



Biofilms Research—Implications to Biosafety and Public Health

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Key Words

Biofilms, Antimicrobial resistance, Public health, Risk assessment, Biofilm resistance

Abstract

Biofilms have been implicated in a variety of nosocomial infections associated with medical devices, hospital equipment, and other hard surfaces. In addition, household and workplace surfaces such as sinks, countertops, toilets, and cutting boards can act as reservoirs. This study's objective was to identify and evaluate literature reporting resistance to antimicrobial agents in biofilm populations. These review findings suggest that the research evaluating resistance in biofilms could be grouped into the following three mechanisms: (1) physicochemical barriers; (2) biological factors; and (3) phenotypic changes. Current research has identified potential mechanisms of antimicrobial resistance, but there is no clear evidence supporting any one mechanism. Moreover, no reported studies examine the potential impact of biofilms on biosafety practices and the public health risk of infectious diseases from biofilms in healthcare facilities and the workplace environment. Future research directions in biofilms are likely to focus on: (a) imaging of biofilms in situ, (b) in vitro and in vivo models of biofilms, (c) genetic, metabolic, and immunologic probes for real-time analysis, (d) antimicrobial resistance in multispecies biofilms, and (e) identification and characterization of phenotypic modifications. In our assessment, these studies will provide the basis to develop guidelines for biofilm-related biosafety and public health risk assessment.

Introduction

A biofilm is a community (population) of microorganisms that may include bacteria, fungi, yeasts, and protozoa, attached to a solid surface. Biofilms are produced by microorganisms and consist of a sticky rigid structure of polysaccharides and other organic contaminants. This slime layer is anchored to a surface and provides a protective environment in which microorganisms grow. Biofilms generally form on any surface that is exposed to nonsterile water or other liquids and are consequently found in many environmental, industrial, and medical environments.

Considerable evidence exists in the scientific literature that implicates biofilms as being responsible for a variety of nosocomial infections associated with medical devices, hospital equipment, and other hard surfaces. In addition, household and workplace surfaces such as sinks, countertops, toilets, and cutting boards can act as reservoirs. There is some evidence linking biofilms to diseases such as otitis media (common ear infection), bacterial endocarditis, and Legionnaire's disease. Biofilms have also been found in patients with cystic fibrosis.

Ongoing biofilms research is primarily focused on: (a) the development of new methodologies to examine the physicochemical characteristics of biofilms, (b) identification of barriers that limit diffusion of antimicrobials, and (c) understanding antimicrobial resistance due to phenotypic and genotypic alterations.

Reduced efficacy of antimicrobial agents is a major concern in the biofilm environment. Published

evidence suggests that antimicrobials that have been effective in controlling or eliminating bacteria are less effective on biofilms (Mah & O’Toole, 2001; Stewart et al., 2001).

According to some estimates, the economic burden of infections arising from biofilms is \$6 billion per year in the United States (O’Toole, 2002). The regulatory and scientific communities and health industry are only beginning to realize the magnitude of the impact of biofilms on healthcare costs.

According to a report by the U.S. Government Accounting Office (GAO), available evidence on biofilms and microbial resistance is woefully inadequate to assess health risks and costs to public health systems, although the database is sufficiently extensive on the use of antimicrobial agents in hospital, industrial, agricultural, and even household products linked to the development of resistance (GAO, 1999). Therefore, this review attempts to critically examine ongoing investigations in biofilms as applied to resistance development and changes in the efficacy of antimicrobial agents. Based on these analyses, the authors project future direction of biofilm research and its potential public health impact.

Literature Identification and Analysis

The objective of this literature was to identify the biochemical and molecular properties of biofilms as related to resistance development and changes in the efficacy of antimicrobial agents. The literature identification and compilation process consisted of the following: (a) identification of available literature; (b) preliminary review of literature to identify relevant publications; (c) review of bibliographies to identify additional research papers.

The literature review included publications available in hard copy or electronic format. Industry research and private publications were not included in the review.

The search criteria were based on identifying the latest publications from available resources from the past decade (1990 to present) and identifying additional references cited within those articles. Using this procedure, a total of 352 publications were compiled for a preliminary review. Based on the study objective, results, and summary, only 14% of the publications were selected for a more detailed review.

Table 1

Major Physicochemical Factors Linked to Changes in Diffusion of Antimicrobial Agents in Biofilm Matrix

Physicochemical Factors	Process Description	Reference
Barrier limiting diffusion	Biofilm exterior matrix (glycocalyx) composition limit diffusion	Stewart (1996)
Sorption	Sorption [of antimicrobial agents] with components of biofilm. Referred to as Aconsumption within the matrix	De Beer et al. (1994) Suci et al. (1994)
Penetration	Models based on penetration across semipermeable membrane demonstrated effect on diffusion	Hoyle (1992) Andrel et al. (2000)
Chemical composition of biofilm matrix	Exopolysaccharide matrix may serve only as a partial barrier against some antimicrobial agents.	Cochran et al. (2000)
Adsorption	Bacterial external surface glycosaminoglyans-mediated absorption to biomaterial	Nichols et al. (1989)
Biofilm thickness	Changes in diffusion due to differences in biofilm thickness reported may involve more than one process.	Cochran et al. (2000)

Discussion

This review and analysis of the published literature indicates that the research evaluating antimicrobial resistance in biofilms can be grouped into three categories: (1) physicochemical barriers, (2) biological factors, and (3) phenotypic changes.

Physicochemical Barriers

Table 1 summarizes published evidence linking physicochemical factors with antimicrobial resistance. These physicochemical factors apply to biofilms on solid surfaces and biomaterials. Depending on the architecture and physicochemical composition of the biofilm, more than one process may modify diffusion of antimicrobial agents. For example, absorption may be the rate-limiting factor in the diffusion of an antimicrobial agent.

Investigators have applied modeling techniques to investigate physicochemical mechanisms involved in antimicrobial resistance (Dodds et al., 2000). Computer models based on *Pseudomonas aeruginosa* biofilms indicated that resistance mechanisms may inhibit the penetration of antimicrobial agents within the biofilm matrix. Catalytic conversion either through an enzyme-driven biochemical or degradative chemical reaction in the aqueous component of the biofilm was also considered a key physicochemical process associated with the development of resistance. However, there is no single model that takes into account the wide variations in biofilm structure and related chemical interactions.

Dodds et al. (2000) developed a computer model to integrate physicochemical mechanisms underlying biofilm resistance to disinfection. The transport model was based on the assumption that the antimicrobial agent neutralizing capacity was uniformly distributed within a narrow region of the microbe-biofilm interface and the reaction proceeded in a stoichiometric manner to either completely neutralize the antimicrobial agent or to have no effect at all.

Other theoretical model algorithms used to mathematically describe resistance mechanisms in biofilms include a catalytic transport model based on the reactivity of chemical components and a physiologic limitation model based on subpopulations of susceptible and resistant microbes. Biofilm resistance

models demonstrate a nonlinear relationship between viable cell numbers versus antimicrobial agent levels, indicating a heterogeneous biofilm population. In contrast, efficacy models in planktonic populations yield a linear dose-response relationship.

Investigators reported considerable differences in the penetration of chlorosulfamates with its lower capacity to react with constituents of biofilms. For example, biofilm test systems challenged with a 1,000 mg/liter concentration of alkaline hypochlorite or chlorosulfamate for 1 hour experienced 0.85 and 1.3 log reductions, respectively. However, treatment of planktonic and resuspended biofilm bacteria was reduced to nondetectable levels (> 6 log reduction) within 60 seconds under identical test conditions (Stewart et al., 2001).

Diffusion of ciprofloxacin through *P. aeruginosa* biofilms (liquid/solid interface) was considerably longer (> 21 minutes) compared to 40 seconds through broth medium (Suci et al., 1994). Similar results were reported with other antimicrobial agents and biofilm test systems (Hoyle et al., 1992; Nichols et al., 1989).

Biofilm thickness is reported to produce a barrier-like effect limiting transport of antimicrobial agents (Stewart et al., 1998). An artificial biofilm system was utilized to evaluate the effect of transport on the efficacy of antimicrobials (chlorine, glutaraldehyde, an isothiazolone, and a quaternary ammonium compound). Transport through the thick biofilm matrix resulted in resistance to test compounds evaluated in the study.

Physicochemical factors alone may not be sufficient to modify diffusion (Tuomanen et al., 1986; Wentland et al., 1996). Several reports indicate that successful diffusion of antimicrobial agents through the biofilm matrix may not destroy microbes but may impact the organization of microbial populations within the biofilm matrix (DuGuid et al., 1992a; Stewart et al. 2001).

Biological Factors

Table 2 summarizes the biological characteristics linked to resistance and response to antimicrobial disinfectants.

Research indicates the interaction of genetic and epigenetic mechanisms in resistance development.

In particular, the organization of microbial populations within the biofilm matrix may cause a physical barrier limiting the rate of diffusion of antimicrobial agents (Donlan, 2000; O’Toole et al. 2000; Stewart & Costerton, 2001). Although several biochemical systems were identified in the multicellular nature of biofilm population, the underlying genetic and epigenetic molecular mechanism(s) are unclear (Mah and O’Toole, 2001; Stewart & Costerton, 2001).

The multicellular characteristics of biofilm population were considered as part of stress-response dynamics introduced under nutrient-limiting conditions, resulting in the increased resistance to antimicrobials (Brown & Baker, 1999). In contrast to a relatively higher susceptibility of rapidly growing planktonic populations, slow-growing microbes in a dense nutrient-limited environment demonstrate resistance to antimicrobial agents.

Studies have focused on what is referred to as quorum sensing (QS)—a biochemical signaling system in microbial organizations (König et al., 2001).

It is unclear whether QS systems are based on genetic or epigenetic changes in slow-growing biofilm populations.

Biocide-Specific Phenotypic Changes

Table 3 summarizes the genomic elements associated with phenotypic modifications associated with resistance.

Stress responses in microbial subpopulations may induce phenotypic modifications resulting in the expression of gene products not observed under normal conditions (Drenkard & Ausubel, 2002; O’Toole, 2002).

While evaluating the role of the multi-antibiotic resistant operon, *mar*, in biofilms, Maira-Litrán et al. (2000) found elevated phenotypic modification (expression of *mar* operon) in biofilms when growth rates were suppressed. The mechanistic link between suppressed growth (nutrient-limiting conditions) and expression of the multi-antibiotic resistant operon was not determined.

Table 2

Biological Factors Associated with Biofilm Population Response to Antimicrobial Agents

Biological (Stress) Factors	Biofilm Process Description	Reference
Nutrient-limitation	Slows growth rate and modifies response to other external stressors.	Brown and Baker (1999)
Population density	Outcome of microbial growth cycle constrained by resources in biofilm. Modify response to external stress.	Wentland et al. (1996); Tuomanen et al. (1986)
Population heterogeneity	Outcome of selection pressure under external stress; subpopulations more resistant to external stress.	Evans et al. (1991); Duguid et al. (1992a, 1992b)
Population organization	Rudimentary features of a multicellular, interfunctional system in biofilm population; response to external stress.	Donlan (2000)
Genetic factors	Structure-function changes in subpopulation possibly due to genetic factors. Role of genetic factors yet unclear.	Maria-Litrán et al. (2000); Thein-Fah et al. (2001)
Quorum sensing	Cell-to-cell signaling system in slow-growing, aged biofilm population; represent rudimentary organization; link to higher resistance yet unclear.	Davies et al. (1998)

Phenotypic alterations in the biofilm populations may be temporary rather than permanent genetic alterations (i.e., mutation). These changes are regarded as “phase variation,” which is a random stress-response process allowing microbes to express phenotypes that may be advantageous in a particular environment. Phase variation may result in multiple phenotypes with varying levels of phenotypic expression allowing selection pressure to prevail (König et al., 2001). The appearance of temperature-dependent surface structures in *E. coli* is one of the more extensively investigated systems. The surface structures, referred to as Pap Pili, enable *E. coli* phenotypes to attach to the host. Similar stress response phenotypes have been reported in biofilms in the lungs of cystic fibrosis patients (Drenkard & Ausubel, 2002).

A common phenotypic modification is the multidrug efflux pumps (Table 3). Upregulation of efflux pumps has shown to increase resistance to antimicrobial agents. For example, phenotypes expressing multidrug efflux pump release chemically unaltered antimicrobial agents from the cell, thereby providing protection against the antimicrobial agent.

Drenkard and Ausubel (2002) reported that antibiotic-resistant phenotypic variants of *P. aeruginosa* formed biofilms in patients with cystic fibrosis. This study identified a regulatory protein (PvrR) that controlled the conversion between antibiotic-resistant and antibiotic-susceptible forms. A similar study on *E. coli* linked expression of a regulatory protein (acrAB) with protection against ciprofloxacin (Maira-Litrán et al., 2000a). Similarly, mutation in the

AmbB loci, which regulates gene encoding of the outer membrane porin proteins *OmpF* and *OmpC*, was linked to increased resistance to beta lactum antibiotics (Jaffe, 1982).

Existing evidence suggests that phenotypic modifications in biofilm populations are transient, and reflect the nutrient-deficient conditions of the biofilm matrix and disappear when reintroduced into a nutrient-rich medium.

Published evidence indicated that genetic elements are less likely to determine the appearance of resistance in biofilms. Instead, epigenetic factors such as microbial population density and biochemical factors related to stress response may result in the appearance of QS and resistance development to antimicrobial agents. Additional studies are required to delineate these differences in the establishment of molecular events leading to phenotypic modification and the appearance of resistance.

Potential Impact of Biofilms on the Efficacy of Antimicrobial Agents

Physicochemical factors were shown to limit, or even prevent, antimicrobial agents from coming in direct contact with the microbial population embedded in the biofilm (Table 1). König et al (2001) reported reduced efficacy of antibiotics and antimicrobial agents when bacterial biofilms were used as inocula, compared to planktonic bacteria. Investigators implicated high bacterial counts in biofilms and the barrier effect as likely reasons for reduced efficacy of antimicrobial agents.

Table 3

Genomic Elements Associated with Phenotypic Changes in Biofilm Population Response to Antimicrobial Agents

Phenotypic Modifications	Organism	Biofilm Media	Reference
<i>mar</i> Operon	<i>E. coli</i>	Hard surface	Maira-Litrán et al. (2000a, 2000b)
<i>PvrR</i> Operon	<i>P. aeruginosa</i>	Multidrug resistance	Drenkard and Ausubel (2002)
<i>AcrAB</i>	<i>E. coli</i>	Multidrug resistance	Maira-Litrán et al. (2000a)
<i>MexAB</i>	<i>P. aeruginosa</i>	Multidrug resistance	Brooun et al. (2000)
<i>oprM</i>	<i>P. aeruginosa</i>	Multidrug resistance	Brooun et al. (2000)
<i>ompB</i> , <i>ompC</i> , <i>ompF</i> ,	<i>E. coli</i>	Multidrug resistance	Prigent-Combaret et al. (1999)

Differences in the efficacy of antimicrobial agents to biofilm bacteria were attributed in part to differences in penetration through biofilm systems (Stewart et al., 2001). Using microelectrode technologies, differences in efficacy were measured in terms of log reduction when the bacterial test system was embedded in test biofilms. Other similar studies on ciprofloxacin and tobramycin have provided additional evidence confirming reduced efficacy of antimicrobial agents on bacterial test populations in biofilms (Hoyle et al., 1992; Suci et al., 1994). Although penetration of antimicrobial agents is an important prerequisite, it is not the sole requirement determining the efficacy of antimicrobial agents.

Published studies reveal rather tenuous evidence linking the potential impact of QS in biofilms on the efficacy of antimicrobial agents. Only preliminary evidence exists suggesting that high microbial density and aging biofilms are likely to demonstrate resistance due to the expression of QS (Stewart & Costerton, 2001). Nevertheless, published evidence linking QS systems with the development of resistance are generally less conclusive (Brooun et al., 2000; Davies et al., 1998).

Preliminary data are available only on a few mutant strains from experimental microbial systems and more data are required to conclusively establish phenotypic changes in biofilm microbial population and molecular links, if any, to the appearance of QS systems. For instance, studies on mutant strains of *P. aeruginosa* have linked phenotypic changes and QS systems with resistance development (Davies et al., 1998), while studies on other mutant strains have yielded contrary results (Brooun et al., 2000). No genetic markers are available for the QS as it relates to phenotypic changes and the development of resistance to antimicrobial agents.

Genetic elements are less likely to determine the appearance of resistance in biofilms. Instead, epigenetic factors such as microbial population density and biochemical factors related to stress response may result in the appearance of QS and developing resistance to antimicrobial agents. More studies are required to delineate differences in the establishment of molecular events leading to phenotypic modification and the appearance of resistance.

Future Directions in Research

Following the publication of the 1999 GAO Report, the U.S. Department of Health and Human Services (DHHS) established in the same year an Interagency Task Force on Antimicrobial Resistance (Task Force) to undertake a strategic research and development plan on the antimicrobial resistance threat (DHHS, 2001). The action plan established four major program components. Research on the role of biofilms in antimicrobial resistance was a key objective. The National Institutes of Health (NIH) is the lead agency in these federal research and development efforts to incorporate the Task Force objectives on antimicrobial resistance research and has initiated an array of biofilm research initiatives. The authors consider this federal research and development program to serve as the catalyst that will provide strategic direction and funding support.

Review of the published literature delineated areas for future research to better understand physicochemical, phenotypic characteristics of antimicrobial-resistant biofilms. Although current research has identified the potential mechanisms of antimicrobial resistance, there is no clear evidence supporting any one mechanism.

Based on the NIH's research priorities and health and public health program priorities, future research directions in biofilms are likely to be in the following areas: (a) imaging of biofilms *in situ*; (b) *in vitro* and *in vivo* models of biofilms; (c) genetic, metabolic, and immunologic probes for real time analysis; (d) antimicrobial resistance in multispecies biofilms; and (e) identification and characterization of phenotypic modifications. The prevalence of biofilms and their impact on public health will drive future research.

In the authors' assessment, the federal government agencies with a mandate in the health, public health, and environmental programs will recognize the increasing importance of biofilms-related problems in biosafety and health risk assessments and will develop biofilm-specific guidelines that are protective of public health.

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