills cells at the surface or at a distance.[110] 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. 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 metabolic activity or other properties of the microbes. As with the other test methods, live-dead staining has many limitations. Firstly, it has a limited dynamic range (usually from 5-95 percent 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, thereby swamping the bacterial signal. Bacterial adhesion to the surfaces is discussed in detail in the following chapter.

Bioluminescence has also been exploited for testing antimicrobial properties of devices, though usually it is used for biofouling and biofilm experiments[60,111]. Typically, these experiments genetically engineered lux-reporter strains, and the bioluminescence is a measure of the cellular respiration. Following the initial transfection of the bacteria with a gene for bioluminescent process, these bacteria are handeled in a similar manner to other bacterial strains. In principle, this method could be applied to any bacterial strain to create a lux-version of that strain. However, once created this is a new strain, and some of it’s energetics and architecture will be devoted to maintenance of the bioluminescence, potentially impacting the ability to compete and defend against antimicrobial therapies.
Critical Discussion and Conclusions

Without a doubt, one of the most detrimental activities to the proper interpretation of the immobilized AMA efficacy has been the use of ZOI testing as a rubber stamp verification that the AMA is not eluting. While these methods are good for identifying massive elution from loaded pads, the numbers do not support ZOI application to monolayer or even multilayer modified samples. The next most hazardous activity has been the use of JIS style direct inoculation methods to obtain quantitative efficacy for agents that are presumed immobilized as a result of ZOI data. These direct measurements sometimes provide extremely high log reduction values, and it has been suggested in the literature, that these high values correspond to an immobilized agent that was exceptionally efficacious eventhough bound to the substrate. Depending upon cell adhesion and the experimental details these results may be accurate, but there may also be a serious flaw, which was outlined above, and the data may in fact support eluting efficacy. These two factors may have misdirected researchers away from truly immobilized efficacious agents, because of apparently low efficacies and toward agents whose efficacy is bolstered by the freedom of diffusion from elution.

As with any test method, the perceived efficacy will be convoluted with the capabilities of the test method, and since many of the above methods exploit the growth of viable microbes in a growth medium, these growth based methods will be limited when the microbe is viable but non-culturable (VBNCS). 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, while it actually remains viable, awaiting on an appropriate trigger or medium to reactivate. The assessment of an immobilized AMC's ability to kill VBNCS will be strengthened by the development of methods capable of monitoring the activity of VBNCS. This field is relatively new, and new VBNCS appropriate methods will likely require detailed understanding of particular species, strain or even phenotypic properties.

One of the key potential drawbacks for immobilized AMAs comes from the fact that the surface may become fouled by the very bacteria that the surface kills. This is expected to be especially true for the surfaces designed to operate by the positive charge density threshold mechanism of action. These are expected to adhere the negatively charged bacteria and upon inactivation, the negatively charged charged cellular material will likely remain to obscure the surface, thereby inactivating the efficacy in that region. Furthermore, low charge density regions that might allow live cells to adhere can act as islands which may propagate, and grow colonies on top of a monolayer of dead cells. This is not a fatal flaw for iAMAs, but is a key limitation, that should be considered when evaluating the immobilized agent for a realistic application.

One other interesting limit to immobilized AMA is the expected upper limit for efficacy. Unlike eluting agents, immobilized agents will require that the microbe comes into close contact with the surface, and while surfaces may be regenerated (via a cleaning protocol), it seems reasonable that the efficacy may be limited by the total surface area of the agent normalized by the size of the microbe. In one example researchers challenged their samples with increasing inoculum, and observed an interesting limit to the efficacy of their surfaces (∼1x10⁸ E. coli/cm²). This value is approximately equal 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 to provide further mechanistic insight into the test method and mode of action.

As with many technologies, the performance of materials are sometimes oversold, with best-case instead of typical conditions being presented. From discussions with numerous other researchers in the field, it is clear that a fair fraction of published work is very difficult to reproduce. This does not implicate the published work, so much as perhaps highlights the number of variables that may change from sample to sample, lab to lab, strain to strain, slant to slant, etc. It should; however, caution the reader and researcher to plan for additional resources and testing when embarking on an iAMA project.

A multitude of strategies and materials have been used to create surfaces with immobilized antimicrobial agents. 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 to minimize biofilm formation. Future developments of this textured aspect might generate added functionality. As new materials are added to surfaces for biomedical devices, the cytotoxicity will also 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.

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