Bacterial biofilms

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Over the review period, a significant amount of literature has been published documenting the impact of biofilms on engineered and biomedical systems. Reactor systems and analytical techniques have evolved to study the molecular chemistry and microbial ecology within biofilm layers only tens of micrometers thick, and various protocols have been developed to control cell adhesion and biofilm formation.

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Introduction

Biofilms are collections of microorganisms, predominantly bacteria, that are enmeshed within a three dimensional gelatinous matrix of extracellular polymers secreted by the bacteria [1–3]. In an aqueous environment, an inert support surface, termed a substratum, will be immediately biased by dissolved organic macromolecules that adsorb rapidly from the liquid phase. Bacterial cells present in the fluid contact the substratum by a variety of transport mechanisms. Once at the substratum, the cells can adsorb either reversibly or irreversibly. Provided they spend sufficient time at the interface, the adherent cells secrete extracellular polymers that serve to attach the cells tenaciously to the substratum. Attached cells metabolize prevailing energy and carbon substrates, consume electron acceptors, grow, replicate, and produce more insoluble extracellular polymers, predominantly polysaccharides, thus accumulating a viable biofilm community. Inert particles and cells of the same or different species continue to be deposited onto the biofilm. As a result of hydrodynamic forces and stresses exerted by replication, there can be a continual erosion of cells and extracellular material from the biofilm interface into the surrounding fluid phase. A more random, stochastic process, known as 'sloughing', occurs where either large sections or the entire biofilm become detached from the substratum and enter the liquid phase.

Fundamental biofilm processes

In summary, the processes governing biofilm formation, persistence and reactivity include: conditioning of the substratum by macromolecule adsorption [4–10]; cell transport to the substratum surface by sedimentation [4,11,12•], motility [4], convection or molecular diffusion [12•]; cell adsorption to and desorption from the substratum [12•]; irreversible cell attachment to the substratum [11,12•,13•,14,15]; attached cell metabolic processes, including substrate and nutrient metabolism [16–19], cell metabolism, growth and replication [13•,20,21•,22•,23], extracellular polysaccharide production [21•,23], and endogenous cell decay, cryptic growth, and lysis; and finally, biofilm removal processes [21•], such as detachment and sloughing.

Biofilms can be formed both intentionally or inadvertently and may either be detrimental or beneficial. Biofilm research is rather broad in that it comprises investigation of the factors affecting any one or a combination of the fundamental processes above, studies of the detrimental effects of biofilm formation and presence, performance studies of biofilm reactor systems, and the development of methods for biofilm analysis. The remainder of this critique will be organized according to these last three research focus areas.

Detrimental biofilms

Uncontrolled biofilm formation within natural, engineered, and biomedical systems can be detrimental, as detailed in several recent, definitive reviews [1–3]. Problems arise by way of a biofilm's influence on the transport of mass, momentum, and energy. Detrimental effects attributed directly to bacterial biofilms, documented in the recent literature, include: material deterioration and corrosion [24–26,27•,28–32]; increases in both frictional and heat-transfer resistances [30,33•]; attachment to and infection of biomedical implant devices [5,7,15,34–38]; and plague bioreactors with operational problems that range from nuisance (performance instability, poor product quality) to catastrophe (reactor instrument failure, culture contamination, equipment failure) [2,3,39].

Abbreviations

ATR—attenuated total reflectance; **CLSM**—confocal laser scanning microscope; **CTC**—5-cyano-2,3 ditolyl-tetrazolium chloride; **PCR**—polymerase chain reaction.

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Control of detrimental biofilms

Control of biofilm formation can be either a positive or negative exercise depending on whether the biofilm is detrimental or beneficial to the operation of the system in question. Methods to control unwanted, detrimental biofilms attempt to prevent bacterial adhesion, retard biofilm formation, or eradicate the existing biofilm.

Recent articles investigating the prevention of cell adhesion to biomaterials have focused on modification of surface chemical properties of the substratum by: photochemical coupling of benzephenone derivatives of polyethylene glycol, polyacrylamide, and poly vinyl pyrrolidone [40]; passive adsorption of pluronic surfactants (copolymers of polyethylene oxide and polypropylene oxide) to polystyrene [41]; incorporation of polyethylene oxide into the upper layers of polyethylene terephthalate by solvent swelling [42]; and adsorption of a series of neutral, anionic, and cationic surfactants onto stainless steel or glass [43]. Rather than dissuade adhesion, an alternative control approach for biomedical materials is to retard surface microbial activity by incorporating within the substratum a slow-release antibiotic agent, such as gentamicin within polymethyl methacrylate [15,44], methyl and propyl paraben (*p*-hydroxy-benzoic-acid esters) in polyurethane [45], cefazolin adsorbed onto intravascular catheters [46], silver-coated stent material [47], and antiseptic (chlorohexidine, mandelic acid, or mandelic and lactic acid combined) rinses of silicone surfaces [48,49].

Remedial approaches to eliminate or eradicate biofilms existing in engineered systems consist of either mechanical cleaning, materials or unit replacement, or chemical biocide challenge [50]. A novel alternative to chemical antagonism of a biofilm has been to eliminate a nutrient essential for microbial growth; Bakke *et al.* [51] elected to control microbial oil 'souring', mediated by sulfate-reducing bacterial biofilms, by limiting the sulfate concentrations dissolved in the water used in flooding operations. In the petrochemical, refining, and the power industries, either oxidants (chlorine, ozone, bromides) or biocides are employed regularly to suppress biofouling of water-cooled heat exchangers. Biocides have proved to be mostly ineffective in that they inactivate the bacterial cells but, unlike oxidants, they are incapable of eliminating the source of the system inefficiency: the biofilm matrix. Blenkinsopp *et al.* [52•] report that, when applied within a low strength electrical field (12 V cm^{-1}) with a low current density (2.1 mA cm^{-2}), several industrial biocides exhibited enhanced killing action against *Pseudomonas aeruginosa* biofilms.

Elimination of bacterial infections of indwelling prostheses, orthopedic implants, or various tissue infections, consists specifically of antibiotic challenges including: gentamicin [53], rifampin alone or rifampin plus combinations of cefazolin and vancomycin [54], clindamycin and trospectomycin [55], and ciproflaxin [56], although biofilm-bound cells have proved to be quite resilient to such chemical antagonism [57•,58]. Regrettably, in studies on antibiotic challenges and substratum chemistry modification, all too often experiments are performed without paying much attention to the mechanism controlling bacterial accumulation on the substratum. Fluid conditions are often quiescent, leaving the system susceptible to mass transfer limitations. Under significant mass transfer limitations, the rate of transport of limiting growth substrate, or cells depositing at a substratum, or the diffusion of an antibiotic to an attached cell, may be much slower than the process of interest, such as attached cell replication, cell adhesion rates or antibiotic killing rates. Any data collected under such conditions that are not corrected for mass transfer limitations are erroneous. Both fluid and liquid phase concentrations of system variables (such as cell concentration, nutrients and oxygen) are often monitored only once in an experiment, if at all. Care should be taken in any extrapolation of 'adhesion kinetics' determined from such experiments to 'real world' conditions.

Beneficial aspects of biofilms

Benefits afforded by biofilms in a reactor situation arise chiefly because the cell population is immobilized and thus the residence time of the cells in the reactor is independent of the fluid phase residence time. In continuous suspended culture bioreactors (chemostats), the mean residence time of the system cannot be less than the generation time of the bacterial species, otherwise cells are not allowed sufficient time to replicate within the reactor and will eventually be diluted from the system.

Immobilized and biofilm-bound cells remain in a continuous reactor system independent of the fluid phase, so the mass loading of limiting substrate (or influent pollutant in the case of a wastewater treatment reactor) can be increased well beyond the growth rate limit imposed on suspended cultures. Consequently, immobilized cell or biofilm reactors provide added volumetric reactivity, more stable operating performance, inherent biomass:fluid separation, and the possibility of staging different bioconversion processes in sequential reactors. Because of these inherent advantages, biofilm reactors are not confined to just bacterial cells but also favour plant and animal cell applications.

Immobilized cell reactors are available in a number of operating geometries but can be naively divided into artificially immobilized cell reactors and naturally formed biofilm reactors. Readers are directed to a number of recent excellent reviews in *Current Opinion in Biotechnology* for more details of artificially immobilized microbial [59], plant [60] and animal [61] cell reactor systems.

Bacterial biofilm reactors are employed either in the production of commodities or in wastewater treatment applications. During the review period, biofilms have reportedly been used to produce acetic acid [62], L-ly-

sine [63], gluconic acid [64], kojic acid [65,66], ethanol [67,68], and in the epoxidation of propene [69]. Such biofilm reactors are operated either as packed- or fluidized-bed reactor systems, with cells either attached to inert support particles or artificially immobilized within a gel matrix.

The one application that relies on a microbial culture's ability to form biofilms is wastewater treatment. Biofilm reactor (or the redundant colloquialism, 'fixed-film' reactor) geometries, applied in both pilot- and full-scale wastewater treatment, include packed-bed 'trickling filters', high-rate plastic media filters, rotating biological contactors, fluidized-bed biofilm reactors, and membrane immobilized cell reactors. Examples of biofilm reactors employed for wastewater treatment reported in the literature during the review period include: polychlorinated hydrocarbon degradation [70], toluene degradation [71], denitrification [72,73], cadmium removal [74], anaerobic butyrate degradation [75], nitrification [16,17,76], glyphosphate degradation [77], anaerobic propionate degradation [78], phenol removal [79], uranium removal [80], and anaerobic carbon removal [81,82]. Often the performance of such biofilm systems is dependent on the dynamics of a selectively enriched microbial culture, metabolizing multiple substrates (electron donors and acceptors) that must be transported from the bulk liquid to the biofilm interface, then transported internally into the lower layers of the accumulating biofilm. Clearly, to mathematically model, predict, and analyze such a dynamic ecology in a spatial dimension of ten to hundreds of micrometers, requires a complex mathematical model and solution technique, the most rigorous being that of Gujer and Wanner [83].

Techniques for biofilm analysis

Historically, the study of biofilms has required the evolution of both reactor systems in which to cultivate biofilms under controlled fluid phase and surface conditions and the analytical methods to assess biofilm presence, reactivity, and the resultant system response. Twenty years ago, the design and operation of a biofilm reactor or the analysis of microbial activity in an alpine stream or heat exchanger, was achieved by measuring attached biomass concentrations (in mass dry weight or total cell numbers) and indirect biofilm activity (dissolved oxygen uptake rates, ATP content, heterotrophic potentials). Such diagnosis was, and for the most part still is, dependent upon destructive sampling of the biofilm.

Biofilms can be pure or mixed strain, pure or mixed species, or mixed cell line (bacteria, platelets, neutrophils, endothelial cells) systems. Recently, research has recognized the need to focus on the heterogeneous nature of biofilm ecology with regard to: first, temporal changes and spatial distributions in cell populations; second, local concentrations of electron donor, acceptor, and pH; and third, spatial differences in biofilm physical, chemical, rheological, and mass transport properties. Fortunately, the advent of several molecular and cellular probes and diagnostic tools has had a tremendous impact on our ability to probe biofilm ecology non-invasively *in situ*.

Huang *et al.* [84], Buret *et al.* [85], and Mittleman *et al.* [86] all describe the use of a rectangular fluid flow cell in which bacterial adhesion and the early stages of biofilm formation under flow conditions can be studied. These flow cells allow microscopic surveillance of the substratum, and provide access for microelectrode probes and portions of substratum to be removed for destructive sampling of the biofilm. The advantages of flow cells are that they mimic the fluid flow hydrodynamics inherent in many systems plagued by biofilm formation, they provide controlled, well-defined hydrodynamic conditions, and access for visual observation of biofilm formation. The disadvantages of such flow cell devices include limited sample surface area and, if operated on a one-through flow basis, the potential exists for gradients in both biofilm amount and fluid phase concentrations to develop in the flow direction. Flow cell reactors are not novel, having been reported on numerous occasions since the 1970s, and they are by no means the only suitable reactor configuration for biofilm studies [1]. What makes these articles [84–86] distinctive is their emphasis on the need for bacterial adhesion studies to be carried out under defined fluid flow conditions at known substratum conditions, rather than the arcane use of quiescent liquid in undefined conditions.

Kinniment and Wimpenny [87] describe a different study system in which biofilms are cultivated, under controlled fluid phase conditions receiving a continuous supply of substrate, within recessed wells in the bottom plate of a laboratory reactor. A wiper blade constantly stirs the fluid phase above the plate, thus eliminating biofilm everywhere in the reactor except within the recesses. Removable, small circular discs form the bottom of each recess, allowing periodic sampling of biofilm of a known thickness. In this study, *Pseudomonas aeruginosa* biofilm samples were periodically removed, quick frozen, and thin-sectioned in the parallel plane to the substratum. The sections of biofilm were then analyzed for adenylate concentrations and adenylate energy charge as a function of biofilm depth.

Analysis of the heterogeneity of biofilms is concerned with spatial gradients of total cell concentrations, individual species concentrations, and local concentrations of various dissolved nutrients and select ions. Kuhn *et al.* [88] used mithramycin to fluorescently stain the total DNA of *Escherichia coli* immobilized with strontium-alginate beads. Cell concentration profiles as a function of radial position in the bead were determined periodically by microfluorimetry on thin sections of the beads. Stewart *et al.* [89] applied a pulse chase of $^{35}SO_4^{-2}$ and subsequent liquid emulsion autoradiography to thin sections of hollow fiber membranes containing immobilized *E. coli*. Sections illustrated viable

cell concentrations as a function of radial position at different locations, and under different operating conditions, in the hollow fiber reactor.

Rogers and Keevil [90] report the destructive sampling of a multispecies biofilm, intentionally inoculated with *Legionella pneumophilia,* followed by both immunogold and fluorescein immunolabelling for this species. Episcopic differential interference contrast microscopy was employed to visualize the total biofilm community and the labelled *Legionella* species simultaneously. This technique provided observations of variations in the biofilm community from one position to another (parallel to the substratum), but was unable to discern such variations with biofilm depth (perpendicular to the substratum).

Similarly, Rodriguez *et al.* [91] have described the potential of a fluorescent redox probe, 5-cyano-2,3 ditolyl-tetrazolium chloride (CTC), for direct observation of actively respiring bacteria. Oxidized CTC is colorless but upon reduction by electron transport activity, the insoluble CTC–formazan fluoresces at 365 nm excitation. Respiring marine bacteria within thin (10–50 μm) biofilms attached to optically opaque polysulfone substrata, were rapidly and easily enumerated. Respiring cell counts using CTC were also helpful in directly assessing the efficacy of various biocidal control agents.

The advent of modern molecular biology has also provided useful diagnostic methods with which to analyze biofilm ecology. Amann *et al.* [92] have characterized the population structure of sulfidogenic biofilms, established in anaerobic bioreactors, by selective polymerase chain reaction (PCR) amplification and fluorescent microscopy. The 16S rRNA common to the sulfate-reducing bacteria in the biofilm was selectively amplified by PCR and used to design both general and specific fluorescent hybridization probes. Biofilms (5–10 μm thick) on glass cover slips immersed in laboratory anaerobic biofilm reactors, were fixed in formaldehyde and dehydrated prior to hybridization with the RNA probes. Hybridized biofilm samples were then viewed by epifluorescence microscopy.

McCarter *et al.* [93•] report a very elegant series of genetic techniques to investigate the bacterial responses to various stimuli, one being the adhesion to a surface. Using a transposon (mini-Mu*lux*), the authors were able to incorporate recombinant reporter gene insertions at sites adjacent to target gene promoters that then code for light production as a function of the target gene expression. Light production is convenient to measure by exposure of X-ray or photographic film, visual examination, chemiluminescence or photometry. Such techniques also obviate the need to disrupt biofilms prior to quantification. McCarter *et al.* [93•] employed a *lux* reporter gene adjacent to the chromosomal gene encoding for swarmer cell differentiation that *Vibrio parahaemolyticus* experiences upon association with a solid surface. The resultant mutants emit light upon growth at a surface but not in suspension. Dagostino *et al.* [22•] also employed transposon mutagenesis to insert a marker gene that lacks its own promoter into an appropriate recipient bacterium. The premise is that if a suitable target gene is 'on' at the surface, then expression of the marker will be observed only in the presence of the surface. Dagostino *et al.* [22•] employed *E. coli* C600 (pRK2013: mini-MuTetr *lacZ*) as the donor and two marine bacteria as recipients. They were able to isolate a transposon-generated mutant in which the *lacZ* gene was not expressed in either liquid or agar but was expressed when cell growth was associated with a polystyrene substratum.

Several citations report the use of invasive but non-destructive analysis of solute concentration profiles, by microsensor chemical probes, as a function of spatial dimension within the developing biofilm. Microsensors (tip size ≤ 15 μm) exist that can detect various dissolved solutes, including glucose, oxygen, pH, sulfide, and ammonia. Lewandowski *et al.* [94] report using oxygen microsensors (tip size 15 μm) to estimate oxygen profiles, local oxygen uptake rates, and oxygen diffusion coefficients in a mixed culture biofilm community cultivated within a laboratory biofilm reactor. Kühl and Jørgensen [95] employed O_2, S^{2-}, and pH microsensors (tip size 20–25 μm) to study O_2 respiration, H_2S oxidation, and SO_4^{-2} reduction in compact, aerobic, trickling-filter biofilms. O_2 respiration was found in only the upper 0.2–0.4 μm of the biofilm, whereas SO_4^{2-} reduction occurred in the deeper anoxic parts of the biofilm. All H_2S produced in these anoxic depths was re-oxidized by O_2 in a narrow zone in the biofilm. Cronenberg and van den Heuvel [96] report the use of glucose oxidase immobilized to a platinum electrode (tip size 20 μm) to determine *in situ* the glucose diffusion coefficient and glucose uptake rates of yeast cells immobilized within agar beads.

The greatest advances in biofilm analyses have arisen in the area of non-invasive, non-destructive diagnosis. Lewandowski *et al.* [97•] employed spin-echo and dual spin-echo nuclear magnetic resonance imaging to visualize laminar flow fluid velocity profiles within an artificial porous medium system cultivating bacterial biofilms. The combined magnetic resonance imaging techniques provide estimates of local fluid velocities, as well as indications of 'dead' water (protons) where nuclei relax at a faster rate than free water, indirectly indicating a biofilm colony.

Bremer and Geesey [27•,98•] cultivated biofilms of a freshwater, copper-corroding bacterium on attenuated total reflectance (ATR) waveguides of germanium, within which an evanescent infra-red wave is transmitted. Spectra of materials that adsorb in the infra-red at the interface of the ATR crystal are collected, fast Fourier series transformed and the infra-red spectra of water subtracted to provide a continuous on-line measure of the molecular chemistry occurring at the cell–substratum interface. Accumulation of infra-red spectral intensities at these wave numbers implies that several proteins and polysaccharides have accumulated on the substratum. As the infra-red wave cannot penetrate more than 0.6 μm above the crystal

interface, this accumulation does not imply an increase in biofilm thickness.

Perhaps the single most significant advance to affect our understanding of biofilm ecological processes will undoubtedly be the confocal laser scanning microscope (CLSM) coupled with digital image analysis and a variety of fluorescent stains. Lawrence *et al.* [99•] illustrate the ability to section fully hydrated biofilms optically, both horizontally and sagitally, producing optical sections of undisturbed biofilms with a spatial thickness of 2 μm. Any fluorescent probe (cellular DNA stain, immunofluorescent stain, pH, ion, and redox-sensitive stains, viability redox stains) or any combination of probes can be recorded simultaneously by the CLSM with great clarity and little background interference, as a result of the confocal exclusion of any fluorescence originating from an excited fluorochrome above or below the focal point. Three dimensional reconstructions of biofilms are possible by displaying several biofilm optical thin sections as stereo pairs. CLSM is a critical tool in population community structure and in the analysis of mixed strain, species, and cell lines, and will allow detailed examination of the relationships between biofilm structure, adaptation, reactivity and response to external stress.

Conclusion

Historically, the foundations of microbiology were based on decades of experimentation and observations of the behavior of bacterial species or mixed cultures in suspension. Over the past fifteen years, research has shown that over 90% of the microbiological activity in an ecosystem is associated with interfaces. Bacterial biofilms can serve beneficial and detrimental roles within both natural and engineered systems. Analysis of biofilms of pure or mixed cultures combines the disciplines of microbiology, heterogeneous reaction engineering, surface chemistry, and mass transfer phenomena. Unlike commercially fabricated biocatalysts (immobilized enzymes or whole cell systems), biofilms are non-uniform in both space and time with the cells in the biofilm being responsible for generating their own support matrix. Research over the past year has recognized the physical, chemical, and biological heterogeneity that is inherent to a biofilm system. Progress in mathematical concepts as well as analytical methods and instrumental tools has been significant over the review period, clearly heralding an increase in our understanding and in our ability to control biofilm systems.

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