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Continuous nondestructive monitoring of microbial biofilms: a review of analytical techniques

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A fundamental requirement for the understanding and control of biofilms is the continuous nondestructive monitoring of biofilm processes. This paper reviews research analytical techniques that monitor biofilm processes in a continuous nondestructive manner and that could also be modified for industrial applications. To be considered 'continuous' and 'nondestructive' for the purpose of this review a technique must: (a) function in an aqueous system; (b) not require sample removal; (c) minimize signal from organisms or contaminants in the bulk phase; and (d) provide real-time data. Various microscopic, spectrochemical, electrochemical, and piezoelectrical analysis methods fulfill these criteria. These techniques monitor the formation of biofilms, the physiology of the microorganisms within biofilms, and/or the interaction of the biofilms with their environment. It is hoped that this review will stimulate development and use of biofilm monitoring techniques in industrial and environmental settings.

Keywords: biofilm; on-line monitoring; nondestructive monitoring; microscopy; Fourier-transform infrared spectrometry; bioluminescence; microelectrode; quartz crystal microbalance

Introduction

A biofilm has been defined as ' . . . cells immobilized at a substratum and frequently embedded in an organic polymer matrix of microbial origin . . . which is not necessarily uniform in time or space . . . composed of a significant fraction of inorganic or abiotic substances held together by the biotic matrix' [24]. Microbial communities at gas–solid, gas–liquid, and liquid–liquid interfaces also exist [94], but are not considered here. Bacteria and microeukaryotes (fungi, algae, and protozoans) readily attach to surfaces to form biofilms, and in nature most microorganisms are attached rather than free in the bulk–liquid phase. The importance of microbial biofilms has been established in, for example, aquatic, soil, industrial, and clinical environments [1,26,45,73]. Advantages for microorganisms within biofilms include increased availability of nutrients concentrated on substrata, fixation in a flowing system increasing the availability of dissolved nutrients, and the creation of 'microniches' such as anaerobic sites within an aerobic environment. Attachment can prevent transfer to a hostile environment such as, for oral microorganisms, from the hospitable conditions of the mouth to the acidic anaerobic gut. The disadvantages of life within a biofilm can include competition for nutrients and the availability to predators of a concentrated, sessile biomass. The structure, function, and physiology of biofilms is beyond the scope of this work, and has been extensively reviewed [23–25,94].

The characteristics of individual biofilms are extremely variable. The cell density ranges from the extremely sparse communities of oligotrophic bacteria within municipal water delivery systems (1×10^1 to 2.2×10^6 colony forming units per cm^2) [79] to the centimeters-thick sludge blankets in sewage treatment systems. A biofilm in a biotechnological treatment process may be a bacterial monoculture, while natural biofilms such as the periphyton covering streambed surfaces tend to be made up of many species. The composition of a biofilm typically includes (besides the organisms themselves) trace organics which concentrate at surfaces, extracellular polysaccharides elaborated by the bacteria, and particulate matter trapped in the polysaccharide matrix. The complexity and variability of biofilms can significantly increase the difficulty of analysis.

Application of classical bacteriological techniques to the study of industrial process problems such as biofouling and corrosion has demonstrated that microorganisms can play a significant role in the corrosion of metals [89], the deterioration of concrete [40], and the clogging of pipelines and filters [116,132]. Heterogeneous distribution of microbes and/or their metabolic activity can promote microbiologically influenced corrosion. This multibillion dollar problem [106] is not well understood even after decades of research. Microbial biofouling is ordinarily the primary step in macrofouling (eg barnacle attachment) and results in a substantial decrease in the hydrodynamic efficiency of ship hulls [11]. Biofilms can seriously decrease the efficiency of heat transfer units [23]. Planktonic organisms in ultra-pure water are often a result of biofilm growth elsewhere in the system [107]. Biofilm formation on medical implants can be life-threatening because the body's natural defenses, even when augmented by potent antibiotics, are usually ineffective against these infections [26].

In other biotechnological or bioremediation processes, it is an advantage to promote biofilm formation and maintain the active biomass. Municipal wastewater treatment is

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largely dependent upon biodegradation of organic waste by microbial biofilms and aggregates [83]. *In situ* biodegradation of organic wastes in subsurface aquifers requires the development of sufficiently active subsurface microbial biofilms [61]. The stability and productivity of many industrial bioprocessing and food fermentation procedures [139] is increased by the presence of appropriate microbial biofilms. Biofilms are even implicated in the maintenance of health in vertebrates—the microbiota attached to the inner lining of the gut produce vitamins and aid in digestion [127].

Consequently, it is important that biofilm microbial ecology be understood so that biofilm development can be prevented or advantageously manipulated. Unfortunately, the most commonly applied methods are destructive, requiring either samples of the biofilm-substratum system, or removal of the biofilm from the substratum. This often results in disruption of the biofilm and usually requires the biofilm to pass through an air–water interface which can damage and/or contaminate the sample. The three-dimensional architecture of a biofilm will be lost if analysis procedures require fixed and dried samples. Methods for removing biofilms from the substratum are inefficient and may cause lysis of the cells. If organisms recovered through biofilm removal are regrown *ex situ*, they are then subject to severe selection by the medium [1,117,135]. However, the greatest problem with destructive analyses is that the inherent variability of successive samples may completely mask the development of the biofilm over time.

The problems outlined above can best be overcome by use of continuous nondestructive monitoring techniques. For the purpose of this review, a technique is classified as continuous and nondestructive if it allows direct measurement of biofilm parameters in an aqueous system in real time. Furthermore, the technique should be noninvasive and should minimize interferences from microorganisms in the bulk phase. In an industrial situation, these techniques could be used on-line (monitoring of a process via a sampling tube) or in-line (direct monitoring of the process).

Microscopic techniques

Visualization of microorganisms has always been critical to understanding microbial interrelationships, particularly those that occur on surfaces. In many industrial applications, it is the interaction of microorganisms with surfaces that creates process problems. Despite the huge variety of techniques available, microscopy remains underutilized in biofilm studies. However, the potential for on-line microscopic investigation of industrial problems has never been greater, especially in light of rapid advances in computer hardware and software. For example, the field of view in a microscope can now be rapidly converted to a digital image at a resolution approaching that obtained by photomicrography. The digital images can be manipulated with image analysis software, thereby permitting cells in a field of view to be counted automatically in a fraction of the time needed for other enumeration procedures. The high sample throughput and compact data storage offered by digital microscopy have been complemented by rapid advances in the number and selectivity of dyes used to detect biochemical and physiological characteristics of cells. Many of these dyes have a low toxicity and can be washed out of the cells (eg fluorescein, certain rhodamines) thereby making repetitive staining (temporal measurements) possible (R Palmer and J Almeida, Univ. Tennessee, unpublished).

Some of the earliest microscopic observations that could be termed on-line were *in situ* microscopy of algae and bacteria in a pond [126], and temporal studies of soil bacteria in capillary channels [5,110]. Staley [126] utilized a partially immersed microscope to observe the colonization of a slide by bacteria and algae. The capillary technique, which was used to follow the growth of bacteria with distinctive morphologies such as *Caulobacter* and *Pedomicrobium*, has never been widely used because it requires specialized glassware and because conditions within the capillary (Eh, pH, oxygen tension) could not be controlled or monitored.

The problems of microscopy under field conditions are difficult, and a more convenient way to examine attached microorganisms is through the use of flowcells. Laminar flow conditions can be created in parallel-plate flowcells, and microscopy of the biofilm that develops on a glass substratum can be performed [21]. The flowcells, which are simple, versatile, and inexpensive modifications on a design originated by Berg and Block [9], can be adapted for microscopy of biofilms under a wide variety of conditions. Movement of *Pseudomonas fluorescens* within the hydrodynamic boundary layer was examined photomicroscopically [75]. Images were analyzed using the 'turnkey' IBAS system (Kontron, West Germany). The same techniques were used to examine the effect of laminar flow velocity on attachment and growth rate of a wild-type *P. fluorescens* and of a motility-mutant that lacked flagella [70]. Also, the attachment, colony formation, and motion of *Vibrio parahaemolyticus* strains on a glass surface was followed under high- and low-viscosity conditions using similar techniques [77]; a wide range of phenotypic variability was seen among the various strains. Long-working-distance optics have been used in conjunction with thicker flowcells to examine the influence of particle (streptococcal cells, polystyrene lattices) surface characteristics on attachment to glass [122]. Automated data collection for this system was realized by integration of a charge-coupled-device camera with an image analysis system and continuous, real-time measurements of streptococcal deposition were performed [123]. The effects of different substrata (fluoroethylene-propylene copolymer, polymethylmethacrylate, glass, mica) on attachment of streptococci [124] were also analyzed. Measurement of microbial co-adhesion in a flowcell has been described [12]. *Actinomyces* cells were allowed to adhere to the glass substratum, images were captured after an adherence phase, then streptococcal cells were introduced and images were captured after these cells had subsequently adhered. Subtraction of the *Actinomyces*-only image from the *Actinomyces*-plus-*Streptomyces* image gave an image that was then used to determine the number of *Streptomyces* cells inside a 0.16-μm shell around each *Actinomyces* cell (co-adherence).

These studies demonstrate the uses of microscopy, flowcells, and image analysis. It is important to recognize that use of a flowcell establishes certain parameters, such as the hydrodynamic regime, that may not exist in the system to be examined or that exist only at certain locations, such as at a pipeline wall. Despite this constraint, flowcells have many practical advantages as on-line test systems including simplicity of construction, low cost, and ease of integration into industrial systems. For example, microbial community structure within a bioreactor can be examined simply by attaching the flowcell to a loop in the reactor line [69]. Integration of flowcells into other systems could be as simple. This type of on-line investigation provides data on the actual community as it develops *in situ* under the conditions within the cell, not on the potentially artifactual simulations of microbial biofilms created by use of pure cultures or of defined cocultures.

Geesey and colleagues recently used plasmid-borne β-galactosidase (*lacZ*) activity fused to an alginate promoter (*algC*) for analysis of alginate production during attachment of *Pseudomonas aeruginosa* [29,30]. Beta-galactosidase activity and, therefore, *algC* promotion can be followed at the level of a single cell by providing nonfluorescent methylumbelliferone-tagged β-galactopyranoside, a fluorogenic substrate that is cleaved by the *lacZ* gene product to yield the fluorescent compound, methylumbelliferone. Methylumbelliferone appears not to diffuse out of cells, thereby permitting detection of *lacZ* expression with an epifluorescence microscope. Using this single-cell fluorescence assay of gene expression, it was demonstrated that attaching cells appeared to undergo a lag in *lacZ* translation, and that the majority of cells that detached from a biofilm no longer expressed *lacZ* [30]. These results obtained using a flowcell system were the first report of transcriptional control at the single-cell level.

For nearly a decade, confocal laser microscopy (CLM) has been a tool in the biological sciences, primarily in neurological studies. The instrument uses a physical barrier system (confocal apertures or 'pinholes') to create a thin (0.4-μm) plane-of-focus in which out-of-focus light has been eliminated. A laser light source provides the intense, highly coherent, collimated light necessary to penetrate deep into a thick specimen. The laser light is used to excite fluorophores, either intrinsic biomolecules such as chlorophyll or chemicals added to the sample such as fluorescein. The resulting fluorescence is detected by photomultiplier tubes, and a digital image is obtained. Images of the x–y plane (parallel to the surface) are collected automatically as the z dimension (depth) is altered by a computer-controlled stepping motor. A series of optical sections through the specimen is thus obtained. Sections in the x–z plane (saggital sections) can also be displayed. The individual optical sections can be processed using standard image-analysis software, and the entire set of images can be computer-processed to create a three-dimensional reconstruction. The ability of CLM to resolve three-dimensional structures in the micrometer range gives microbiologists a tool for exploration of biofilm architecture in the native state: hydrated, living cells within an exopolymer matrix. Fluorescent probes can be used to localize and measure intra- and extracellular conditions (pH, ionic concentration, membrane potential) in three dimensions within living biofilms in real time. Detail on these varied topics is outside the scope of this article. The reader is referred to excellent sources on theory and practice [13,108]. A multitude of fluorochromes exist for neuro- and cell biological purposes [59]. The potential utility of these probes in biofilm studies is not known, although some stains have been used to investigate bacteria in a manner analogous to the eukaryotic applications [95].

The pioneering work of Caldwell, Korber and Lawrence established the value of CLM in microbiological research [19]. Using a simple exclusion-staining method [20], biofilms grown in flowcells were optically sectioned, and the spatial arrangement of organisms within biofilms was visualized in three dimensions [76,138]. Physical parameters, such as diffusion coefficients [78], can be measured within the biofilm matrix. Palmer and Caldwell [106a] have used CLM to examine the three-dimensional structure of a dental-plaque-analogue biofilm (Figure 1). In this 'on-line *in vitro* mouth', natural saliva was used to establish undefined, mixed-species biofilms similar to those occurring *in vivo*. Three-dimensional interrelationships of fluorescein-stained organisms within these films were analyzed, and differences in the intensity of staining between morphologically identical cells were interpreted as differences in cellular chemistry. Fluorescence Recovery After Photobleaching (FRAP) has been used to measure accumulation of a fluorescent substrate in the extracellular polymer of a mixed-species biofilm; the uptake of the substrate was inhibited in the presence of KCN [137].

Spectrochemical techniques

Light can be absorbed, scattered, and/or emitted by matter. These types of interactions contain qualitative and quantitative information on biological systems. Absorption of high-energy radiation (gamma-rays, X-rays, and ultraviolet) can lead to photodecomposition of biomolecules and cell death, but radiation in the visible, near-infrared, mid-infrared, and radio frequencies have been used in absorption spectrometry to detect living microorganisms in aqueous environments. Using a 50-μm diameter fiber optic probe, profiles of visible and near-infrared light (400–900 nm) penetration were measured at different depths within microbial mat and sediment cores [72,74]. Near-infrared radiation (700–1100 nm) has been used to monitor cell density in bioreactor fluid [49]. Absorption of radio frequencies in the presence of magnetic fields (nuclear magnetic resonance spectrometry) was employed to study substrate consumption and intracellular metabolites in dense suspensions of bacteria [119]. NMR has also been used to measure the effect of biofilms on flow velocity profiles [84]. These as well as other spectrometric techniques such as Raman scattering [28] and membrane-inlet mass spectrometry [90] may also have attributes that allow continuous monitoring, but have not yet been directly or extensively applied to biofilm analysis. The following discussion pertains to light-sensing technologies that have been directly applied to biofilm research.

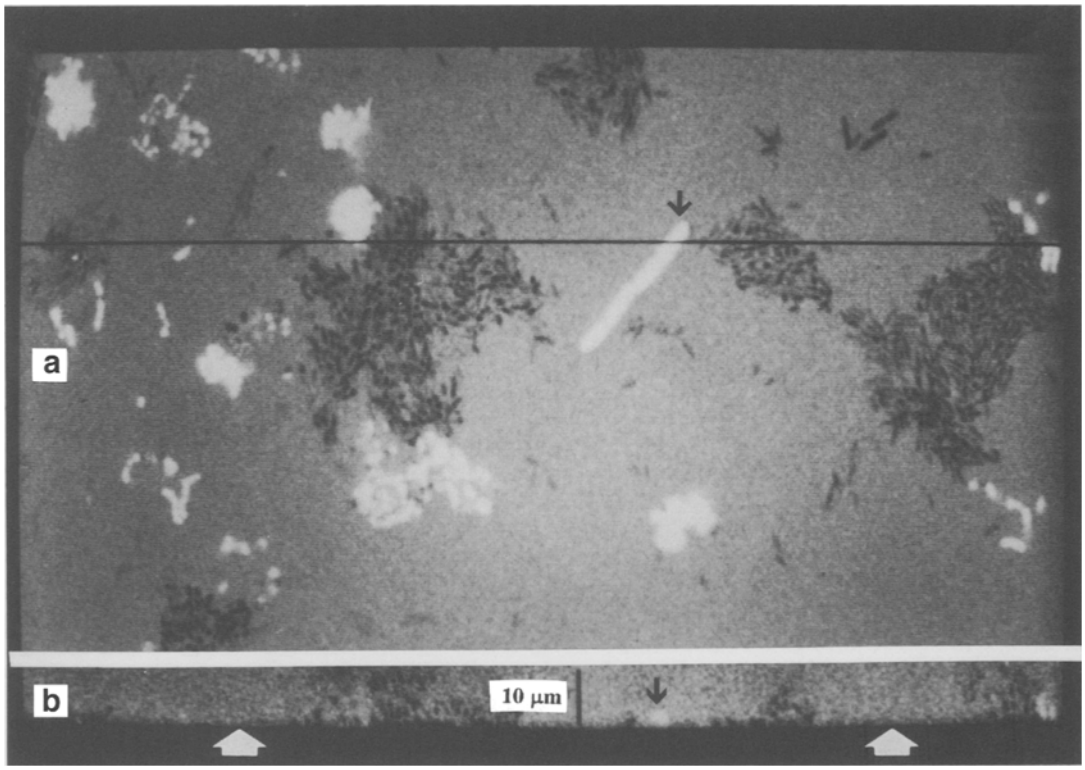

Figure 1 Confocal laser microscope images of a fluorescein-stained biofilm on a glass coverslip. (a) Top view, in the plane of the substratum. Composite of twelve 0.3-μm *x-y* planes collected at 1-μm intervals. The effective depth-of-field is thus 12 μm. Four distinct bacterial morphologies can be discerned: large bright rods, dark cocci, tightly-packed bright cocci, and loosely-packed bright cocci. The position at which image b was acquired is indicated by the thin black line across the image. (b) Side view through the biofilm (saggital section, in the *x-z* plane). The white arrows point to the interface of the biofilm with the glass substratum (black area). Black arrows indicate where the large bright rod-shaped bacterium appears in each image. The scale bar = 10 μm, and it applies to both parts of the figure

Infrared absorption spectrometry

In condensed phases (solids and liquids), infrared (IR) spectrometry is used to study the interaction between IR radiation and vibrational modes of molecules. In general, IR radiation is absorbed by a molecule when the energy of the radiation is equal to that required to promote the molecule to an excited vibrational state. Absorption occurs only at discrete frequencies when a molecule is exposed to a continuum of IR radiation. The amount of radiation absorbed is proportional to the number of molecules present. This frequency-dependent absorption results in a unique absorbance pattern or spectrum that is defined by the structure of the molecule. For complex systems such as biofilms, the spectrum is the sum of the spectral signature of each biomolecule in the sample. The frequency or wavenumber at which a molecule absorbs radiation is mainly determined by specific groups of atoms (functional groups) within the molecule and is less dependent on the remaining structure. The individual wavenumber range at which a specific group of atoms absorbs radiation is referred to as the characteristic, or group, frequency. Tables of characteristic frequencies have been compiled to aid in the identification of IR absorbance bands [87,100]. For example, the group-frequency range for the carbonyl stretch of esters is from 1750 to 1715 cm^{-1} [87]. The ester groups in alginic acid and in poly-β-hydroxyalkanoates absorb radiation within this range at 1732 and at 1740 cm^{-1}, respectively [46,101]. Thus, differences in molecular structure may be identified as well as quantified in a complex sample, making IR absorption spectrometry an attractive method to study biofilms.

Fourier transform-infrared (FT-IR) spectrometers became affordable in the 1980s and offered numerous advantages over older dispersive instruments. Dispersive instruments used monochromators to scan narrow bands of frequencies sequentially, whereas FT-IR spectrometers use interferometers to measure all frequencies simultaneously with the signal modulated over time [56]. The advantages of FT-IR over dispersive instruments include increased analysis speed, improved signal to noise ratios (because spectra are easily added and averaged with time), better wavenumber accuracy, and greater signal throughput at similar resolution. Although many types of commercially available FT-IR instruments have been used to study aqueous biofilms, a FT-IR spectrometer has been specifically designed for biofilm studies that incorporates three optical channels, a mercury-cadium-teluride detector for sensitivity, and a refractively scanning interferometer for stability [102]. This multichannel instrument can be used to measure either the spectra of biofilms exposed to three different treatments or the spectra of triplicate biofilms over time within a single experiment.

Attenuated total reflection (ATR) spectroscopy is a sampling technique used in FT-IR spectrometry to examine aqueous environments near the surface of a special substratum called the internal reflection element (IRE, Figure 2) [58]. The IRE must reflect internally as well as be transparent to the IR radiation and, for biofilm studies, must be insoluble in water. Materials such as zinc selenide and germanium meet these requirements. Transparency is a necessity because the IR radiation must reach the detector. With total internal reflection, a standing wave of radiation penetrates out from the IRE into the water, and the intensity of the radiation decays exponentially with distance from the IRE [58]. The decaying wave, termed an evanescent wave, consists of the same frequencies as the reflected light, and can be absorbed by molecules near the outer surface of the IRE. The distance that the evanescent wave penetrates into the outside water (dependent on the refractive index of the IRE, the wavelength of the light, and the angle of incidence) can be estimated using the parameter depth-of-penetration [58] which is defined as the distance from the IRE surface at which the intensity of the wave decreases to e^{-1} (approximately 37%) of the initial level. Depth-of-penetration is 0.4 μm for water in contact with a germanium crystal, at an incident angle of 45°, and with radiation at 1550 cm^{-1} (amide II band). Only water molecules and biofilm components within the evanescent-wave region contribute to the absorption spectrum.

Conditioning films are layers of organic-rich material adsorbed to a substratum and are important in the formation of biofilms [7,52]. Conditioning film formation has been monitored in flow-through systems using ATR/FT-IR spectrometry. In an early study, the adsorption of substances (possibly phosphate) from filtered sea water was monitored using germanium IREs [101]. More recently, the technique was used to monitor the adsorption/desorption of alginate and dextran to uncoated and to protein-coated germanium IREs at different pH levels [64]. Three different proteins (albumin, β-lactoglobulin and myoglobin) were coated separately on germanium IREs. Alginate, an acidic polysaccharide, adsorbed in greater amounts to protein-coated IREs than to uncoated IREs. In contrast, dextran, a neutral polysaccharide, adsorbed in greater amounts to uncoated IREs than to the protein-coated IREs. Because attachment of bacteria to solid surfaces is thought to be facilitated by extracellular polymers, these results support the hypothesis that the attachment of a bacterium to a conditioned surface will be different than to an unconditioned surface.

A study monitoring living cells elucidated the chemical nature of an extracellular polymer that facilitates adhesion [131]. Suspensions of *Catenaria anguillulae* zoospores in high-purity water were pumped through flowcells containing a germanium IRE. Infrared spectra showed a time-dependent increase of bands at 1645 (amide I), 1550 (amide II), 1450 (CH_2 deformation), and 1400 (carboxylate ion) cm^{-1}. The bands, indicative of proteins, suggested that the

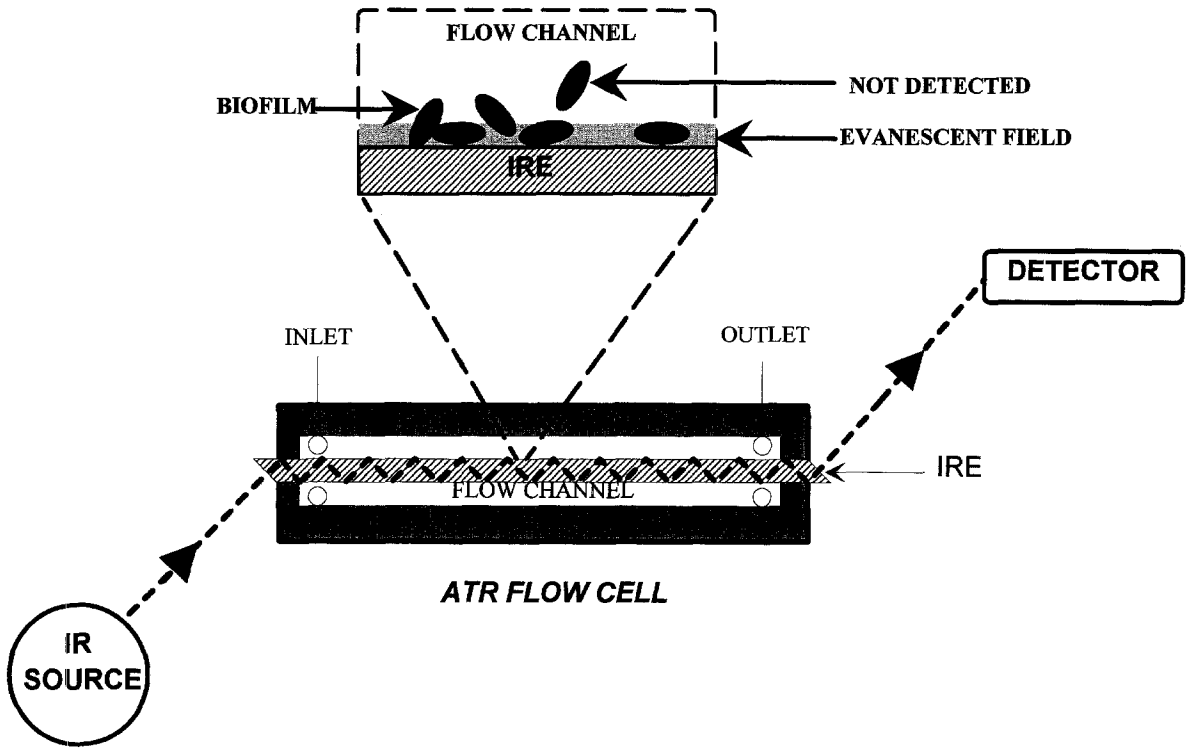

Figure 2 Schematic diagram of attenuated total reflectance (ATR) flowcell. Infrared radiation from the source is reflected on the inside surfaces of the internal reflection element (IRE). At each reflection, some of the radiation penetrates out from the IRE and into the biofilm (see expanded view). This radiation is the evanescent wave and is depicted as the shaded region. Biofilm material (eg cells, extracellular polymer) as well as interfacial water absorbs radiation and is detected by an infrared detector

extracellular polymers contacting the surface were proteinaceous.

Bremer and Geesey [15] monitored the development of a biofilm with ATR/FT-IR. Pure cultures of bacteria originally isolated from corroded copper tubing were inoculated into flowcells containing enriched growth medium. In these experiments, sterile and inoculated flowcells were monitored with time. The bacteria formed a biofilm on the surface of the cylindrical germanium IRE and the resultant IR spectra contained bands that increased in intensity with time.

The contributions of bacterial cells to the IR spectrum have been distinguished from those resulting from medium components [103]. In this study, *Caulobacter crescentus* cells suspended in high-purity water, in minimal medium, or in bioreactor fluid were analyzed with the ATR/FT-IR technique. Examination of the sterile medium showed a time-dependent increase in the intensity of a single absorbance band at 1080 cm^{-1}. The band originated from the P-O stretch mode of inorganic phosphate, which was probably precipitated on the surface of the IRE as the calcium and/or iron salt. Although this band overlapped the biofilm C-O and P-O stretch bands, the amide I and amide II (as well as other biofilm bands) were unaffected, and therefore amide I and II were used to follow the development of the biofilm. At the end of each bioreactor experiment, parallelogram IREs (less efficient at capturing the IR beam than cylindrical IREs, but more practical for microscopic examinations) were removed from the flowcells, and the number of attached cells was determined by light microscopy using acridine orange staining. A plot of attached cells per unit area versus amide II absorbance values was generated to estimate a limit of detection of 5×10^5 bacteria cm^{-2}.

The development of biofilms with *C. crescentus* or *Pseudomonas cepacia*, and their subsequent production of poly-β-hydroxybutyrate, has been monitored (Nivens *et al*, in preparation). Two types of IREs were used to obtain spectra from biofilms at different depths-of-penetration. Because the refractive index of zinc selenide is lower than that of germanium, evanescent waves from the zinc selenide IREs penetrate twice as far as those from germanium IREs. Production of poly-β-hydroxybutyrate was monitored by increases in the intensities of bands at 1739 (carbonyl stretch of the ester band), and 1188 and 1059 (C-O stretches) cm^{-1}(Figure 3). The spectra of *P. cepacia* biofilms on the zinc selenide IREs were similar to but more intense than the spectra obtained with the germanium IREs. However, for *C. crescentus* cells, the poly-β-hydroxybutyrate (1739 cm^{-1}) to amide II (1550 cm^{-1}) ratio increased with penetration distance of the evanescent wave. Assuming that *C. crescentus* biofilms on germanium are similar to those on zinc selenide, the results indicated that *P. cepacia* probably grows proximal to the surface, whereas much of the biomass of the *C. crescentus* cells, that are tethered to the surface with a stalk, is located away from the surface of the IRE. These results support theoretical calculations that the depth-of-penetration is less than one micrometer for germanium IREs.

In a medical application, the ATR/FT-IR technique has been used to monitor not only the formation of *P. aeruginosa* biofilms, but also to follow the penetration of an antibiotic (100 $\mu g\ ml^{-1}$ ciprofloxacin) into the evanescent-wave region located near the base of the biofilm [130]. Transport of the antibiotic was monitored using the area of IR bands at 1303 and 1270 cm^{-1}. This study demonstrated that the biofilm impeded transport of the antibiotic through the biofilm and to the IRE. Also noteworthy is the appearance of bands at 1729, 1400, 1250, 1059 cm^{-1} after the exposure of the biofilm to the antibiotics. It was suggested that the bands may be associated with changes in RNA and DNA vibrational modes. However, these bands are also present in the spectrum of alginate which is produced by *P. aeruginosa* [46].

Some investigators have expanded the applications of ATR/FT-IR spectrometry by coating IREs with thin films of industrially relevant materials. A prerequisite of such research is deposition of a continuous film on the IRE and, at the same time, transmission of the IR radiation to the aqueous environment. Using copper-coated germanium IREs, ATR/FT-IR spectrometry was used to monitor adsorption of biomolecules in a stagnant cell [51,66] and biofilm-mediated corrosion of copper in a flow-through system [14,50]. Corrosion or dissolution of the copper film was monitored as an increase in absorbance of the water band at 1640 cm^{-1}. In control experiments, the absorbance of the water band remained nearly constant in a sterile medium indicating that the copper films were stable. Recently, Ishida and Griffiths [65] showed that copper-coated IREs (8 to 12-nm nominal thickness) with three reflections could be used to obtain adequate signal-to-noise ratios (IREs with ten reflections are typically used). However, when these continuous films were exposed to saline solutions (0.15 M NaCl at a pH of 7.0 or 4.8), a shift in the water band was discovered. This shift, which could cause spectral artifacts in the water-substracted spectra of biofilms, was attributed to the dissolution and reorganization of the copper film (formation of metal islands). In addition to copper, 316-stainless-steel films have also been successfuly deposited on germanium IREs [109], and were also used in a preliminary microbial influenced corrosion study [129].

Attenuated total reflection/Fourier transform-infrared spectrometry has demonstrated efficacy as a technique in understanding biofilm composition and the effects of biofilms on substrata. ATR/FT-IR spectrometry provides a unique 'inside out' view of biofilms. However, limitations to the technique exist and should be recognized. The technique analyzes only the base layer (approximately 1 μm) of biofilms and provides an average picture of the chemistry transpiring over the entire area exposed to the aqueous environment. In addition, the technique produces spectra containing vibrational information from all the molecules within the evanescent-wave region; the data are coincidental and convoluted. Even with this enormous amount of information, determinations such as distinguishing dead biomass from living biomass from a single spectrum cannot be done. In order to realize the full potential of the technique, further progress is needed in data interpretation, in instrumentation (stability and sensitivity), and in the development of new, relevant surfaces.

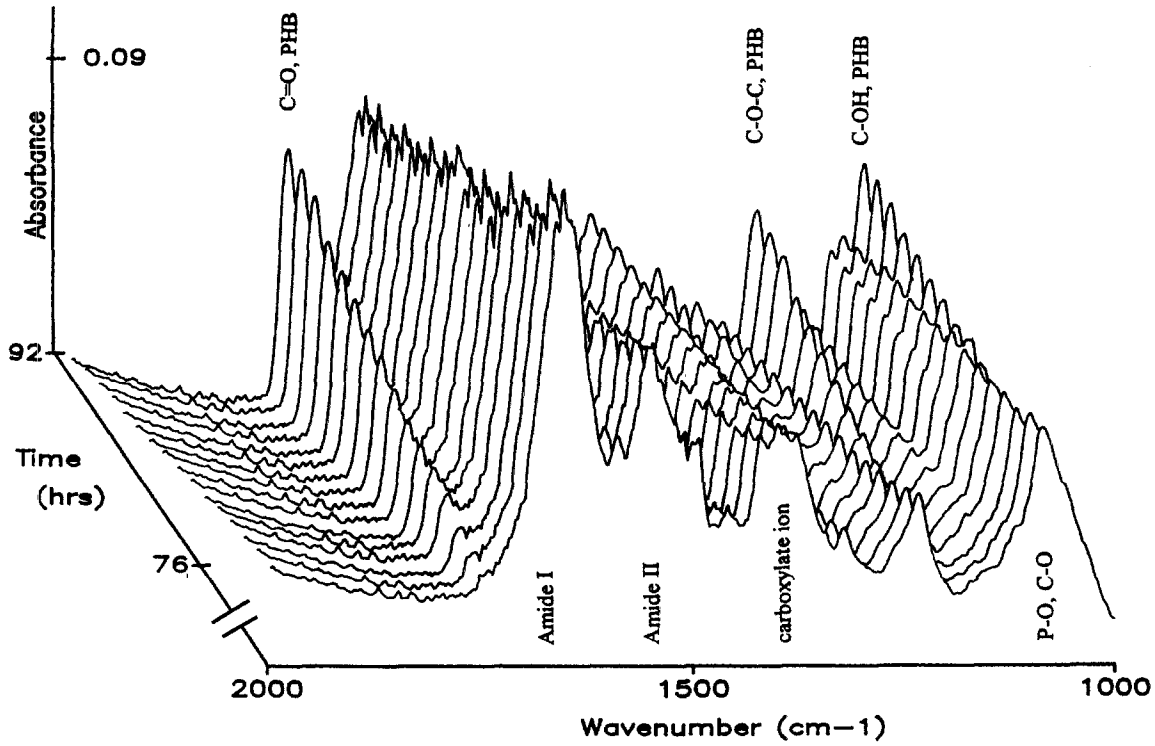

Figure 3 A three-dimensional plot of time-dependent ATR-FT/IR spectra showing poly-β-hydroxybutyrate production by a *Pseudomonas cepacia* biofilm. The biofilm, inoculated at time zero, developed on a germanium crystal over 76 h. The first IR spectrum contains bands (eg amide I at 1650, amide II at 1548, carboxylate ion at 1400, the P-O, C-O stretches at 1080 cm^{-1}) representative of a biofilm grown on a balanced medium. A carbon-rich medium (lower ammonium ion concentration) was pumped through the flowcell to stimulate the production of poly-β-hydroxybutyrate, an intracellular carbon storage product. In this series of spectra, the labeled bands indicative of poly-β-hydroxybutyrate increased with time, while the bands associated with the biofilm remained constant

Fluorescence

Absorption of ultraviolet or visible radiation by a molecule causes the transition of an electron from a ground electronic state to an excited electronic state. Fluorescence is the emission of a photon during the return to the ground state. Typically this photon has less energy (longer wavelength) than the excitation radiation because the molecule experiences a loss of vibrational energy in the excited state. Several biomolecules (including tryptophan, phenylalanine, tyrosine, photosynthetic pigments, riboflavin, the archaeal coenzyme F420, and reduced and oxidized NAD) display fluorescent quantum efficiencies that are favorable for detection. Fluorescence spectra can contain quantitative information as well as some qualitative structural information. Fluorescence measurements have been used to monitor on-line microbial changes in bioreactor fluids. The subject has been reviewed [92] and a table of applications has been reported by Li and Humphrey [85]. Although numerous studies have demonstrated the relationships of fluorescence to biomass (tryptophan fluorescence) and activity (NADH fluorescence), the intensity of the fluorescence signal can be affected by many factors. These include photodegradation of the excited molecule, quenching of the excited state, absorption of the fluorescence (the cascade effect), or a loss of intensity of the source radiation resulting from a high concentration of absorbing molecules (the inner filter effect). These and other problems associated with fluorescence measurements have been discussed [85,125,133] and should be considered when using fluorescence to study biofilms.

Instrumentation used for fluorescence measurements is composed of a radiation source, an excitation wavelength selector, focusing optics, sample apparatus, collection optics, an emission wavelength selector, and a detector. A fluorometer using a xenon lamp source, double monochromators for both excitation and emission, a bifurcated quartz fiber-optic bundle, and a photomultiplier tube for detection has been used to monitor the tryptophan fluorescence of *Vibrio harveyi* biofilms generated on stainless steel substrata in a flow-through system [3]. Flowcells were designed to contain five sample substrata and to operate under laminar flow conditions at a flow rate of 5 ml min^{-1} [97]. Flush-mounted quartz windows were sealed into the flowcells directly above the substrata. Fiber optic probes were used to detect fluorescence from the biofilms through quartz windows. Tryptophan fluorescence correlated linearly with the biomass of the biofilm (from approximately 5×10^5 to 4×10^6 cells cm^{-2}) as determined by direct counts using acridine orange staining. Also with this test system, tryptophan fluorescence was used to monitor the formation of *V. harveyi* biofilms on antifouling coatings [6]. The coatings included vinyl co-polymers that released a biocide at different constant fluxes. Biocide release rates correlated with decreased tryptophan fluorescence

(normalized to a stainless steel substratum located upstream from the antifouling coatings) as well as with a reduction in the number of attached microorganisms as determined at the end of the experiment by acridine orange direct counts.

Application of fluorescence spectrometry to biofilm research can be expanded by the use of fluorescent gene-reporter technology. Transcriptional fusions of promoters for microbial functional genes with the gene coding for a fluorescent molecule are created and incorporated into a test or probe organism. For example, green-fluorescent-protein (GFP, cloned from the jellyfish *Aequorea victoria*) has been expressed in *Escherichia coli* [22]. With excitation at 380–490 nm, fluorescence of GFP can be detected at 509 nm. One advantage of the GFP reporter system is additional substrates or cofactors are not required, in contrast to other reporter methods (such as *lacZ* and bioluminescence). Development of reporter systems having fluorescence at different wavelengths will result in the ability to simultaneously monitor activity of different genes.

Bioluminescence

Bioluminescence, light production by organisms such as fireflies and certain bacteria, is a special case of light-emission spectrometry that, unlike fluorescence, does not require source radiation. Instead, *in vivo* biochemical reactions induce the excitation of biomolecules and the subsequent emission of photons. Light production requires the enzyme luciferase, oxygen, a long-chain fatty aldehyde, and a reduced flavin mononucleotide [128]. Bacterial bioluminescence can be used to detect bacterial biomass (assuming constant light flux per cell), cellular activity (at a given biomass), or gene expression (in genetically engineered bacteria containing the *lux* gene fusion) [128]. An on-line biosensor using genetically engineered bioluminescent *Pseudomonas fluorescens* has been used to detect naphthalene and salicalate in mixed waste streams [60].

Both the bioluminescence and the tryptophan fluorescence of *V. harveyi* biofilms in flow cells were used to evaluate the effectiveness of antifouling and fouling-release coatings [6]. Bioluminescence and fluorescence of biofilms on the coated surfaces were normalized to signals obtained from uncoated stainless steel surfaces. A bioluminescence ratio significantly greater than the tryptophan ratio was used as an indicator of increased photon flux per cell and greater overall cellular activity. It was suggested that the biocide released from the coating disrupted cellular metabolism and that the method could be used to rank relative toxicity of the anti-fouling coatings.

Bacteria harboring bioluminescence reporter plasmids, transcriptional fusions containing the *lux* genes [67], were also utilized in biofilm studies. The attachment to polystyrene surfaces of *E. coli* K12 (containing the *lux* expression plasmid PSB100) was monitored with bioluminescence measurements [38]. In these experiments, the substrata were exposed for a given time, separated from the suspensions, and the bioluminescence determined. In addition to time-dependent attachment curves obtained at different cell concentrations, adherent bacteria were challenged with biocide to determine biocide efficacy. In a flow-through system designed to create a shear gradient, bioluminescence measurements were used to monitor biofilm formation on glass plates by *Pseudomonas fluorescens* containing a *lux* reporter plasmid [96]. Microbial biomass and lipid biosynthesis both correlated with bioluminescence intensity. Additionally, an environmental bacterium harboring a transcriptional fusion between *lux* and the promoter for *algD*, the gene for alginate biosynthesis, was utilized in studies monitoring bioluminescence during biofilm formation [115].

Electrochemical techniques

Large-area metal electrodes—electrode as substratum

One strategy for the nondestructive, continuous monitoring of biofilms involves the measurement of time-dependent changes in the potential associated with large metal electrodes (typically greater than 1 cm in diameter). Potential (sometimes referred to as the redox, open circuit, or corrosion potential) is measured as a voltage difference between the electrode of interest (working electrode) and a reference electrode. Both the interfacial and the electrode chemistries determine the potential of the system. Biofilm activity can alter the interfacial chemistry, and thereby change the potential of the system. The magnitude and direction of the change in potential is dependent on the type of metal, the consortia of microorganisms present, and other environmental conditions (eg light, temperature, salinity). For example, biofilms have been reported to increase (referred to as enoblement), decrease, or have little effect on the potential of stainless steel and platinum electrodes [39,41,42,88,93,98]. Under a defined set of conditions, a change in potential can be used to indicate microbial activity and may correlate with the presence of biomass. However, in general, potential is not a direct or quantitative measurement of microbial activity or biomass.

Other types of electrochemical analysis of large electrodes measure variables such as current (rate of movement of charge carriers) and impedance (frequency-dependent resistance to the flow of current). With many types of metal electrodes, changes in electrochemical variables can be detected as biofilms form on the electrode and, in many instances, these changes are indicators of surface alterations induced by the biofilm. For example, microbiologically influenced corrosion (MIC) is a well-known industrial problem and electrochemical techniques have been employed to monitor variables that assess damage to the metal, and help determine the mechanism of attack [93]. However, many variables (eg polarization resistance, double-layer capacitance, corrosion current density) can only be determined by techniques that apply current to the system and thereby change the biofilm. Small electrochemical perturbations, such as those applied in electrochemical impedance spectroscopy (316 stainless steel, ± 5 mV at various frequencies), do not affect the activity and number of attached bacteria within a biofilm [47]. Although these non-steady-state techniques have been used to provide quantitative information on corrosion phenomena [93], a relation-

ship between the variables and the physical properties of the biofilm can only be inferred.

Analyte-selective microelectrodes—electrode as probe

Analyte-selective microelectrodes (typically, less than 20 μm tip diameter) measure the activities (concentrations) of various chemical species as changes in current or potential within the electrode system (reference and sensing electrode) [2]. Microelectrodes have been prepared which detect oxygen [8,113], pH [111,134], sulfide [111], nitrate [32], ammonium [33], nitrous oxide [114], chlorine [35], and glucose [27]. Ideally, the microelectrode tip should be small (approximately the size of a bacterium), stable, sensitive, selective for only the species of interest, reproducible, accurate, and have a fast response time. In practice, microelectrodes respond to interferences and the signal drifts, they are fragile, must be calibrated, and are difficult to manufacture. Insertion or adjustment of a microelectrode can be destructive to the biofilm, and larger electrodes will cause more damage. These problems aside, the use of microelectrodes in on-line systems has greatly enhanced the understanding of several physiological processes within biofilms by measuring spatial and temporal concentration gradients.

The size of the electrode limits the spatial precision with which measurements can be made. Sulfide electrodes have large tip diameters (generally $\geq$50 μm) and thus the intervals over which concentration measurements can be obtained are large compared to the scale of bacteria [112]. The dental literature has examples of pH measurement within dental plaque using large (>1 mm tip diameter) electrodes. Some of these studies were conducted in flowing *in vitro* systems [62,118,121].

Microelectrodes have been constructed that have a tip diameter $\leq$10 μm which measure concentrations on a scale significant for microorganisms in a biofilm. In an early study [136], dissolved oxygen was measured at 25-μm intervals with a 'microbial slime system' under flowing conditions, although the technique used for vertification of the electrode position (attachment to a microscope objective) was crude. These authors reported calculation of oxygen diffusivities and microbial respiration rates [16]. Revsbech, Jørgensen, and colleagues applied microelectrodes to measure oxygen concentrations and pH at 100-μm intervals through microbial mat cores [112]. This study demonstrated that such electrodes, while fragile, could be used under harsh circumstances. The construction and calibration of electrolyte-filled pH microelectrodes is not trivial, but can be accomplished without a large investment in special equipment [111]. A less fragile solid-state iridium oxide microelectrode has been used for measurements of pH at intervals of 50 μm within biofilms [134]. This promising ion-selective electrode has advantages over electrolyte-filled electrodes with respect to rigidity, range, calibration, and reproducibility. The iridium oxide electrode is, however, not useful in situations where sulfide is present. Polarographic oxygen microelectrodes can be constructed that have a tip diameter of less than 1 μm [8], but construction and use of these electrodes is very complicated. They have been used to measure oxygen gradients within a microbially colonized alginate bead 1 mm in diameter [10]. Dissolved oxygen and pH have been measured at 100-μm intervals through a 2.5-mm-thick mixed species biofilm on corroding steel [80,81]. The open system was under flow and additional measurements (open circuit potential, cathodic and anodic polarization, AC impedance) were made on the stainless steel to describe the corrosion process. Recently, a J-shaped oxygen microelectrode was used with an open flow system under microscopic examination at depth intervals of 10 μm [31]. The location of the electrode tip was confirmed using confocal laser microscopy thereby yielding very accurate information on the depth of the electrode within the biofilm and on electrode position relative to pores in the biofilm matrix. Local gradients of oxygen were measured within cell clusters and pores. An example of the use of the concentration data obtained with these microelectrodes and the explanatory power which is gained by performing complementary analyses in parallel is a study of an aerobic trickling-filter biofilm from a sewage treatment plant [71]. Microelectrodes for oxygen, sulfide, and pH were applied in parallel to derive depth profiles of oxygen and sulfate respiration rates from concentration data.

Aggregates are a special case in which the biofilm is attached to itself rather than to a solid substrate, and floats free in the liquid medium. Examples of the use of microelectrodes to probe the metabolic activities within aggregates include *Bacillus laevolacticus* in pure culture [36], ethanol-producing aggregates from potato starch wastewater treatment [82], and nitrifying aggregates grown in a fluidized bed reactor [34]. The detailed concentration gradients obtained under different regimes of nutrient supply allowed the localization of the various biotransformations involved. For example, microelectrodes for ammonium, oxygen, nitrate, and pH provided concentration profiles within the nitrifying aggregates, which showed that ammonium consumption and nitrate production were localized in the outer 100–120 μm of the 1 to 3-mm particles.

Scanning vibrating electrode technique

Recently, small platinum microelectrodes have been used to scan the surface of metal coupons with attached biofilm in order to map the potential field. The scanning vibrating electrode technique (SVET), vibrates a platinum electrode vertically 100 μm above the surface to create an alternating potential signal that can be filtered to increase the signal to noise. The microelectrode is scanned at intervals 200 μm in the x–y plane to map the potential field. The map shows anodic and cathodic sites on the surface, and the anodic sites have been shown to correspond to the location of pitting corrosion. The technique was used to show that biofilms facilitated pit propagation on carbon steel electrodes [48]. Expanding the technique, a SVET system has been coupled to a microscope to provide both a potential map and a visual image of the biofilm [4]. This application demonstrates that many of the techniques presented in this review are complementary in nature, and when coupled can enhance

our understanding of complex problems such as microbiologically influenced corrosion.

Piezoelectric techniques

Application of electric fields to some materials (eg some crystals, ceramics, polymers, and plastics) causes realignment of the atoms resulting in macroscale deformation—twists, bends, contractions, and expansions. This phenomenon is referred to as the converse piezoelectric effect [18]. An alternating electric field causes a back-and-forth deformation or vibrational motion in the bulk material. This vibrational motion is central to the function of all piezoelectric sensors.

A quartz crystal microbalance (QCM) is a piezoelectric sensor that measures frequency shifts due to changes in the mass of material on its surface. QCMs typically use a thin circular plate (~0.3 mm oscillates at 5 MHz) from a specially cut crystal [91]. An alternating electric field is applied to the metal electrodes to stimulate a mechanical oscillation. Mass deposited on the surface of the electrode will cause a decrease in the frequency of the vibration, monitored with a simple frequency counter. If the deposited materials have similar acoustic properties (similar rigidity) to those of quartz, the change in mass is directly proportional to the frequency shift [120]. The technique was originally employed in vacuum deposition systems to measure the thickness of deposited films [91]. In the 1980s, it was discovered that a QCM can function when one side of the crystal is exposed to liquid [68,105]. In aqueous environments, QCMs have been applied to investigate electrochemical processes [17,37] and have been used as transducers for biosensors [43,44,99] such as for the attachment of African green monkey kidney cells to a metal surface [57].

QCMs have been used to monitor biofilm formation [104], and the study revealed a number of experimental complications. Changes in either hydraulic pressure or temperature resulted in signal drift that could be misinterpreted as mass changes. Using a flow-through system designed to maintain constant temperature and pressure, the frequency of the oscillations was monitored both for sterile oligotrophic medium and for *P. cepacia* biofilms over 48 h of development. The frequency decreased as the biomass increased on the surface of the QCM. Formation of the biofilm was terminated at different times, and the number of attached cells per unit area was determined by light microscopy with acridine orange staining. A plot of the frequency shift versus the numbers of microbial cells per unit area was used to estimate the limits of detection (3×10^5 cells cm^{-2}). Using data near the origin of the resultant curve and a frequency-to-mass conversion equation developed for rigid films, the average wet mass of a single attached *P. cepacia* cell was calculated to be 0.8 pg, which is similar to an estimate of 1 pg for *E. coli* [63]. Nonlinearity in the curve was related to the viscoelastic (non-rigid) behavior of the biofilm during maturation. Surface roughness, chemical adsorption, non-uniform mass sensitivity, and longitudinal standing waves affect the frequency response of the QCM [17,86,91]. Biofilms are non-rigid; and the viscoelastic properties of biofilms (due to production of extracellular polymers, pili, fimbria) are probably time-dependent. Furthermore, the formation of biofilms on substrata involves attachment of microoganisms and development of the attached microorganisms into three-dimensional microcolonies. Mature biofilms contain cells within the microcolonies that do not contact the surface, and the percentage of cells that contact the substratum probably decreases during biofilm formation. In liquid and viscoelastic films, molecules at the surface of the substratum move with the surface, but the shear wave produced by the vibrational motion is dampened with distance from the surface. For example, the shear wave has been calculated to penetrate only 250 nm into pure water [53]. Thus, cells not directly attached to the substratum will be less affected by the shear wave and might not be detected.

Piezoelectric devices actually belong to a broader class of devices termed acoustic wave microsensors [54]. To our knowledge, only quartz crystal microbalances have been applied to microbial biofilm research. However, acoustic wave microsensors are presently being used in liquids [55] and may have similar potential for on-line sensors in biofilm research. Problems associated with long-term industrial or environmental monitoring of biofilms with acoustic wave microsensors are not insurmountable. If methods of pressure and temperature compensation are devised, the QCM technology could be employed as a low cost detector to indicate the formation of biofilms. Since the frequency change is not linear with the mass of the attached biofilm, a QCM could be used to study the viscoelastic properties of biofilms. As with the ATR/FT-IR technique, the active surface of the QCM can be coated with industrially relevant surfaces and formation of biofilms could be compared. As advancements are made in the theory of nonrigid films and in transducer technology, piezoelectric sensors should find more application in biofilm research.

Conclusion

The techniques reviewed here can be used to measure the biomass, metabolic activities, or the chemical composition of biofilms—continuously and nondestructively. The availability of such techniques will allow engineers and microbial ecologists to: 1) make on-line measurements of other important biofilm systems; 2) couple complementary techniques to obtain more detailed understanding of biofilm processes; and 3) modify or develop devices for routine industrial and ecological on-line or in-line monitoring.

The vast majority of biofilms are unexplored, offering many research opportunities. Selection of techniques that answer relevant questions and that function within the constraints of the environment are critical. The interpretation of the resulting data requires consideration of both the biofilm and the instrumental system. However, a single analytical technique may not provide sufficient information to understand the system.

Coupling of an on-line technology with another method may synergistically increase explanatory power. For example, in microbially influenced corrosion of steel, it is known

that bacterial biomass correlates with pit formation. However, it has not been proven whether bacteria induce pit formation or incipient pits accumulate bacteria. Coupling microscopy with the scanning vibrating electrode would provide a means of simultaneously monitoring bacterial accumulation and pit formation, thus observing the process.

The evolution of these techniques into new devices with improved ruggedness, sensitivity, and stability will require more interdisciplinary communication. Analytical chemists and engineers need to understand the complexity of biofilm systems; microbial ecologists need to appreciate the power and limitations of the techniques. We hope that this review has helped to extend this dialog.

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