BIOFILMS: MICROBIAL LIFE ON SURFACES WITH EMPHASIS ON USAGE OF ACOUSTIC ENERGY

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Abstract

Microbial biofilms are a major impediment to the use of indwelling medical devices, generating device-related infections with high morbidity and mortality. Major efforts directed towards preventing and eradicating the biofilm problem face difficulties because biofilms protect themselves very effectively by producing a polysaccharide coating, reducing biofilm sensitivity to antimicrobial agents. Techniques applied to combating biofilms have been primarily chemical. These have met with partial and limited success rates, leading to current trends of eradicating biofilms through physico-mechanical strategies. Here we review the different approaches that have been developed to control biofilm formation and removal, focusing on the utilization of acoustic energy to achieve these objectives. Microorganisms attach to surfaces and develop biofilms. Biofilm-associated cells can be differentiated from their suspended counterparts by generation of an extracellular polymeric substance (EPS) matrix, reduced growth rates, and the up- and down-regulation of specific genes. Attachment is a complex pro-cess regulated by diverse characteristics of the growth medium, substratum, and cell surface. An estab-lished biofilm structure comprises microbial cells and EPS, has a defined architecture, and provides an optimal environment for the exchange of genetic material between cells. Cells may also communicate via quorum sensing, which may in turn affect biofilm processes such as detachment. Biofilms have great importance for public health because of their role in certain infectious diseases and importance in a variety of device-related infections. A greater understanding of biofilm processes should lead to novel, effective control strategies for biofilm control and a resulting improvement in patient management.

Key words: Anti-microbial agents; Biofilms; Biofilm prevention; Acoustic energy; Ultrasonication.

1. Introduction

Indwelling medical devices have become major tools in the clinical management of hospitalized patients, particularly those requiring life supporting devices. Urinary, intratracheal, central vein, peritoneal dialysis nephrostomes and other indwelling devices are becoming increasingly frequent in medical practice and are applied to more than 25% of hospitalized patients. However, as the duration of their placements become prolonged, risk factors related to microbial infections and biofilm formation culminate in higher morbidity and mortality rates among hospitalized patients, and sending the costs of hospitalization spiraling upwards. Most circulatory and urinary tract infection cases are associated with indwelling medical devices [1]. Microbial biofilms develop on the surfaces of medical devices and proceed to cause full blown bacterial infections and sepsis. In patients with urinary catheters, infection rates increase with the duration of catheterization at rates of 5-10% per day with virtually all of those who undergo long-term catheterization (>28 days) becoming infected [2-4]. Estimates by Costerton attribute more than half of bacterial infections in immuno-compromised patients to slime encased microbial colonies (biofilms) [5]. The U.S. National Institutes of Health mention infection rates as high as 80% that are due to microbial biofilms [6]. The magnitude of the biofilm

problem and its impact on medical and financial aspects of modern hospital medicine have triggered the investment of major efforts to develop novel anti-biofilm strategies based on thorough, in depth analyses of the microbial transformation cascades. The rationale has been to develop means for disruption of colony formation at multiple sites of these transformation cascades and for eradication of existing biofilms. The composition of inert surfaces and intersurfaces has come under review as these form substrates to which unicellular planktonic microorganisms attach to enable their transformation into the multicellular sessile forms.

The encasing slimy exopolysaccharide matrix which is secreted by the microorganisms and in which developing colonies become encapsulated, is another major target. This unique architecture regulates exchange of ions, chemicals and nutrients with the surrounding environment. It thus protects biofilms from external insults by blocking entry of biocides, surfactants and predators and renders them 1,000 times more resistant to antibiotics compared to free floating bacteria. [7,8]. In addition to acting as transport barriers to agents harmful to biofilms [9], exopolysaccharide matrix polymers also bind to and neutralize antibiotics prior to their interaction with bacteria [10]. Even if the antibiotics are successful in penetrating into the biofilm other researchers suggest that bacteria within biofilms are dormant and do not actively metabolize antibiotics [11].

Biofilm is an assemblage of surface-associated microbial cells that is enclosed in an extracellular polymeric substance matrix. Van Leeuwenhoek, using his simple microscopes, first observed microorganisms on tooth surfaces and can be cred-ited with the discovery of microbial biofilms. Heukelekian and Heller (1) observed the "bottle effect" for marine microorgan-isms, i.e., bacterial growth and activity were substantially enhanced by the incorporation of a surface to which these organisms could attach. Zobell (2) observed that the number of bacteria on surfaces was dramatically higher than in the sur-rounding medium (in this case, seawater). However, a detailed examination of biofilms would await the electron microscope, which allowed high-resolution photomicroscopy at much higher magnifications than did the light microscope. Jones et al. (3) used scanning and transmission electron microscopy to examine biofilms on trickling filters in a wastewater treatment plant and showed them to be composed of a variety of organ-isms (based on cell morphology).

2. Biofilms and Anti-Microbial Immunity

Microbial cells within biofilm colonies are also much less susceptible to host immune mechanisms. Key antigens are either repressed or concealed from effector immune cells [12], and bacteria in colonies are highly resistant to phagocytosis by immune system phagocytes [12]. Deposition of complement C3b and IgG on bacterial surfaces has also been shown to be prevented as demonstrated for *Staphylococcus epidermidis* [13], contributing to protection of bacteria from killing by polymorphonuclear leukocytes. Furthermore, in airways of cystic fibrosis patients the presence of polymorphonuclear leukocytes has even been found to enhance *Pseudomonas auruginosa* biofilm formation due to bacterial binding to F-actin and DNA polymers [14]. Thus, the various arms of anti-microbial immunity are neutralized by the biofilm exopolysaccharide protective matrix, leaving affected patients fully vulnerable to the problem.

Of interest are results of studies which have evaluated the effects of effector molecules of innate immune mechanisms on formation and survival of various types of microbial biofilms. Molecules such as lactoferrin, a constituent of human external secretions, have been found to inhibit development of *Pseudomonas aeruginosa* biofilms at lactoferrin concentrations lower than those that kill or prevent growth of the planktonic cells. The authors suggest that by chelating iron lactoferrin stimulates twitching surface motility, causing dispersion of bacteria rather than formation of the cell clusters required to form biofilms [15]. The importance of these observations is in the principle which suggests existence of a specific anti-biofilm formation protective mechanism. It acts at the stage in which bacteria begin to aggregate to form communities that subsequently transform into the sessile form of life [15]. Lactoferrin, however did not prevent fungal biofilm formation. This could be partially achieved by using oxidative and non-oxidative antimicrobial molecules produced by phagocytic cells such as PG-1, β-defensin-1, and β-defensin-3, which significantly reduce the metabolic activity in the biofilm [16]. Altogether immune reactions do not effectively inactivate biofilms but rather

further invigorate the unique and highly resistant properties of microbial biofilms. The compromise of anti-microbial immune mechanisms is the basis for the difficulties in eradicating this major source of microbial infection, explaining the severity, persistence and high morbidity associated with biofilm-derived infections.

2.1. Biofilm Defined

A biofilm is an assemblage of microbial cells that is irre-versibly associated (not removed by gentle rinsing) with a sur-face and enclosed in a matrix of primarily polysaccharide material. Noncellular materials such as mineral crystals, corro-sion particles, clay or silt particles, or blood components, depending on the environment in which the biofilm has devel-oped, may also be found in the biofilm matrix. Biofilm-associ-ated organisms also differ from their planktonic (freely suspended) counterparts with respect to the genes that are tran-scribed. Biofilms may form on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or potable water system piping, or natural aquatic systems. The variable nature of biofilms can be illustrated from scan-ning electron micrographs of biofilms from an industrial water system and a medical device, respectively (Figures 1 and 2). The water system biofilm is highly complex, containing corrosion products, clay material, fresh water diatoms, and fil-amentous bacteria. The biofilm on the medical device, on the other hand, appears to be composed of a single, coccoid organ-ism and the associated extracellular polymeric substance (EPS) matrix

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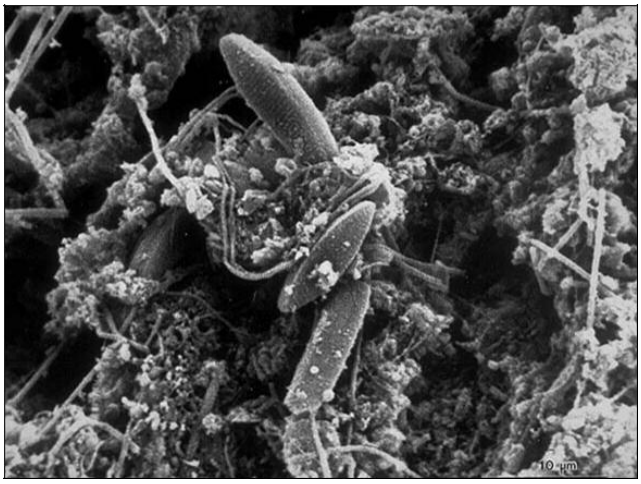

Figure 1. Scanning electron micrograph of a native biofilm that devel-oped on a mild steel surface in an 8-week period in an industrial water system. Rodney Donlan and Donald Gibbon, authors. Licensed for use, American Society for Microbiology Microbe Library. Available from: URL: http://www.microbelibrary.org

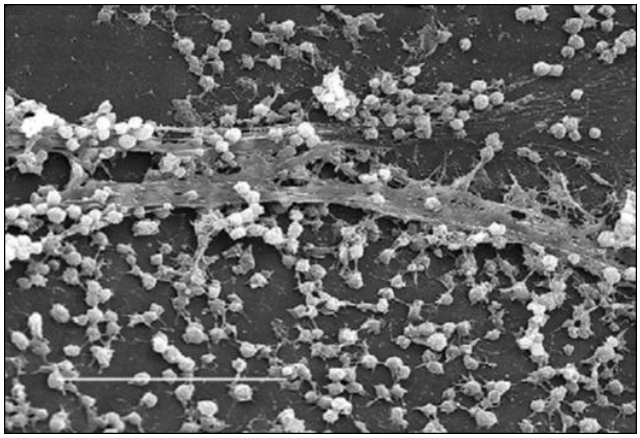

Figure 2. Scanning electron micrograph of a staphylococcal biofilm on the inner surface of an indwelling medical device. Bar, 20 □m. Used with permission of Lippincott Williams & Wilkins.

2.2. Attachment

The solid-liquid interface between a surface and an aque-ous medium (e.g., water, blood) provides an ideal environment for the attachment and growth of microorganisms. A clear pic-ture of attachment cannot be obtained without considering the effects of the substratum, conditioning films forming on the substratum, hydrodynamics of the aqueous medium, character-istics of the medium, and various properties of the cell surface. Each of these factors will be considered in detail.

2.3. Substratum Effects

The solid surface may have several characteristics that are important in the attachment process. Characklis et al. (6) noted that the extent of microbial colonization appears to increase as the surface roughness increases. This is because shear forces are diminished, and surface area is higher on rougher surfaces. The physicochemical properties of the surface may also exert a strong influence on the rate and extent of attachment. Most investigators have found that microorganisms attach more rap-idly to hydrophobic, nonpolar surfaces such as Teflon and other plastics than to hydrophilic materials such as glass or metals (7–9). Even though results of these studies have at times been contradictory because no standardized methods exist for determining surface hydrophobicity, some kind of hydrophobic interaction apparently occurs between the cell surface and the substratum that would enable the cell to over-come the repulsive forces active within a certain distance from the substratum surface and irreversibly attach.

2.4. Characteristics of the Aqueous Medium

Other characteristics of the aqueous medium, such as pH, nutrient levels, ionic strength, and temperature, may play a role in the rate of microbial attachment to a substratum. Sev-eral studies have shown a seasonal effect on bacterial attach-ment and biofilm formation in different aqueous systems (17,18). This effect may be due to water temperature or to other unmeasured, seasonally affected parameters. Fletcher (19,20) found that an increase in the concentration of several cations (sodium, calcium, lanthanum, ferric iron) affected the attachment of *Pseudomonas fluorescens* to glass surfaces, pre-sumably by reducing the repulsive forces between the nega-tively charged bacterial cells and the glass surfaces. Cowan et al. (21) showed in a laboratory study that an increase in nutri-ent concentration correlated with an increase in the number of attached bacterial cells.

2.5. Predation and Competition

Bacteria within biofilms may be subject to predation by free-living protozoa, *Bdellovibrio* spp., bacteriophage, and polymorphonuclear leukocytes (PMNs) as a result of localized cell concentration. Murga et al. demonstrated the coloni-zation and subsequent predation of heterotrophic biofilms by *Hartmannella vermiformis*, a free-living protozoon. Predation has also been demonstrated with *Acanthamoeba* spp. in con-tact lens storage case biofilms.

James et al. noted that competition also occurs within biofilms and demonstrated that invasion of a *Hyphomicrobium* sp. biofilm by *P. putida* resulted in dominance by the *P. putida*, even though the biofilm-associated *Hyphomicrobium* numbers remained relatively constant. Stewart et al. investigated biofilms containing *K. pneumoniae* and *P. aeruginosa* and found that both species are able to coexist in a stable commu-nity even though *P. aeruginosa* growth rates are much slower in the mixed culture biofilm than when grown as a pure culture biofilm. *P. aeruginosa* grow primarily as a base biofilm, whereas *K. pneumoniae* form localized microcolonies (cover-ing only about 10% of the area) that may have greater access to nutrients and oxygen**.** Apparently *P. aeruginosa* can com-pete because it colonizes the surface rapidly and establishes a long-term competitive advantage. *K. pneumoniae* apparently survives because of its ability to attach to the *P. aeruginosa* biofilm, grow more rapidly, and out-compete the *P. aeruginosa* in the surface layers of the biofilm.

3. Interference with Inter-Bacterial Signaling for Biofilm Formation

Following attachment to solid surfaces bacteria secrete several classes of small, diffusible quorum sensing molecules also known as autoinducers. These molecules form concentration gradients that convey inter-bacterial signaling capable of modulating bacterial gene expression to patterns that transform the planktonic lifestyle into a sessile form. Their activity promotes biofilm development and differentiation. Oligopeptides and *N*-acylhomoserine lactones are involved in group-specific communications within gram-

positive and gram-negative bacteria respectively, and boronated-diester molecules in communication among both gram-positive and gram-negative bacteria. Disruption of autoinducer signaling pathways have been hypothesized to help prevent biofilm formation and differentiation. Two approaches have recently been proposed for interfering with these biofilm differentiation-promoting signaling mechanisms: one is biological and the other is mechanical. The biological approach identified naturally occurring products such as furocoumarins, found in grapefruit juice, that are capable of inhibiting cell-to-cell autoinducer signaling between bacteria, inhibiting biofilm formation. The authors reported >95% inhibition of all types of autoinducers, gram negative or gram positive specific, as well as interspecific classes. The efficacy of these compounds, were analyzed in a *Vibrio harveyi* based autoinducer bioassay and in biofilm formation assays by *Escherichia coli*, *Salmonella typhimurium* and *Pseudomonas aeruginosa*. Although they appear to be effective *in vitro*, these products have yet to prove their effectiveness *in vivo;* their routes of administration must also be delineated more precisely, as grapefruit juice consumption is abundant and not known to prevent life threatening infections due to bacterial biofilms.

Quorum sensing signaling in bacteria has also been tackled through mechanical approaches aimed at disruption of the concentration gradients of the small molecule mediators. Ultrasonic acoustic energy is being investigated as a mean for disrupting quorum sensing signaling through induction of chaos in the concentration gradients. The aim is to prevent bacterial cell migration and assembly at sites of colony formation, interfering in this way with biofilm differentiation as well as with induction of several additional anti-biofilm effects that are discussed later in this review.

4. High Energy Ultrasound

Ultrasonic energy used in the combat against microbial biofilms is divided into two categories with respect to the effects it produces: power intensities that cause cavitation are energy levels in excess of the cavitation producing energy thresholds. These are wave frequencies f $\geq$ 100 kHz generated at acoustic power intensities of 0.5-2x10^3 mW/cm^2. A second category includes the lower power intensities that do not form cavitations . The high acoustic energy power intensity levels are more suitable for eradication of existing biofilms rather than preventing their formation. Biofilm removal was found to strongly depend on the intensity of acoustic energy and to a far lesser extent on the frequency . The author reported that coupling the acoustic energy with convective fluid flow dramatically improved biofilm removal at acoustic intensity of 27 W/cm^2 (removal of up to 80% of biomass in two minutes and close to 100% when intense ultrasonication was coupled with gas bubbles in the fluid) . Unlike these power intensity doses prevention of biofilm formation is effective upon transmission of much lower acoustic energy intensity levels ($\leq$ 0.35 mW/cm^2).

5. Future Outlook and Conclusions

Despite significant breakthroughs in biofilm prevention and eradication technologies, current relief is only short lived, limited to certain types of catheters and unsuitable for others. In many cases efficacy is seen on some types of bacteria and not on others. The likelihood for broadening the spectrum of chemical anti-biofilm specific reagents may depend on the identification of the entire spectrum of saccharides, proteins, mucins and lipids that the various bacteria can target for microbial adhesion. One would then be required to develop combinations of inhibitors that can counteract each of the receptors. The current outlook for the general and non-specific anti-biofilm arena is more likely to employ mechanical means in the form of various types of acoustic energy. Arrays of modifications and adjustments that will render the low acoustic energy conditions suitable to different indwelling medical devices, are likely to expand its scope of use and may render this technology suitable for utilization in central vein catheters and possibly also in endotracheal as well as other catheters. There is also the potential of utilizing acoustic energy to prevent biofilm formation on organs and not only on devices. One example which we have begun to evaluate is prevention of intra-tracheal biofilm formation, a utilization that may be of importance for patients with cystic fibrosis. Preliminary studies on sheep trachea reveal the complexity of applying this concept even before addressing the problem of the high intolerance of the airways to foreign bodies.

Research on microbial biofilms is proceeding on many fronts, with particular

emphasis on elucidation of the genes specifically expressed by biofilm-associated organisms, evalu-ation of various control strategies (including medical devices treated with antimicrobial agents and antimicrobial locks) for either preventing or remediating biofilm colonization of medi-cal devices, and development of new methods for assessing the efficacy of these treatments. Research should also focus on the role of biofilms in antimicrobial resistance, biofilms as a reservoir for pathogenic organisms, and the role of biofilms in chronic diseases. The field of microbiology has come to accept the universality of the biofilm phenotype. Researchers in the fields of clinical, food and water, and environmental microbi-ology have begun to investigate microbiologic processes from biofilm perspective. As the pharmaceutical and health-care industries embrace this approach, novel strategies for biofilm prevention and control will undoubtedly emerge. The key to success may hinge upon a more complete understanding of what makes the biofilm phenotype so different from the plank-tonic phenotype.

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