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15 Monitoring Biofilms by Fourier Transform Infrared Spectroscopy

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The use of infrared spectroscopy (IR) for chemical characterization of microbial biofilms developed from the success of this spectroscopic technique in detection and identification of the chemical constituents of organic conditioning films that formed upon submersion of a clean solid surface in aqueous media. Infrared spectra, collected in the attenuated total reflectance (ATR) mode using a Fourier transform infrared spectrometer (FT-IR), contain absorption bands that are contributed by the chemical constituents of surface-associated microorganisms. The unique chemistry of different bacterial species has been resolved by FT-IR, to the extent that the approach is used to validate other methods of bacterial identification. Both diffuse reflectance and ATR modes have been used to follow biofilm development under a variety of aqueous conditions. ATR/FT-IR offers the opportunity to follow bacterial attachment to and biofilm development on a variety of solid surface materials non-destructively in real-time. This approach has been particularly fruitful in assessing the contribution and identification of mechanisms of biologically influenced corrosion of metal surfaces. By combining ATR/FT-IR with microscopic techniques, it is now possible to relate biofilm structure to chemical reactions in developing biofilms.

KEY WORDS: biofouling, corrosion, attenuated total reflectance, surface chemistry

INTRODUCTION

Microbial biofilms have become an important biological component, intentionally or unintentionally, in many industrial water systems. The accumulation of microbial biomass on equipment surfaces in contact with aqueous media poses a variety of problems for water system operators ranging from under-deposit corrosion, to loss in heat transfer efficiency or hydraulic valve malfunction. Historically, industries have had to take a biofouled system off-line for periods of time either to mechanically remove the fouling layer or to replace the equipment when damaged beyond repair. Frequently, chemical biocides are used to "control" biofilm accumulation on industrial surfaces, but more often than not, a biofouling layer eventually appears, and if the layer cannot be subsequently removed by application of alternative biocides, the system must be taken off-line and mechanically cleaned (Strauss, 1985).

Monitoring techniques for microbiological growth have been developed primarily for measurements in the bulk aqueous phase. Since biofouling is a surface-localized

phenomenon, conventional monitoring techniques are typically insensitive to most biofilm processes. A few on-line monitoring devices have been developed to measure surface biofouling which are based on fluid flow resistance or heat flux (Johnson and Howells 1981). Bryers and Characklis (1981) developed mathematical models based on system engineering parameters to predict biofouling and biofilm accumulation. Considerable biofilm development is required, however, before these devices detect surface fouling. By the time these devices sense that biofouling has occurred, the system may have become sufficiently fouled that subsequent remedial treatments are ineffective (Atkinson, 1979). Implementation of a sensitive and accurate biofilm monitoring system, on the other hand, permits operators to adjust control parameters to achieve long-term surface protection against biofilm accumulation. Accurate and sensitive monitoring facilitates prediction of when the system will deteriorate to an unacceptable level, so that corrective procedures or a scheduled maintenance activity can be effectively implemented.

Many industries faced with biofouling of equipment surfaces have monitored biofilm accumulation using surrogate coupons that are exposed to the same conditions as the equipment surfaces. In practice, these are sacrificed at intervals and analyzed for biofilm biomass by a variety of destructive or non-destructive methods. Coupons have been used in conjunction with side-stream loops to monitor surface fouling in parts of a system that are inaccessible (Poje *et al.*, 1982; McCoy *et al.*, 1981). While an improvement over gross system operational parameters, periodic sampling of coupons to assess biofouling is often inadequate and loss of system control can occur between periods of scheduled coupon sampling and analysis.

Some water systems, such as those used in the microelectronics and pharmaceutical industries, have such low tolerance for biofouling that coupons offer little benefit to an effective monitoring program (Patterson *et al.*, 1991). In other industries coupon retrieval poses problems. Coupons deployed in normally-accessible, spent nuclear fuel wet storage facilities have become so heavily contaminated with radionuclides through scavenging reactions of associated biofilms that they can no longer be recovered and analyzed due to safety regulations (Roberto, INEEL, personal communication). Thus, there is a real need for biofilm monitoring equipment that permits remote, sensitive, non-destructive, real-time analysis of surface biofouling. This will enable system operators to have a good understanding of the condition of the system surfaces at all times, as well as sufficient warning of a deteriorating condition in the system to implement effective remedial procedures that minimize costly, unscheduled shut-downs.

The same features of a biofilm monitor described above for practical surveillance in industry are also useful for more fundamental studies of microbial surface colonization. Most investigations of microbial surface colonization in the laboratory have involved the exclusive use of some form of visual observation or culture technique. The earliest biofilms studies utilized direct light microscopic techniques to qualitatively examine stained cells attached to transparent surfaces (Henrici, 1933). Later, phase contrast and transmitted differential interference contrast (DIC) optics were employed to examine microscopically unstained preparations in a non-destructive manner (Surman *et al.*, 1996). High resolution scanning electron microscopy (SEM) and transmission electron microscopy (TEM) offer additional information on biofilm structure and adhesive structures and matrix materials produced by microorganisms

associated with biofilms (Mack *et al.*, 1975; Beech *et al.*, 2000). These methods are destructive in nature, however, and introduce distortions in spatial relationships between structures during sample dehydration. They are typically not amenable to routine biofilm monitoring due to the labor-intensive sample preparation and the non-quantitative nature of the information provided.

Commercialization of confocal scanning laser microscopy (CSLM) in recent years has made it possible to qualitatively describe biofilm structure, and quantitatively describe local spatial biofilm phenomena such as thickness, cell density and distribution non-destructively, in real time under fully hydrated conditions (Lawrence *et al.*, 1991). Environmental scanning electron microscopy (ESEM) and tapping mode atomic force microscopy (AFM) offer high resolution information on the outermost features of biofilms maintained under hydrated conditions (Bremer *et al.*, 1992; Collins *et al.*, 1993; Beech *et al.*, 2000). Spatially-resolved elemental information can also be from hydrated biofilms using ESEM.

Spectroscopic techniques offer unique opportunities to monitor biofouling at the molecular level. In contrast to most of the cellular and structural information provided by microscopic techniques, spectroscopy provides quantitative chemical information that offers new insight into the physiological state of biofilm populations, as well as the molecular composition and molecular interactions between their metabolic products and other constituents of the system. Reference databases containing infrared (ir) spectra of many molecules of biological importance are available to facilitate band assignment and identification of molecular structure.

A technique such as ir spectroscopy, when used in the attenuated total reflectance (ATR) or multiple internal reflectance (MIR) mode, offers the opportunity to monitor real-time chemical changes during various stages of biofilm development on a variety of different substrata. Recent developments in sample cell design permit concatenation of chemical spectroscopy, light microscopy and computer-assisted spectral and image analysis to establish structure-function relationships in biofilms.

In this chapter, the use of ir spectroscopy in the field of biofilm microbiology is described. While a brief review of the application of ir spectroscopy to the study of biofilms has recently been published (Naumann *et al.*, 1996), the present chapter emphasizes the novel, on-line, ir spectroscopic methods that have been applied to biofouling assessment and biofilm characterization.

EVALUATION OF SURFACE CONDITIONING FILMS BY ATR IR SPECTROSCOPY

ATR or MIR ir spectrometry, using a high refractive index, infrared-transparent internal reflection element (IRE) such as germanium (Ge), provides a thin sampling region at the surface of the IRE where it contacts the surrounding medium. Ir spectra of material deposited on the IRE surface can be obtained by this sampling technique. In one of the earliest applications of ir spectroscopy to biofilm monitoring, Baier (1973) employed a grating spectrometer and a rectangular, Ge IRE to characterize the chemical nature and adsorption kinetics of material that deposited on a clean surface upon immersion in aqueous environments. After only 10 min exposure to the water of Biscayne Bay, FL, protein-like molecules had adsorbed to the surface, based

on the appearance of amide bands in the mid-ir (Goupil *et al.*, 1980). In other applications, the rate of adsorption of blood proteins to surfaces bathed in serum was even faster, with a uniform coating of protein of average thickness 50 nm forming within 5 s and a coating of average thickness of 100–200 nm forming after 60 s (Baier, 1973). These macromolecules appeared to be irreversibly adsorbed, as vigorous rinsing with distilled water failed to eliminate the respective ir peaks.

Using MIR ir spectroscopy in combination with ellipsometry and critical surface tension determinations Baier (1973) showed that surfaces containing conditioning films formed after only short periods of immersion in aqueous medium exhibit critical surface tensions similar to surfaces with conditioning films formed over longer periods of time. The similarities of the ir spectra, suggested that substratum surface properties did not influence the nature of the conditioning film.

Immersion of IREs in natural water for longer periods of time allowed Baier (1973) to follow colonization of the IRE surface by bacteria and other microorganisms. Band broadening and the appearance of peaks centered at 1050 cm^{-1} were common spectral features observed during bacterial surface colonization. The appearance of absorption bands around 1050 cm^{-1} suggested the presence of carbohydrate material. As a result of this information, Baier (1973) suggested that a glycoprotein layer is the first acquired modifying film obtained by a clean surface after immersion in aqueous media. In spite of the appearance of whole cells on the surface at later time periods, the spectra remained similar to that obtained initially in the presence of organic molecules adsorbed from the bulk aqueous phase (Baier, 1980). This early application of ATR ir spectroscopy established the now widely accepted paradigm that microbial attachment and biofilm formation on an inert substratum, regardless of composition, is preceded by the deposition of a spontaneously adsorbed, glycoprotein-dominated conditioning film.

FOURIER TRANSFORM IR SPECTROSCOPY

Ir spectroscopy became a more useful analytical method for biologists when Fourier transform techniques were developed to collect and process spectra. The same high quality spectra obtained with grating spectrometers could be collected and processed in about one-thousandth of the time using a Fourier transform infrared (FT-IR) spectrometer. The increased number of replicate spectra collected by FT-IR over that collected with a grating instrument leads to an increase in signal-to-noise ratio, which in turn increases sensitivity by a factor of 10–100. A key component of a FT-IR spectrometer is a Michelson interferometer. The interferometer consists of a fixed and moving mirror and a beamsplitter that are used to produce a path difference or a phase shift between all wavelengths of energy scanned, giving an interferogram which is related to the spectrum through its Fourier transform. In practice, a single-beam background transmittance spectrum is first measured and then a sample is placed in the beam path and another single-beam transmittance spectrum is measured. The ratio of these 2 spectra yields the sample transmittance spectrum, and the negative logarithm of this is usually computed to produce the absorbance spectrum. Details of how a FT-IR spectrum is obtained from an interferogram are provided by Griffiths (1983) and Griffiths and de Haseth (1986).

Additional advances in technology that contributed significantly to the application of FT-IR to the study of biological phenomena included 1) the development of the fast Fourier transform algorithm, 2) the laboratory minicomputer for rapid scanning, 3) incorporation of small reliable He-Ne lasers for high spectral resolution, 4) new detectors that allowed faster scan speeds and increased sensitivity, and 5) and mini- and micro-computer-based data systems that rapidly processed the spectral information (Griffiths, 1983). When affordable, FT-IR spectrometers with these features became commercially available in the mid-1970s, they quickly established their niche in microbiology and biofilm research.

Taxonomy and Identification of Microorganisms by FT-IR

FT-IR has been used to characterize microorganisms (Naumann *et al.*, 1991b; 1994). The sum or combinations of 50–60 spectral bands, resolved through enhancement techniques, has been used to establish strain-specific fingerprints to differentiate and identify unknown microorganisms. Among the bands that have been proposed to be of value in this regard is one centered at 1088 cm^{-1}, which is often assigned the symmetric vibration of the phosphodiester bond. That this particular molecular vibration can be used to distinguish microorganisms is unexpected since DNA, RNA and phopholipids all absorb in this region. In addition, the region between 900 and 600 cm^{-1} exhibits extremely rich information that has led to its designation as the "bacterial fingerprint region". Rarely are valid vibrational assignments made in this region, however.

The success of FT-IR in differentiating bacteria resides in the treatment of spectral fingerprints by such methods as principle-component analysis and artificial neural networks for data reduction and objective assessment of complex and composite data structures (Helm *et al.*, 1991). Grouping of bacteria on the basis of spectral similarity involves cluster analysis and other multivariate techniques using spectral reference libraries. These techniques all depend on the ability to establish pure cultures of the unknown microorganism.

In one of the early applications of combined FT-IR spectroscopy and microscopy, Naumann *et al.* (1991a; 1991b) identified microcolonies of bacteria containing less than 10^4 bacteria, using an ir-transparent plate as a stamp to transfer locally-separated microcolonies from an agar culture plate to an ir-sample holder. This replica technique permitted detection, enumeration and differentiation of populations from mixed cultures. Unlike some of the newer molecular techniques used to distinguish different microorganisms, ir microscopic differentiation necessitates the culture and isolation of populations before analysis. This bacterial fingerprinting technique is most useful as a complement to other approaches for bacterial differentiation and classification.

FT-IR Band Assignments from Intact Bacterial Cells

Naumann *et al.* (1996) point out that, with few exceptions, ir spectra of intact bacteria do not provide information on a single or a few specific cellular compounds because of the difficulty in making specific band assignments to specific structures from a complex assemblage of molecules. Nevertheless, the exceptions are worth noting

inasmuch as they offer physiological information difficult to obtain by other techniques.

Bacteria produce a number of compounds not found in other biological systems. These compounds have become "signatures" of the microbial world. Naumann (1984) recognized fingerprint-like ir spectral features of the peptidoglycan from cell wall preparations from different bacteria. In Gram-positive bacteria, 90% of the cell wall consists of peptidoglycan (Brock and Madigan, 1991). In Gram-negative bacteria, only 5–20% of the cell wall is peptidoglycan, with LPS, phospholipid and protein contributing 30%, 20–25% and 45–50%, respectively to an outer membrane wall component (Boyd, 1988). Peptidoglycan contributes only 2% of the total cell mass of the Gram-negative bacterium *Escherichia coli* in balanced growth with a mass doubling time of 40 min (Niedhardt *et al.*, 1990). The contribution of cell wall components to total cell mass varies considerably, however, depending on the physiological status of the cell.

Using specular reflectance or internal reflectance sampling mode, dehydrated preparations of peptidoglycan from a variety of bacteria yielded common absorption bands (Naumann *et al.*, 1982). Furthermore, bands near 1730 cm^{-1}, 1600 cm^{-1}, and 1400 cm^{-1} afforded the opportunity to differentiate peptidoglycan among different species. Band intensities also offered information on degree of cross-linking and other structural features. By this approach, changes in peptidoglycan induced by the culture medium and chemical treatment to intact cells, or changes associated with stages of cell growth, can be detected by FT-IR (Naumann, 1984).

In other studies, freeze-dried preparations of different strains of oral streptococci, pressed into KBr pellets, when analyzed by transmission ir absorption spectroscopy, all exhibited similar absorption bands, but each could be distinguished on the basis of differences in the relative intensities of the bands (van der Mei *et al.*, 1989). Among the absorption bands detected, those at 1236 and 1082 cm^{-1}, arising from the asymmetric stretching mode of phosphates and carbonyl stretch of carbohydrate, respectively, were assigned to teichoic acids in the cell wall. Such assignments should be viewed with caution since phosphate groups in nucleic acids and carbohydrates in other cell structures also absorb in these regions. In *Bacillus subtilis*, teichoic acids represent 50% of the cell wall dry weight (Boyd, 1988). Teichoic acids are not found in walls of Gram-negative bacteria.

By normalizing the absorbance of bands contributed by teichoic acids as well as the amide I and amide II bands contributed by protein against the CH stretching region centered at 2930 cm^{-1}, van der Mei *et al.* (1989) were able to resolve the different strains of oral streptococci tested. An important conclusion drawn from the investigation was that transmission ir spectroscopy of freeze-dried bacterial cells yields surface-sensitive information comparable to x-ray photoelectron spectroscopy. However, these conclusions can only be justified if the contribution of intracellular and cytoplasmic membrane material is negligible in comparison to that of cell wall components. The insignificance of intracellular and membrane components to the ir spectrum of whole cells of Gram-positive bacteria remains to be confirmed.

FT-IR spectroscopy has been used to monitor metabolic events in bacterial cells relevant to agricultural, environmental and health sciences. For example, reversible spectral changes attributed to quantitative and structural changes in cell wall peptidoglycan were observed as a result of transfer of *Bradyrhizobium japonicum* from liquid

to solid medium (Zeroual *et al.*, 1994). Spectra were obtained from samples dried onto zinc selenide (ZnSe) IREs to avoid water interference. Fourier transform self-deconvolution was used to resolve peptidoglycan bands in the presence of other biomolecules, which contribute a large numbers of vibrations in samples of whole cells. Absorption bands centered at 1154 cm^{-1} and 1027 cm^{-1}, assigned to the C-O stretching vibrations of carbohydrates, were shown to increase on the solid medium. These spectral changes were suggested to represent changes in the peptidoglycan structure based on electron microscopy of thin section preparations. Capsular polysaccharides, synthesized during growth on solid medium, may also have contributed to the changes in band intensities observed in this region of the spectrum. The difficulty in eliminating contributions from other cellular molecules present in whole cell preparations from a particular absorption band, even after application of spectra processing software, has complicated spectral interpretation and limited the use of FT-IR as a means of following the fate of specific microbial metabolites. Furthermore, since samples were manually deposited on the IRE and dehydrated before acquisition of spectra, it was not possible to observe the molecular changes in living cells in real time.

In addition to cell wall components, FT-IR spectral assignments have been made from whole cell preparations for dipicolinic acid during endospore formation, poly-β-hydroxybutyrate and glycogen-like storage material, and capsular polysaccharides (Naumann *et al.*, 1996). As spectrum deconvolution algorithms become more sophisticated and spectral data are matched to chemical data obtained by other independent analytical approaches, resolution of other metabolic products in whole cell preparations may be possible in the future.

FT-IR Analysis of Protein Conditioning Films and Microbial Biofilms Deposited *in situ*

Early studies with grating spectrometers suggested that irreversible adsorption of proteins from aqueous solutions to submerged surfaces is a key process in the formation of the conditioning film with which early surface-colonizing microbes interact. Protein interactions with the substratum may influence subsequent adhesion of surface-colonizing microbes. Conditioning film proteins may also control biofilm processes established at much later stages of surface biofouling. FT-IR offers the opportunity to resolve protein-surface interactions, not previously achievable with dispersive instruments, that may be important to biofouling.

Using near grazing incidence reflection-absorption FT-IR, where a dehydrated surface is sampled by a single external reflection of the incident beam, Taylor *et al.* (1996) observed little effect of substratum wettability (critical surface tension) on the quantity of a model protein, ribulose-1-5,-bisphosphate carboxylase-oxygenase (Rubisco) that adsorbed from solution. These results supported earlier work using a grating spectrometer and ATR sampling geometry (Baier, 1973). However, natural conditioning films deposited on surfaces of Fe, Al and Ti in nearshore tropical seawater yielded distinctly different spectra, although a protein signature was common to the conditioning films formed on all three substrata (Taylor *et al.*, 1996).

Using the reflectance-absorbance FT-IR spectroscopic technique above in combination with ellipsometry, Taylor *et al.* (1993, 1994), showed that the secondary

structure of Rubisco was dependent on the extent of protein surface coverage, protein film thickness and the properties of the substratum to which the protein had adsorbed. The coverage and thickness of the adsorbed Rubisco was shown to have a significant influence on the surface properties of the substratum (Taylor *et al.*, 1994). The critical surface tension and surface free energy of Ti oxide and copper surfaces were significantly influenced by the amount of protein adsorbed.

Diffuse reflectance infrared Fourier transform (DRIFT) spectroscopic sampling of freeze-dried biofilms was used to identify molecular vibrations contributed by biomolecules that served as tracers of microbial biofilm development on surfaces (White *et al.*, 1988). Although the ability to monitor living biofilms is sacrificed, resolution is enhanced by this sampling mode. DRIFT was able to detect two physiological changes associated with the nutritional state of a biofilm population on a Ge IRE. The appearance and increase in intensity of bands assigned to vibrational modes of poly-hydroxyalkanoic acid (PHA) and acidic exopolysaccharides, two polymers associated with nutritional imbalance in bacterial cells, exemplify the utility of FT-IR to monitor the physiological state of a bacterial population. DRIFT has also been used to demonstrate an increase in biofilm protein and carbohydrate concentrations with increased applied shear on 316 stainless steel coupon mounted in a cell adhesion measurement module (Mittelman *et al.*, 1990).

Although these applications of FT-IR yielded important information on the nature and formation rates of organic conditioning films and the physiological response of surface-associated bacteria to environmental conditions, the samples required dehydration before acquisition of an ir spectrum. Dehydration has recently been shown to have a profound effect on the structure and topography of protein conditioning films (Baty *et al.*, 1997). Dehydration has long been known to disrupt the native structure of biofilms (Mack *et al.*, 1975). Utilization of a sampling technique that permits acquisition of high quality ir spectra of fully hydrated biofilms should provide the possibility for on-line monitoring of surface biofouling.

Integrating Attenuated Total Reflectance Sampling Geometry with FT-IR Spectroscopy

Because water absorbs strongly in the region of the ir spectrum where useful information on biologically-important molecular vibrations reside, the water bands typically mask the more subtle absorbance bands of proteins and other biological molecules. In the past, this problem was solved by several different approaches. Mattson *et al.* (1975) were successful in characterizing proteins adsorbed to multiple internal reflection elements under fully hydrating conditions by processing spectra collected with a grating spectrometer using a computer data processing system interfaced to the spectrometer. Water interference has also been overcome by substitution of D_2O for H_2O in the aqueous phase when grating spectrometers were used to collect spectra (MacCarthy *et al.*, 1975).

It is easier to subtract water absorbance from spectra collected with an FT-IR spectrometer than from those collected with grating spectrometers. Even with their superior scan speed, signal-to noise ratio and energy throughput, modern FT-IR spectrometers still required short path lengths to successfully minimize water inter-

ference. Short path lengths can be achieved with a conventional transmission liquid cell. Although transmission cells create path lengths of < 15 μm, it is difficult to reproduce this when reassembling the cell for replicate or comparative experiments. Alternatively, ATR sampling geometry utilize IREs that have a well defined path for a given angle of incidence and refractive indices of the IRE and surrounding medium (see Eqn 1, p. 264). The ATR sampling mode does not give rise to interference fringes common to transmission liquid cells, avoiding the masking of spectral features.

By limiting ir sampling to a thin region immediately adjacent to an IRE surface immersed in an aqueous medium, as is the case for the ATR beam path, it is possible to avoid the total loss of energy in the spectral region where maximum water absorption occurs. The depth of penetration, d_p, is defined as the distance into the solution where the evanescent field amplitude decays to e^{-1} of its magnitude at the IRE-solution interface. For Ge-water at a 45° angle of incidence, this distance is 0.064 λ_o where λ_o is the *in vacuo* wavelength (Mattson *et al.*, 1975). The d_p is approximately 0.4 μm for the water band at 1640 cm^{-1}, providing an effective path length of 4 μm, assuming the number of reflections is 10 (Iwaoka *et al.*, 1986). This sampling geometry, thus, provides the opportunity to isolate surface chemical information from bulk aqueous phase chemistry without surface dehydration. Further information on the ATR sampling mode and its applications to surface chemical characterization is provided by Wragg and White (1991).

ATR is a particularly useful sampling technique for non-destructive chemical characterization of protein behavior at a hydrated surface and chemical characterization of biological processes localized to the bottom few cell layers of a fully-hydrated biofilm. By combining ATR with FT-IR, it is possible to collect useful spectra rapidly from surfaces that require no dehydration. Water can be efficiently subtracted from the spectrum of the thin aqueous layer adjacent to the substratum containing the surface-associated conditioning film and/or microbial biofilm. This offers new opportunities to chemically characterize biological systems in their native state, non-destructively, in real-time.

The early IREs developed for ATR sampling were optimized for the beam geometry of grating spectrometers. Consequently, the rectangular-shaped ir beam entered the IRE across a rectangular surface. In contrast, FT-IR instruments produce a circular beam, which is not efficiently captured by or transferred through IREs with a rectangular entrance geometry. In 1983, SpectraTech Incorporated (Stamford, CT) introduced the CIRCLE accessory which efficiently captured the circular beam of the FT-IR spectrometer and transmitted it to a circular target in the detector (Wilks, 1982). With proper water subtraction, this cylindrical geometry permitted collection of FT-IR spectra of an organic phosphonic acid at solution concentrations as low as 100 mg l^{-1}. By the mid-1980s, ATR/FT-IR had evolved such that it could also offer new insights to chemical phenomena at hydrated surfaces. The situation improved even more with the availability of computer software that simplified water subtraction from the spectrum. Powell *et al.* (1986) described an algorithm that could be used to subtract water from FT-IR spectra of proteins adsorbed to an ATR crystal. Dousseau *et al.* (1989) described an algorithm, which allowed quantitative subtraction of water from transmission FT-IR spectra of proteins in aqueous solutions. All the technology was in place at this time to exploit non-destructive ATR/FT-IR chemical spectroscopy to characterize conditioning films and biofilms in ways never previously possible.

ATR/FT-IR of Hydrated Surface Conditioning Films

Nichols *et al.* (1985) were among the first to demonstrate the use of ATR/FT-IR to follow, in real time, the deposition of organic material from filtered seawater on a rectangular IRE under flow conditions. Their results showed that carbohydrate rather than protein was the predominant material adsorbed to the surface during the first 5 h of seawater immersion. These results contrast with those reported previously in which protein was the major component of the conditioning film on surfaces immersed in seawater (Baier, 1973). A possible explanation is that the carbohydrate fraction of adsorbed material, due to its lower affinity for the substratum, was lost during the dehydration procedure used in earlier ATR ir studies. Dehydration has also been shown to cause an increase in the ir absorbance of biomolecules adsorbed to surfaces over that obtained under fully hydrated conditions, as well as change the relative absorbance intensities of different chemical species associated with the surface (Geesey and Bremer 1990). Thus, the chemical nature of conditioning films needs to be reassessed using techniques that accommodate the presence of water.

Interactions between proteins and polysaccharides on surfaces in the presence of water have been monitored in real-time using ATR/FT-IR (Ishida and Griffiths, 1990). The acidic polysaccharide, alginic acid, adsorbs rapidly to a clean Ge surface from a flowing 1% aqueous solution at pH 7.4, based on changes in intensity of the 1034 cm^{-1} absorption band assigned to the C-O stretching of the sugar subunits. Desorption of the alginic acid from the Ge surface occurred slowly during surface rinsing over a 4-h period. Only 60% of that initially adsorbed desorbed over that period. Establishment of a protein film of β-lactoglobulin accelerated the rate of alginic acid adsorption to the Ge substratum compared to that observed in the absence of the protein film. More polysaccharide adsorbed to the "protein conditioned" surface than to the bare Ge surface. Protein was not displaced from the surface as a result of polysaccharide adsorption. The polysaccharide but not the protein desorbed from the surface during subsequent surface rinsing. A similar polysaccharide residual remained with the protein-conditioned surface as with the bare Ge surface. The neutral polysaccharride, dextran, unlike alginic acid, was excluded from the protein-conditioned Ge surface (Ishida and Griffiths, 1993a). Dextran exhibited a greater affinity for bare Ge than a protein-conditioned Ge surface. These studies suggest that neither acidic nor neutral polysaccharides, which resemble the matrix biopolymers of microbial biofilms, establish strong interactions with the protein conditioning film formed upon immersion of surfaces in aqueous media.

Proteins take on a different secondary and possibly tertiary structures when adsorbed to surfaces as compared to their structure in solution. Ishida and Griffiths (1993b) described how amide I/amide II ratios of proteins changed when spectra collected by a liquid transmission cell and a CIRCLE ATR cell were compared. They also described how to calculate protein film thickness and showed how solution phase pH influenced protein film thickness on a Ge IRE. Thicknesses of 5.6 nm, 7.2 nm and 6.7 nm were calculated for bovine serum albumin adsorbed at pH 7.0, 4.1 and 9.0, using spectra collected under fully hydrated conditions.

The interactions of a hydrated Ge surface and bulk aqueous phase detergents, used to control biofilm accumulation on membrane material, was investigated by ATR/FT-IR. The aliphatic tail of the surfactant was more firmly bound to the Ge surface

than the ethoxy substituents (Ishida *et al.*, 1998). Studies of interactions between surfaces and compounds in the bulk aqueous phase are now a routine application for ATR/FT-IR.

Characterization of Fully Hydrated, Intact Microorganisms by ATR/FT-IR and Assignment of Ir Absorption Bands to Specific Biomolecules

One of the earliest applications of ATR/FT-IR to evaluate whole cells of microorganisms in aqueous medium was performed by Hopkinson *et al.* (1987). Using a flat, six-reflection ZnSe IRE (Specac, Orpington, Kent, UK), they suggested that the spectrum of an aqueous suspension of the yeast *Candida pseudotropicalis* was dominated by cell wall components. The broad band centered at 1070 cm^{-1} was suggested to be contributed by the C-O stretching vibrations corresponding to a composite of chitin and the main cell wall polysaccharides mannan and glucan. Their conclusions were based on evidence that these bands were significantly reduced in protoplast preparations, which preserve membrane envelope components (*i.e.* protein and lipids) but not these polysaccharide cell wall components. While this off-line application of ATR/FT-IR permits chemical characterization of hydrated intact microorganisms and other living material, it does not demonstrate the true analytical potential of the spectroscopic method for on-line monitoring of surface-associated biological processes.

Characterization of Microbial Biofilm Development on Surfaces in Real Time by ATR/FT-IR

The use of ATR/FT-IR to follow microbial biofilm development nondestructively, in real-time under fully hydrated conditions was first demonstrated by Geesey and Bremer (1990). Unlike earlier applications of ATR/FT-IR to biological systems which are short-term in nature (Gendreau *et al.*, 1981; Winters *et al.*, 1982), biofilm processes often require days or weeks to develop. To overcome spectral artifacts contributed by long-term fluctuations in energy throughput and temperature fluctuations, a double beam instrument was employed which could ratio out these effects on bacterial biofilm spectra. Two CIRCLE cells containing similar IREs were placed parallel to each other in the optical bench of a Perkin Elmer Model 1800 FT-IR spectrometer containing a medium-band HgCdTe (MCT) detector (Bremer and Geesey, 1991a). Both flow cells were sterilized with ethylene oxide. One CIRCLE cell was inoculated with microorganisms while the other was maintained in a sterile but otherwise identical state as the inoculated cell. The spectrum of the inoculated cell was then ratioed against that of the sterile control to eliminate the spectral artifacts that appear over the long term. Using this approach, it was possible to detect and identify the major chemical components of a developing microbial biofilm, as well as monitor changes in chemical composition during biofilm maturation under static and continuous flow conditions over a 196 h time span (Geesey and Bremer, 1990; 1991).

Nivens *et al.* (1993a) described a multi-channel ATR/FT-IR spectrometer that utilized a mid-ir liquid-cooled source, a Transept III interferometer, an optical system containing collection and focusing mirrors for three channels, three Harrick rectangular flow cells (Harrick Scientific, Ossining, NY), and a narrow-band MCT

detector. They showed that reproducible ir spectra could be obtained from the three channels.

Nivens *et al.* (1993a) described two methodologies using the multi-channel spectrometer to obtain ATR/FT-IR spectra of living *Caulobacter crescentus* cells attached to flat trapezoidal Ge IREs. Spectra were obtained for attached bacteria in high purity water, which provided details of the attachment process without spectral interference from components of the bulk aqueous medium. They also followed growth of attached bacteria by using a culture medium that did not contribute ir absorption in the region 2000–1200 cm^{-1}. Using the amide II band as a marker for biofilm biomass, they determined the detection limit to be approximately 5×10^5 cells cm^{-2}. Sterile controls gave rise to an ir absorption band at 1080 cm^{-1}, which was attributed to precipitation of inorganic salts onto the IRE. Adhesion of *C. crescentus* to the IRE produced ir absorption bands at 1648, 1550, 1306 cm^{-1} (amide I, amide II and amide III, respectively), and at 1454, 1397, 1246, 1080 cm^{-1} (C-H bend, C-O stretch, P = O stretch or amide III, C-O stretch of alcohols and carbohydrates, respectively). It should be noted, however, that there is a strong contribution from the phophodiester linkage in DNA and RNA cellular constituents in the region 1080–1088 cm^{-1}, and this is likely to dominate vibrations contributed by the C-O stretch. The intensity of all bands increased during the first 3 h, suggesting that bacterial cells had accumulated on the surface over that period of time. In other studies, White *et al.* (1991) used ATR/FT-IR to monitor colonization of a Ge IRE surface by *C. crescentus*. They related the intensities of ir bands assigned to amide I, amide II and the C-O stretching region of carbohydrates to bacterial cell densities on the surface determined by acridine orange direct count microscoppy over an 80 h period.

The sampling depth of the evanescent wave of radiation D_p can be calculated using the equation,

$$D_p = \lambda/2\pi n_1[\sin^2\theta - (n_2/n_1)]^{1/2} \quad (1)$$

where λ is the wavelength of the radiation, n_1 is the refractive index of the IRE, n_2 is the refractive index of the medium in contact with the IRE, and θ is the angle of incidence. For water in contact with a Ge IRE cut at a 45° angle, D_p is calculated to be 0.5 μm at the wavelength where amide II absorbs. Thus, by using a Ge IRE as a substratum, the sampling depth is optimized to detect only the layer of bacterial cells in a biofilm that is in contact with the substratum.

By comparing intact biofilm spectra with those of isolated microbial products, it was possible to time-resolve the biosynthesis and deposition of the carbohydrate matrix polymer glue that anchored the bacterial cells to the substratum (Geesey and Bremer, 1990). According to the Beer-Lambert Law, the absorbance of a component in the spectrum is proportional to the concentration of the component at a given path length and an extinction coefficient. Quantitative as well as qualitative information can therefore be extracted from the spectra. Fink *et al.* (1987) demonstrated a good correlation between amide I or amide II band intensities and amount of adsorbed protein. Correspondingly, the amide II peak area from the ATR/FT-IR spectra provided a good measure of bacterial cell density on the surface of an IRE (Suci *et al.*, 1997).

Use of ATR/FT-IR to Characterize Interactions between Microbial Adhesins and Surfaces

Few studies have established a clear role for conditioning films in microbial adhesion to and biofilm formation on substrata immersed in aqueous environments. Using [^{3}H]-leucine labeled cells of *Pseudomonas fluorescens* and an *Acinetobacter sp.*, Pringle and Fletcher (1986) determined that conditioning films comprised of the protein bovine serum albumin or bovine glycoprotein or lipopolysaccharide from *Escherichia coli* inhibited attachment to polystyrene. Similarly, Frolund *et al.* (1996) found that a conditioning film comprised of a mussel adhesive protein (MAP) inhibited attachment of cells of *Hyphomonas* MHS-3 to a Ge substratum. These results were consistent with the affinity of purified polysaccharide adhesin from this bacterium for the Ge surface in the presence and absence of MAP conditioning films. The binding data were obtained by ATR/FT-IR. While these results suggest that some model monomolecular conditoning films interfere with the adhesion process in some bacteria, extrapolation to natural conditions is premature.

The differences in the ATR/FT-IR spectrum of *C. crescentus* cells attached in high purity water and growth medium enabled Nivens *et al.* (1993b) to detect different holdfast organelles under different adhesion conditions. Such information would be difficult to obtain using other analytical techniques. While these investigators did not pursue the identity of the holdfast molecules responsible for the spectrum differences, these and the studies described above demonstrate that ATR/FT-IR offers many new opportunities to better understand the molecular basis of microbial adhesion and biofilm development (Suci and Geesey, 1998).

Use of ATR/FT-IR to Characterize Interactions between Antimicrobial Agents and Biofilm Populations

Microbial cells within biofilms are more resistant or recalcitrant to antimicrobial agents (AA) than when suspended as individual cells in aqueous media (Nickel *et al.*, 1985; Evans and Holmes, 1987; Gilbert *et al.*, 1990). The word recalcitrant is preferred since resistance implies that gene mutations have been identified which cause an alteration in the AA target, or genes have been acquired that produce products which inactivate the AA. Biofilm recalcitrance to AA is not as well understood as classical resistance mechanisms utilized by suspended cell populations. Theories of biofilm recalcitrance fall into two categories that are non-exclusive. The first is based on the hypothesis that microorganisms in biofilms have physiological characteristics that make them less susceptible to the lethal dose. For example, biofilm microorganisms replicate and/or metabolize more slowly than cultures of individual cells in aqueous suspension (Gilbert *et al.*, 1990). Alternatively, transport of the AA may be hindered in biofilms such that the lethal dose does not reach certain portions of the biofilm (Nichols *et al.*, 1988; Hodges and Gordon, 1991; Hoyle *et al.*, 1992).

Different mechanisms of AA resistance by bacterial cells in biofilms have been evaluated using ATR/FT-IR. Jass (1990) was the first to consider this approach to monitor β-lactam antibiotic penetration in biofilms of *Pseudomonas aeruginosa* and was able to isolate the ir absorbance of the antibiotics from that contributed by the

microbial cells. The results suggested that the biofilm matrix did not impede the transport of either pipericillin or ticarcillin. Suci *et al.* (1994) used ATR/FT-IR to follow the penetration of ciprofloxacin into *P. aeruginosa* biofilms. Vrany *et al.* (1997) used results obtained by ATR/FT-IR to mathematically model transport diffusion coefficients for the fluoroquinolone antibiotics levofloxacin and ciprofloxacin, binding site density, and adsorption and desorption rates for biofilms of *P. aeruginosa*. Like Jass (1990), these investigators concluded that transport of the antibiotics could not explain the recalcitrance of the biofilm population and suggested that physiological factors were responsible. In both studies, ATR/FT-IR was used to quantify non-destructively the accumulation of the antibiotics at the biofilm-substratum interface in the presence of a complex spectral contribution from living bacterial cells, in real time, under fully hydrated conditions.

IRE SURFACE MODIFICATION FOR ATR/FT-IR

Once ATR became the sampling mode of choice for real-time ir studies on surface-associated conditioning film and biofilm processes under hydrated conditions, a need for IREs with different substratum surface properties became apparent. ZnSe, Ge and KRS-5 (thallium bromoiodide) are the only materials used to fabricate commercially-available IREs that offer reasonable ir transparency in the biologically-relevant region of the ir spectrum. Silicon and sapphire IREs offer only limited opportunities in this regard.

The notion that an IRE surface could be modified by coating with an ultrathin film of a different material without compromising IRE performance or spectrum quality was evaluated by Baier and Loeb (1971). They silanized Ge IREs cleaned by radio frequency glow-discharge in air to produce different surface free energies. These surfaces were then exposed to natural and artificial freshwater and seawater for varying periods of time, dehydrated and analyzed both spectroscopically and microsopically to determine the influence of surface energy on conditioning film and biofilm accumulation. The results indicated that differences in substratum surface free energy exerted no detectable influence on the formation of the macromolecular organic conditioning film (Baier *et al.*, 1983). However, very tightly adsorbed and densely compressed interfacial films were preferentially attracted to Ge surfaces displaying an extremely high surface energy, whereas, more loosely attached and easily removed films were associated with highly-methylated, silanized surfaces (DePalma and Baier, 1978).

Thin films of hydroxyapatite (HA), a multicomponent compound which forms the major constituent of the outermost part of the tooth surface, were deposited on a Ge IRE by radiofrequency sputtering and used to characterize the adsorption of salivary components to the tooth surface (Ruckenstein and Gourisankar, 1983). The films were thin enough (< 20 nm) to detect ir absorption bands assigned to protein after dehydration and analysis by multiple internal reflection ir spectroscopy. No attempt was made to evaluate the stability of the HA film in aqueous environments, however.

IRE surfaces have also been modified to display the properties of membrane material. Ishida *et al.* (1998) dip-cast thin films of cellulose acetate (CA) onto Ge IREs in order to study membrane fouling by organic molecular films and microbial biofilms.

They found that the CA film became slowly hydrated when in contact with aqueous media. The unusually high degree of hydration as measured by the increase in intensity of the water absorption band at 1639 cm^{-1} was attributed to a factor other than normal polymer expansion, possibly separation of the film from the IRE. CA film hydration was accompanied by a shift to lower wavenumbers of the carbonyl band from 1749 to 1747 cm^{-1}, and a shift to higher wavenumbers for the C-O-C acetate ester band from 1230 to 1232 cm^{-1}. By exposing the CA film to solutions of alternating ionic strength, these investigators were able to demonstrate the passage of water across the film. The film appeared to act as an osmotic pressure cell. Thus, ATR/FT-IR offers a novel, sensitive way to evaluate membrane flux.

ATR/FT-IR Evaluation of Behavior of Molecules and Cells Adsorbed to Chemically-Modified IRE Surfaces

Silanized Ge IREs have been implanted in rabbits and the chemical nature of the tissue components that grew in contact with the implant surface subsequently evaluated by ATR ir spectroscopy and other surface analytical techniques (Baier *et al.*, 1984). These experiments indicated that protein-dominated films of greater thickness developed on implant surfaces with lower surface energy than the films that developed on intermediate and high surface energy surfaces. Infrared spectral analysis also revealed that material adsorbed to the lower energy surfaces was less substantially altered from its natural solution state configuration than that adsorbed to higher energy surfaces.

The behavior of proteins, polysaccharides and surfactants on IRE surfaces coated with thin films of CA was also evaluated by Ishida *et al.* (1998). Electrostatic interactions were suggested to dominate adsorption behavior of the various organic molecules tested. Feedwater to a reverse osmosis treatment plant consisting of secondary treated wastewater was also flowed across the CA thin film to evaluate the types of compounds that adsorb and contribute to membrane fouling. Adsorption bands contributed by proteins and carbohydrates dominated the ATR/FT-IR spectrum. During a rinse with deionized water, the carbohydrates desorbed rapidly whereas, proteins desorbed more slowly. These results corroborate earlier studies indicating the lower affinity for surfaces of carbohydrates than proteins.

The notion that ATR/FT-IR could be used to evaluate the stability of metal surfaces in the presence of adsorbed biomolecules was demonstrated by Iwaoka *et al.* (1986). Using chemical vapor deposition (PVD), they deposited ultrathin (10–20 nm), continuous films of metallic copper on Ge IREs that were sufficiently thin to permit penetration of the evanescent wave of ir radiation into the surrounding medium. Cu films (6.7 nm nominal thickness) deposited on Ge IREs by PVD were characterized by several surface analytical techniques (Bremer *et al.*, 1991). Films were shown to be continuous and to possess a Cu(I) oxide layer by x-ray photoelectron spectroscopic analysis. The surfaces were not significantly altered by ethylene oxide sterilization or exposure to water.

Ishida and Griffiths (1990) compared protein adsorption at different pHs on bare Ge IREs and Ge IREs coated with a thin film (3–4 nm) of either Cu or Ni deposited by PVD. They determined that the net charge on albumin appears to be more significant than the nature of the substratum in controlling how much protein

accumulates of the surface. Correspondingly, surface charge effects were suggested to control protein adsorption rates on these substrata.

Ishida and Griffiths (unpublished results) conducted a thorough investigation of the influence of different Cu film thicknesses on the optical properties of the stratified media and the resulting ir spectra of water at the Cu film-water interface. Films deposited by PVD < 8 μm thick were found to be discontinuous. Even films > 10 μm in thickness were found to erode in a non-uniform manner in the presence of some aqueous media such that metal islands were produced. Water band shifts were attributed to Cu island effects, whereas, band shape changes were attributed to surface enhanced infrared absorption. These phenomena complicate ir spectrum band assignment and interpretation.

Advances in thin film technology have made it possible to deposit well-defined, stable metal films on IREs for subsequent studies in aqueous environments. Pedraza *et al.* (1989) described the use of XeCl laser treatment to enhance adhesion of 80 nm thick copper films on sapphire substrates relative to as-sputtered films. Laser treatment also improved the smoothness of the films.

Advanced thin film technologies have also been used to deposit alloys of stainless steel (ss) on IREs for biofilm studies. The phase structure of the ss thin film was influenced by the nature of the underlying substratum (Godbole *et al.*, 1993). The phase structure of 316L ss sputtered on Ge substrates was controlled by careful selection of annealing temperature (Godbole *et al.*, 1992). Stability in water of 316L ss thin films deposited on Ge IREs required the deposition of a 2 nm-thick chromium oxide bonding layer on the Ge prior to sputtering the ss (Pedraza *et al.*, 1993). Films deposited in this manner were found to be stable under non-aggressive aqueous conditions for at least 1000 h (Suci *et al.*, 1993). The conditions included exposure to and colonization by a consortium of bacteria.

Comparing ir spectra and microscopic images of biofilms collected on IRE-modified high and low energy surfaces, Baier (1980) found similar densities of surface-associated bacteria. Bacteria associated with the low energy surface appeared to be positioned on top of the glycoprotein matrix rather than embedded within it as was the case on the high energy surface.

Ishida *et al.* (1998) studied the fouling of CA thin films deposited on Ge IREs. Bacterial cell attachment was monitored in real time by the intensity of the amide II band at 1547 cm^{-1} as a solution containing a bacterial inoculum flowed over the CA film surface. Within 20 min, bacterial adhesion could be detected. A cellular adhesion rate of 7.38×10^{-4} h^{-1} was obtained using this approach. The cell accumulation rate on the CA film decreased after the fluid flowing across the film surface was replaced with liquid containing no bacterial inoculum. At the end of the experiment, the bacterial density on the film was determined by direct epifluorescent microscopic enumeration after staining cells with a fluorogenic compound that binds cellular DNA.

Advances in surface chemistry have thus expanded the utility of ATR/FT-IR in assessment of the influence of substratum properties on molecular and cellular interactions. Such investigations can now be carried out in the presence of water in real time, yielding useful thermodynamic and kinetic information.

MONITORING CORROSION BY FT-IR SPECTROSCOPY

Use of Diffuse Reflectance FT-IR to Monitor Microbiologically Influenced Corrosion

White *et al.* (1986) used the off-line DRIFT sampling method to correlate increased electrochemically-derived corrosion rates with the appearance of surface-associated bacterial products on metal coupons exposed to aqueous bacterial suspensions. They related the increased corrosion rates to an increase in accumulation of surface-associated material displaying an ir absorbance centered at 1440 cm^{-1} when a 304 stainless steel surface was exposed to a marine bacterial culture in artificial seawater. The material was subsequently suggested to be extracellular calcium hydroxide, possibly associated with an organic matrix, based on ir spectral comparison with an authentic standard (Nivens *et al.*, 1986).

ATR/FT-IR Using IREs Coated with Thin Metal Films to Monitor Microbiologically Influenced Corrosion

Iwaoka *et al.* (1986) and Jolley *et al.* (1989) showed that the water absorbance intensity measured by ATR/FT-IR was a very sensitive way to monitor the integrity (average film thickness) of thin Cu films deposited on cylindrical IREs by various methods. This approach was able to detect dissolution or ionization of only a few atomic layers of metallic Cu in contact with the aqueous phase. After formation of a Cu oxide layer, the underlying metallic Cu film appeared to be stable for extended periods of time in the absence of aggressive conditions. Such sensitivity allowed these and other investigators to evaluate the aggressive nature of biomolecules towards the hydrated metallic Cu film (Iwaoka *et al.*, 1986; Jolley *et al.*, 1989). The analytical approach proved useful in demonstrating a novel mechanism for microbially influenced corrosion of copper involving different matrix polysaccharides excreted by the biofilm bacterial populations (Geesey *et al.*, 1986; 1987).

Using ATR/FT-IR, Ishida and Griffiths (1990) evaluated the corrosive nature of the protein bovine serum albumin (BSA) when adsorbed to Cu and Ni thin (3–4 nm) films deposited on IREs by PVD. BSA did not exert a corrosive effect on any of the metal substrata to which it adsorbed. The albumin promoted adsorption of acidic polysaccharides to the metal films but their effect on film stability (corrosion) was not assessed.

Cylindrical IREs containing copper thin films were used to demonstrate the aggressive action of specific strains of bacteria growing as biofilms on the copper surface in real time using ATR/FT-IR. Corrosion of the copper film was detected by an increase in water band intensity at 1640 cm^{-1}, following exposure to a aqueous suspension of bacteria which formed a biofilm on the copper film (Geesey and Bremer, 1991; Bremer and Geesey, 1991b). Analysis the spectra revealed that an increase in the C-O stretching bands of carbohydrates at 1062 and 1229 cm^{-1} associated with biofilm development coincided with the increase in water absorption band at 1640 cm^{-1}, suggesting that the polysaccharide matrix was linked to corrosion of the copper. That corrosion occurred *via* localized attack was indicated by the appearance

of discrete areas of discoloration on the copper film upon removal of the biofilm. It was also shown that not all microbial biofilms promote localized attack (Geesey and Bremer, 1991). Some strains of bacteria, if given the opportunity to form the initial biofilm, protect the copper surface from aggressive attack by other biofilm-forming bacterial strains that colonize the surface later (Bremer and Geesey 1991b). This on-line monitoring technique made it possible to characterize interfacial chemical reactions over the long-term without disturbing the living organisms growing at the interface.

Corrosion Product Identification by ATR/FT-IR

Inorganic corrosion products formed on metal coupons have been identified by FT-IR after pressing the dehydrated coupons against multiple internal reflection elements (Borgard *et al.*, 1988). Since this procedure does not protect against sample oxidation and dehydration, corrosion products may become chemically altered prior to analysis. Thus, this analytical approach offers little additional information that could not be obtained by x-ray diffraction or high vacuum spectroscopic techniques.

Electrochemically Modulated Infrared Spectrometry

A technique that enhances the sensitivity of internal or external reflection approaches is electrochemically modulated infrared spectroscopy (EMIRS). A "pseudo double beam" approach is used to normalize spectra and enhance surface sensitivity. Spectra are constructed by difference in reflectivity, ΔR, of a surface at two potentials. The modulation is between two potentials, V_1 and V_2, which causes a change in the adsorbed material. This, in turn, gives rise to changes in the optical constants of the interface affecting the reflectivity, resulting in a signal in phase with modulation. A spectrum is obtained by recording this change in DR as the wavelength of the probing radiation is slowly scanned.

When used in conjunction with FT-IR, this sampling technique is referred to as subtractively normalized interfacial FT-IR spectroscopy (SNIFTERS). For SNIFTERS, the sum of all interferograms taken at V_1 (the reference level, at a level of zero adsorbance for the species under consideration) are subtracted from those taken at V_2 and then divided by V_1 to normalize the spectra. The most useful ΔR corresponds to vibrations of species that cycle through surface adsorption and desorption or oxidized and reduced states at the two potentials. EMIRS, when used in conjunction with the ATR sampling mode, has the potential to identify which biofilm microbial metabolites or adsorbed organic species participate in a particular corrosion reaction at a surface. Although the technique can detect very subtle changes in molecular behavior at surfaces, it is usually necessary to have a good understanding of the reactions anticipated. SNIFTERS can be performed on-line when used in conjunction with ATR sampling mode, although this has not been widely documented. A more detailed discussion of EMIRS and SNIFTERS, and their applications to corrosion reaction characterization and monitoring, is provided by Wragg and White (1991). Although, EMIRS has not yet been used to characterize or monitor biocorrosion, it

represents an extremely powerful alternative approach to current methods used in the field.

CONCATENATION OF IR SPECTROSCOPIC AND LIGHT MICROSCOPIC TECHNIQUES TO INTEGRATE BIOCHEMISTRY AND BIOLOGICAL STRUCTURE OF MICROBIAL BIOFILMS

To date, it has been difficult to obtain chemical information on intact biofilms. Light microscopic techniques typically provide bacterial cell density, diversity and distribution determinations, microcolony size, diversity and density determinations and even associations between populations or cells, but little chemical information in particular on fully hydrated samples. Ir microscopy provides the opportunity to obtain spatially-resolved chemical information on dehydrated samples, but because of the effects dehydration imposes on spatial relationships as well as ir spectra, it has contributed limited insight to biofilm chemistry. Ir microscopy has been used to collect DRIFT spectra of freeze-dried biofilms on a metal corrosion coupon. This approach permitted the mapping of ratios of band intensities contributed by cell protein, PHA and acidic exopolysaccharides at a resolution of spot diameters of 20 μm (White *et al.*, 1988).

Recent developments in Raman microscopy make this technique a potentially more useful tool for resolving spatially-dependent, chemical information on biofilm structures (Schaeberle *et al.*, 1995; Morris *et al.*, 1996). Since water does not absorb strongly in the Raman spectrum, useful, high-resolution biochemical information should be obtainable from fully hydrated specimens. Autofluorescence from biological samples can compromise Raman spectral quality, however.

Another approach to relate chemical and structural information on biofilms involves the combined ATR/FT-IR spectroscopy and light microscopy. The first attempt to combine these instruments was reported by Suci *et al.* (1997). They described a flat plate, flow-channel reactor containing a flat trapezoidal Ge IRE as a surface colonized by microorganisms introduced in an aqueous stream, which passes through the reactor. The reactor also contained a viewing window that accommodated reflected differential interference contrast (DIC) or epifluorescence microscopic observation of the Ge surface through a water immersion objective lens when mounted on the stage of a microscope. The reactor could be transferred to the optical bench of an FT-IR spectrometer housing a Harrick Horizon Cell mirror assembly (Harrick Scientific, Ossing, NY) for collection of ir spectra without disturbance to biological processes taking place on the Ge surface. They demonstrated how surface-associated bacterial cell density determinations by DIC microscopy correlated with surface-associated biomass determinations based on the intensity of the amide II protein band during different stages of biofilm development. A similar system has been developed by Ishida *et al.* (1998) to follow biofouling of reverse osmosis membrane material. While these concatenated techniques do not yet offer the opportunity to obtain spatially-resolved chemical information due to the averaging of molecular vibrations across the IRE surface, they do allow comparisons between biofilms formed on different IREs that are run in parallel.

SUMMARY AND CONCLUSIONS

FT-IR has proven to be a useful analytical approach for on-line monitoring of chemical and biological changes at surfaces in contact with aqueous media. ATR sampling geometries have isolated the acquisition of ir spectra to the solid-liquid interface where organic conditioning films form as a result of adsorption of protein and carbohydrate material from the bulk aqueous phase. The same sampling geometries have been successfully used to detect the attachment of bacteria from the bulk aqueous phase to the substratum surface and to characterize the adhesive holdfast material excreted by the attached microorganisms. Because this sampling approach does not disturb biological processes, it has been used to follow microbial biofilm development over long time periods. It has proved to be very useful in monitoring chemical and biochemical changes at the base of biofilms. Through this approach, new mechanisms of microbiologically influenced corrosion have been demonstrated, proposed mechanisms of biofilm recalcitrance to antimicrobial agents have been evaluated and changes in physiological status of cells observed. None of these discoveries could have been as easily made using other analytical approaches. Moreover, because ir absorbance follows the Beer-Lambert equation, quantitative information can be obtained in many cases for molecules present at an interface. Although, ir spectroscopy has long been used for chemical identification of relatively pure samples, it offers limited capabilities when sampling complex biological systems such as mixed populations of intact bacterial cells growing on surfaces. Nevertheless, FT-IR has proved useful in detecting differences between different types of bacteria and has provided taxonomic information on microorganisms.

FT-IR as an approach to on-line monitoring of microbiological phenomena is still in its infancy. As the need to define biological processes at surfaces expands, new applications of FT-IR will evolve in this area. Since most microbiological processes in nature occur in the presence of surfaces, exceptional opportunities exists for new discoveries to be made by evaluating microbiological reactions at surfaces. The ATR sampling mode, when combined with FT-IR, offers a unique window to the chemical and biochemical reactions underlying the microbiological processes that develop on surfaces.

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