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Investigation of interactions between antimicrobial agents and bacterial biofilms using attenuated total reflection Fourier transform infrared spectroscopy

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Abstract

Biomaterial-centred infections are often difficult to treat. An impaired immune response, acute inflamatory reactions and the presence of recalcitrant attached microorganisms are all contributing factors. A brief review of the role of attached bacteria in biomaterial-centred infections is presented. Two major hypotheses which may explain the recalcitrance of biofilms to antimicrobial agents are discussed. The analytical capabilities of attenuated total reflection Fourier transform infrared (ATR/FTIR) spectroscopy for providing information on both transport of an antimicrobial agent to bacteria embedded in the biofilm and interactions between an antimicrobial agent and biofilm components are described.

Keywords: Antimicrobial agents; Bacterial biofilms; ATR/FTIR

1. Role of attached bacteria in biomaterial-centred infections

1.1. Infections associated with biomedical devices

Lack of tissue integration and associated infections are two of the major factors leading to premature failure of devices implanted in soft tissues as well as catheters and transcutaneous devices [1–5]. The reaction of soft tissues in contact with synthetic materials often involves some extent of chronic inflammation and fibrous encapsulation [6, 7]. The foreign body reaction is characterized by recruitment of leucocytes and macrophages, some of which adhere to the material and merge to form multinucleated giant cells. If the foreign body cannot be degraded it is commonly encapsulated by fibrous tissue. Clinically, a certain level of chronic inflammation is anticipated as an almost inevitable side effect of contact of synthetic materials with soft tissue. However, implanted materials which have become the locus for bacterial infection must usually be replaced, or alternatively the infection must be treated as a chronic illness [8].

Bacteria are common inhabitants of the body which are normally kept under control by the immune system. However, once bacteria adhere to a surface they may form a biofilm in which cells are, for reasons which have not been fully elucidated, protected from many antagonistic agents. Biofilm bacteria have been shown to be protected from complement-mediated opsonic factors, phagocytic cells and antimicrobial agents [9, 10]. Components of the extracellular polymeric substances (EPS) of bacteria can also modulate the cellular immune response [11–13].

Both fungal and bacterial biofilms have been found on a variety of indwelling devices removed from patients with associated infections [14–17]. In vivo studies have indicated that inocula of bacteria which would otherwise be cleared by an animal's immune system cause infection in animals in which biomaterials have been implanted [18]. In these studies the bacteria have been found colonizing the implanted biomaterial.

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In vitro experiments indicate that bacteria colonizing biomedical materials can sometimes withstand many times the dosage of antimicrobial agent sufficient to completely eradicate planktonic (free floating) bacteria [19–21]. Hypotheses which have emerged to explain the reduced susceptibility of biofilm bacteria to anti-microbial agents are summarized in Table 1. They can be grouped into two categories. The first category encompasses origins of recalcitrance related to transport limitation within the biofilm, while the second focusses on physiological or metabolic characteristics which microorganisms assume by virtue of life within a biofilm.

1.2. Transport limitation of antimicrobials through biofilms

Biofilm bacteria are typically enveloped in an EPS matrix [12, 25–28]. This polymeric network connects cells with one another and to the substratum. Intuitively, one might assume that the EPS matrix shields the cells from antagonistic agents including antimicrobial agents, and this interpretation has been invoked to explain biofilm recalcitrance [10, 19, 21, 29].

Qualitatively, it has been shown that a biofilm grown on a filter between two chambers impedes the passive transport of an antimicrobial agent from one chamber to the other [30]. More quantative measurement of transport of antimicrobial agents through the EPS matrix components has been limited mostly to studies of alginates isolated from *Pseudomonas aeruginosa* or from kelp. Alginate is a linear copolymer of (1–4)-linked α-L-guluronic and β-D-manuronic acid residues [31, 32]. Each residue incorporates a carboxylate functionality. The diffusion coefficient for transport of uncharged antimicrobial agents through aqueous solutions of alginate is approximately the same as that for water (or slightly reduced). However, binding of positively charged molecules (e.g. aminoglycosides) can result in substantially reduced (3–40%) 'effective' diffusion coefficients [33–35]. As expected, the binding affinity of these antimicrobial agents becomes smaller as ionic strength is increased [35, 36].

The transport of positively charged antimicrobial agents is impeded by binding to bacterial alginate. However, the calculated delay for exceeding the bactericidal dose for cells at the base of a 100 μm biofilm, attributed to this binding, is relatively small [34]. When uptake and/or adsorption by cells is added to the equation, the increase in delay becomes more substantial. For example, it has been predicted that the time required for the aminoglycoside tobramycin to reach 90% of its bulk concentration at the base of a 100-μm-thick biofilm would be increased from 27 s to between 42 min and 2.4 h (depending on the strain) by the combined effects of binding to the EPS and uptake and/or adsorption to cells in the biofilm [37]. Certain classes of antimicrobial agents (especially β-lactams) can be inactivated by bacterial enzymes. If maximum values for rates of hydrolysis of β-lactams are included as part of a transport model, the model predicts that the antimicrobial agent remains below 90% of the bulk concentration at the base of a 100 μm film indefinitely [37].

Although mucoid strains of *P. aeruginosa* isolated from lungs of cystic fibrosis (CF) patients produce copious quantities of an acetylated form of alginate [38], the EPS matrix surrounding other bacteria implicated in biomaterial-centred infections (*Staphylococcus aureus* and *Staphylococcus epidermidis*) is probably composed of other polysaccharides [39–41]. In addition, EPS typically contains a substantial portion of proteins, and can

Table 1
Possible mechanisms of biofilm recalcitrance to antimicrobial agents

Attribute[a]	Mechanism[b]
Transport-related	
EPS	EPS moieties (e.g. uronic acid groups) may bind charged compounds; EPS can effect diffusion parameters (see text)
Cell surface hydrophobicity	Mobilization of hydrophobic proteins at cell surface may affect transport of polar compounds [22]
pH/eH	Change in pH/eH through the biofilm could alter efficacy [23]
Physiology-related	
Decreased metabolic activity (e.g. respiration, cell wall synthesis)	May impede active transport across walls/membranes; agents which depend on interference with an enzyme involved in repair or regeneration of cellular components may be less effective; agents which interfere with translation or transcription may be less effective
Decreased growth rate	May alter effectiveness of agents which interfere with enzymes involved in replication (e.g. FQs); structure/organization of cell envelope may alter to decrease permeability
Specific enzyme activity	Increased production of enzymes which inactivate antimicrobials [24]

[a] The division into transport and physiology related attributes is non-exclusive.

[b] References have been included in cases in which the evidence supports the listed mechanism. Others are speculative. For a discussion of evidence for metabolic and growth rate effects see text.

also contain other components such as nucleic acids [42] or siderophores [43]. Moreover, it has not been established that the EPS composition of *P. aeruginosa* strains colonizing locations other than the lungs of CF patients (e.g. peritoneal cavity infections) is composed primarily of alginate of identical composition to that found in mucoid CF strains. More importantly, there is growing evidence that the EPS matrix of in vivo biofilms may be structurally organized to optimize biofilm architecture for nutrient transport or antagonist exclusion [44–46]. Therefore, eventually it will be necessary to measure transport of antimicrobials in intact biofilms in order to obtain a global assessment of the role of transport limitation on antimicrobial efficacy.

1.3. Physiological characteristics of biofilm bacteria which may bestow recalcitrance

Survival dictates that bacteria be capable of making appropriate responses to environmental changes [47]. Some well-characterized examples include chemotaxis [48], the SOS response to DNA damage [49] and 'starvation-survivial' response to nutrient limitation [50, 51]. Many of these responses involve regulation of sets of genes. One of the most extensively studied examples of gene regulation associated with transformation from a planktonic to a sessile existence is the CF *P. aeruginosa* infection model. In the environment of the CF lung these strains make the genetically controlled and reversible transition from non-mucoid to mucoid (EPS enclosed) phenotype [52]. By analogy, one can predict that, in general, biofilm bacteria will differ from their planktonic counterparts at the level of genetic regulation, and thus may differ profoundly in many physiological aspects [53]. It would be surprising if some of the physiological changes inherent to surface-associated growth did not, either fortuitously or by design, affect their susceptibility to antimicrobial agents.

With some exceptions [53], the complex physiological changes associated with biofilm formation and their influence on antimicrobial agent susceptibility remain hypothetical. It is evident that both growth rate and availability of nutrients, including trace metals, can influence both the composition and structural organization of the cell envelope [54]. Differences in cell envelope composition have been correlated with differences in sensitivity to chemical lytic agents [55] and susceptibility to antimicrobial agents [56]. Growth rate influences penicillin binding proteins [57] and the susceptibility of *Escherichia coli* to β-lactams was found to be proportional to the growth rate [58].

Gilbert et al. [59] have devised an apparatus for controlling the growth rate of bacteria colonizing a cellulose acetate filter. They have employed this apparatus to compare the efficacy of selected antimicrobial agents against planktonic and sessile bacteria at various growth rates. In general, planktonic bacteria become more recalcitrant as growth rate is reduced [60, 61].

1.4. Biofilm recalcitrance: moderated transport/adaptive physiology

An argument that is commonly expressed to refute the claim that hindered transport through biofilms can be responsible for the observed recalcitrance is the following: even if there is a delay in reaching the minimum bactericidal concentration (MBC) in certain portions of the biofilm, if the bulk concentration is larger than the MBC, given enough time, this concentration will be exceeded. This argument ignores the possibility of physiological adaptation; i.e. the possibility that, given a time period of sufficient duration, bacteria will adjust to a sublethal concentration of a given antimicrobial agent, thus enabling survival during exposure to concentrations exceeding the MBC. Bacteria can adapt rapidly (10–20 min) to environmental stress by altering expression of various proteins [62]. Exposure to sublethal doses of antimicrobial agents causes changes in the protein content of bacterial cell envelopes [63]. Subinhibitory concentrations of various antimicrobial agents have been shown to induce altered protein expression in *Streptococcus sobrinus* within minutes [64].

If the combined high cell density, EPS composition and biofilm architecture can moderate delivery of an antagonist to cells within the biofilm, this may give the cells time to adopt a physiologically protective set of changes. This would be especially possible if biofilm bacteria were already poised to adjust to relatively slowly changing concentration gradients of nutrients and/or antagonists. Perhaps the high levels of recalcitrance exhibited by bacterial biofilms originate from an interplay of regulated transport and physiological adaptation.

2. ATR/FTIR technique for investigation of biofilm recalcitrance to antimicrobial agents

2.1. General principles

Infrared (IR) spectroscopy has been used for approximately a century as an analytical technique in chemistry and especially organic chemistry. Infrared energy (approximate wavelength range of 1 μm to 1 mm) is absorbed by quantum energy levels associated with atomic vibrations (or rotations for far-IR). Infrared absorption spectra of organic molecules are rich in structure and essentially unique: a spectrum of a compound provides a positive identification. Spectral features of a compound may be altered by solvent environment, conformation or association with other compounds or a surface. This can

be used to advantage to monitor changes in one of these properties, but may also obscure identification.

Fourier transform (FT) refers to a pair of mathematical (integral) expressions which essentially relate a continuous function and its decomposition into weighted frequency components. Fourier transform instruments acquire data using interferometry which yields the frequency component form, and then, from this representation, compute a conventional spectrum using Fourier transform mathematics. One advantage of the FT instrument is increased sensitivity compared to dispersive instruments. This increased sensitivity has made IR analysis of compounds in aqueous solution more feasible.

Water absorbs very strongly in the mid-infrared range, a region in which many biological molecules have strong resonance vibrations. The absorption in the regions centred at $1640\,cm^{-1}$ ($\sim 6\,\mu m$ wavelength) and $3300\,cm^{-1}$ ($\sim 3\,\mu m$) is so great that, except for transmission through a sample of small pathlength ($<15\,\mu m$), these regions will be entirely obscured. One way to achieve a micrometre scale pathlength is to use attenuated total reflection (ATR). The infrared beam is guided within a high refractive index prism or internal reflection element (IRE) (usually germanium (Ge) or zinc selenide (ZnSe) by multiple internal reflections. With the appropriate geometry, the beam reflects from internal surfaces almost as if they were a mirror. However, some of the IR energy penetrates through the surface upon each reflection in the form of an evanescent wave which can absorb energy. If the IRE is contained within a flow chamber, the IR absorption of compounds in an aqueous medium adjacent to the surface of the IRE can be measured. Since the evanescent wave penetrates on the order of a micrometre into the adjacent medium, the effective pathlength is small enough that spectra can be obtained within the strong water band centered at $1640\,cm^{-1}$. By combining ATR with FT quite reasonable IR spectra can be obtained of aqueous-phase biomolecules in the entire mid-IR range.

2.2. Lateral growth curve of bacteria colonizing a surface

Bacteria are complex mixtures of many different types of organic molecules which result in a broad band of overlapping IR resonance frequencies. However, the resonance frequencies associated with similar repeating or replicated structures in various biomolecules accumulate in certain regions of the IR spectral range, giving the bacterial spectrum discernable features which can be identified as originating from particular classes of biopolymers. The three main groups of biomolecules which can be distinguished in an IR spectrum of a bacterium are proteins, RNA/DNA and polysaccharides [65, 66]. Aliphatic chains of lipids can also be identified as well as the carbonyl stretch associated with an ester linkage or the salt of a carboxylic acid. Fig. 1 demonstrates that a simple mixture of biopolymers, combined in relative amounts representative of the composition of a bacterium [67], exhibits an IR spectrum having many of the major features of an actual bacterium, i.e. prominent bands associated with biopolymers can be identified. The spectra of all bacteria have these features in common. However, underlying these common spectral features are more subtle differences which can be exploited. For example, using appropriate analysis techniques, IR spectra can be used to identify bacterial species [65].

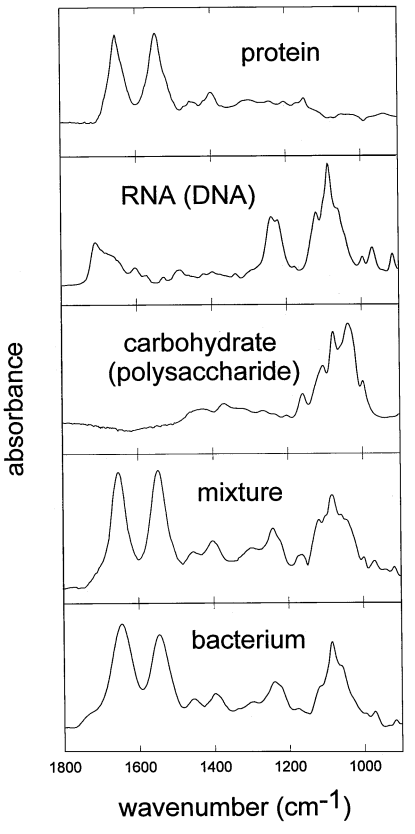

Fig. 1. ATR/FTIR spectra of bulk samples of (from top to bottom): protein (bovine serum albumin); RNA (ribonucleic acid, sodium salt from yeast); carbohydrate, (D(+)-cellobiose); mixture of approximate proportions of protein, RNA and carbohydrate found in a bacterium [67]; bacterium, *Pseudomonas aeruginosa*.

The distinguishing IR characteristics of a bacterium, together with the ATR geometry and FT sensitivity, can be exploited to follow the lateral colonization of a bacterium on the surface of an IRE. Most FTIR spectrophotometers currently manufactured have a sample chamber incorporating a single beam. The Perkin-Elmer 1800 was designed to operate as a double beam instrument. This configuration facilitates culturing of biofilms on two IREs simultaneously, under nearly identical conditions.

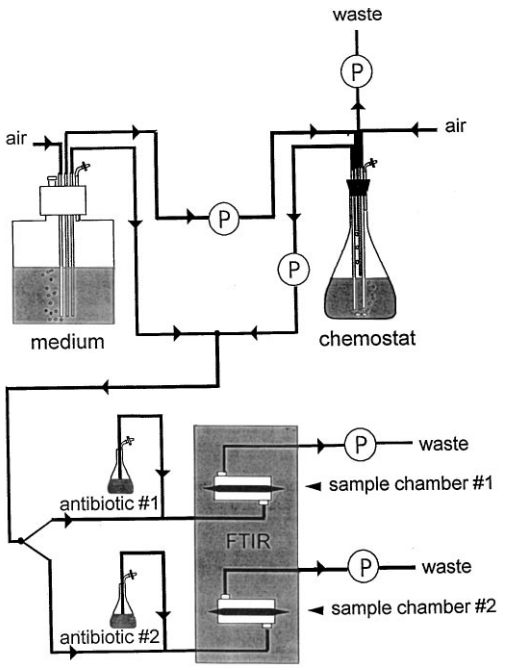

Fig. 2. Experimental set-up for monitoring growth of two biofilms simultaneously under nearly identical conditions. Interaction of two antimicrobial agents with these biofilms can be compared. P, pump.

The experimental set-up used for growing a biofilm and monitoring its growth is shown in Fig. 2. A continuous culture is used as inoculum. This is a suspended culture of bacteria which is being fed continuously at a constant rate and is therefore growing at a known, average growth rate. These bacteria are diluted into a stream of sterile culture medium to initiate biofilm formation. Once growth begins, the biofilm is fed sterile culture medium.

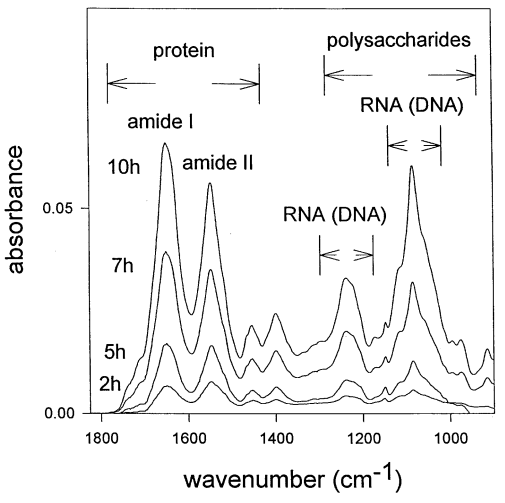

Fig. 3. Spectra of a *Pseudomonas aeruginosa* biofilm colonizing the surface of a Ge internal reflection element (IRE). Regions of absorbance of primary biomolecules are indicated.

Spectra of a biofilm grown on the surface of a Ge IRE are shown in Fig. 3. By taking the area under one of the bands (in this case, the amide II band associated with the peptide linkage of proteins) one can follow the colonization of the surface. The accumulated data are referred to as a 'lateral growth curve', since the absorbance is only being measured in the monolayer of cells immediately adjacent to the surface. Lateral growth curves for biofilms grown in flow chambers known as circle cell assemblies (CCA) (Spectra-Tech, Stamford, CN, USA) are shown in Fig. 4. At the times indicated the flow was stopped, which in this case induced an increase in the bacterial signal. Note that the growth curves from flow chambers 1 and 2 are very similar.

The CCA are designed to enclose a cylindrical IRE which is held in place by two polytetrafluoroethylene (PTFE) O-rings. Flow chambers for trapezoidal IREs have also been designed [68] and some are commercially available (Spectra-Tech, Harrick Scientific Corp., Ossining, NY, USA). The advantage of the CCA is its ease of assembly, lack of leaking, contact with the IRE only via PTFE (an inert material) over a small area, and ease of cleaning. The cylindrical geometry reflects the circular beam symmetrically along the IRE surfaces providing maximum interface for evanescent wave penetration. One disadvantage is that the cylindrical geometry does not lend itself easily to direct analysis of the IRE surface

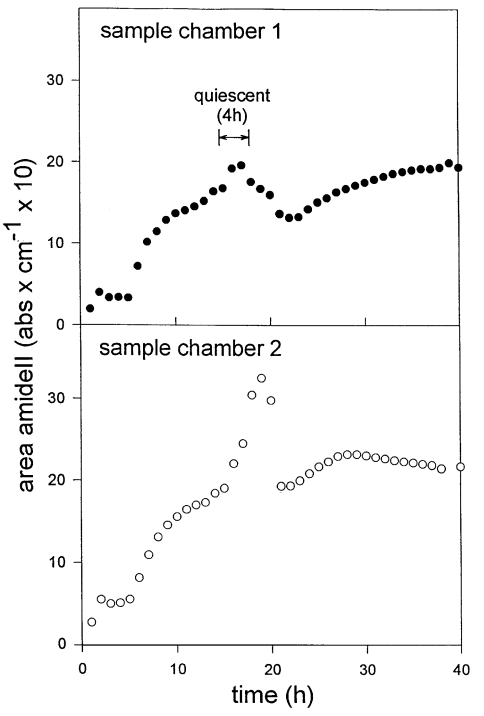

Fig. 4. Lateral growth curves obtained by computing the area of the amide II band for *Pseudomonas aeruginosa* colonizing IREs in the two sample chambers of the spectrophotometer. Flow was discontinued in both chambers during the time period indicated.

by other techniques (e.g. microscopic observation, X-ray photoelectron spectroscopy (XPS) analysis, etc.), which require a planar surface for optimal performance. The PTFE O-rings also result in two large bands (1150 and 1200 cm^{-1}) which vary in intensity due to the deformation of the PTFE during the course of an experiment, and thus cause problems with background subtraction in those regions.

2.3. Time course of antimicrobial penetration

Once a biofilm is established one can use the ATR/FTIR technique to follow the time course of antimicrobial appearance at the biofilm/substratum interface. A spectrum is acquired immediately before introduction of the antimicrobial. With as little perturbation of the flow as possible, culture medium containing an antimicrobial is channelled through the chamber and spectra are acquired periodically. Finally, sterile culture medium is again introduced and the wash-out is monitored. If the spectrum acquired immediately before introduction of the antimicrobial is used as the background spectrum, the spectral features which are the IR fingerprint of the antimicrobial will appear, wax and finally wane during the wash-out period. The technique will work in this simple form only if the change in the relatively large bacterial signal originating from the biofilm remains relatively small. With fluoroquinolones (FQs), the biofilm spectrum seems to change within limits which allow one to follow the time course of FQ appearance and disappearance. In some cases, the change in background is so slight that the bands associated with FQ are clearly distinguishable during the entire time course (Fig. 5). However, in other cases one can only distinguish one or two prominent bands from the FQ, especially at later times.

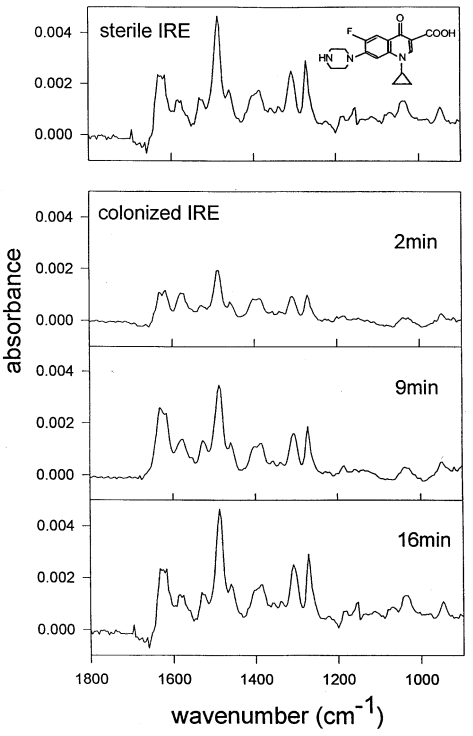

Fig. 5. ATR/FTIR spectra of ciprofloxacin: top, sterile IRE (structure is indicated); bottom three, time course of appearance at the base of a *Pseudomonas aeruginosa* biofilm (times after introduction of the fluoroquinolone into the chamber are indicated).

2.4. Interactions between antimicrobial agents and biofilm components

Besides allowing acquisition of a lateral growth curve and a time course of antimicrobial appearance at (and disappearance from) the biofilm/substratum interface, it is possible to measure interactions between components of the biofilm and the antimicrobial using the ATR/FTIR technique. Fig. 6 shows a set of bacterial spectra acquired after a *P. aeruginosa* biofilm was exposed to the antimicrobial agent ciprofloxacin for 20 min [69]. Three bands appear in the spectra, which are notably absent in the spectra acquired from bacteria not exposed to the antimicrobial. Note that the kinetics of changes induced by the FQ are relatively slow, suggesting a long-term physiological response. The appearance of these bands was interpreted as a possible indirect effect of ciprofloxacin (known to interact with the bacterial DNA gyrase) on the bacterial DNA. However, it is possible

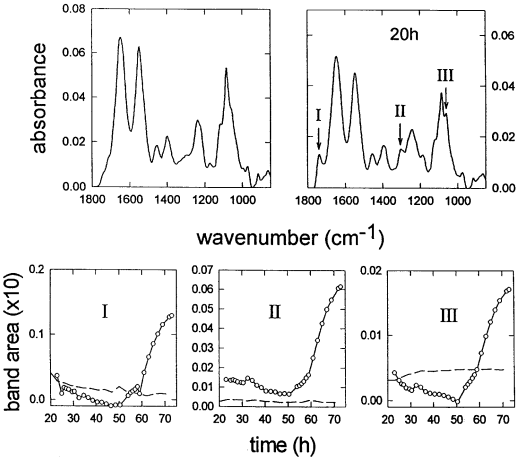

Fig. 6. Appearance of three bands in the spectra of a *Pseudomonas aeruginosa* biofilm after exposure to 100 $\mu g\,ml^{-1}$ of ciprofloxacin for 20 min. (Figure used with permission from *Antimicrobial Agents and Chemotheraphy* [69].)

that the bands may also derive from production of an acetylated form of the bacterial polysaccharide, alginate, in response to the FQ. The positions of prominent spectral bands are indeed similar [38]. Characterization of molecular interactions between antimicrobial agents and biofilm components is one of the most intriguing possi-

bilities of the ATR/FTIR technique. The complexity of the system makes unambiguous interpretation of results challenging. However, the rewards are potentially great, since information on chemical interactions can be obtained non-destructively, in real-time, in the absence of an extrinsic label.

3. Analysis of data acquired by the ATR/FTIR technique

3.1. Mathematical modelling applied to transport kinetics

In a qualitative sense the generalization can be made that, in comparison to a clean, sterile substratum, the biofilm alters the kinetics of both FQ appearance and disappearance at the substratum/biofilm interface. Preliminary experiments indicate that the form of the alteration depends on both the FQ and the biofilm.

Stylized versions of the two extreme types of kinetic curves, which seem to bracket the shapes of data sets obtained experimentally from penetration of FQs into *P. aeruginosa* biofilms, are depicted in Fig. 7. A close approximation to the lower curve was obtained from a 30 μm biofilm (ciprofloxacin/*P. aeruginosa*) [69]. Compared to a clean, sterile substratum, the appearance at the interface is hindered and does not reach the bulk concentration level during the exposure period. The curve has a dependence which is linear with respect to the square root of time. This type of dependence has been associated with diffusion-limited transport [70]. The upper curve depicted in Fig. 7 is merely a representation of the other extreme, which is approximated when thinner biofilms (5 to 10 μm) are exposed to levofloxacin. In this case the FQ accumulates at the interface during the exposure period, following approximately first-order kinetics and finally exceeds the bulk level. In order to demonstrate the direction our modelling efforts are taking, the first type of data curve will be used as an example.

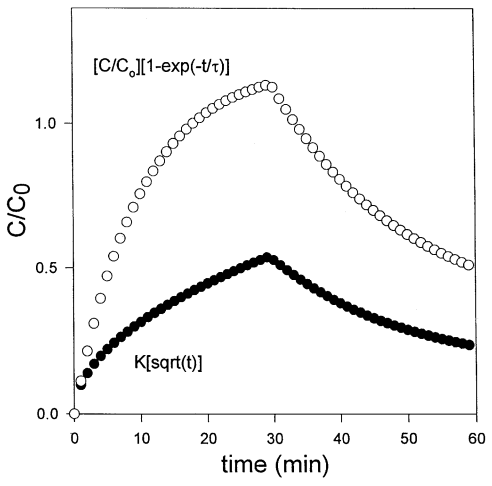

Fig. 7. Stylized versions of two types of kinetic data curves with equations used to generate the curves indicated. (Wash-out portions are first-order decays to a plateau value.)

In order to simplify the discussion, only the data for appearance of the FQ at the interface will be discussed. Assume for simplicity that the biofilm is a thin homogeneous slab, or sheet. Analytical expressions are available which describe the diffusion of a substance through the sheet [71]. If one takes the initial conditions to be a concentration of C_0 (bulk concentration) at distance (l) from the interface, the kinetics of appearance of the substance at the interface can be described by a family of sigmoidal curves represented in Fig. 8a. Note that the coordinates are non-dimensional and that the slope of the middle section of the curve is approximately linear with respect to the square root of time. Although these curves can be generated by an analytical expression, we have obtained a numerical solution to Fick's law of diffusion using a program called AQUASIM [72]. This program allows additional complexity (i.e. mathematical terms) to be introduced into the differential equation in stepwise fashion without relying on availability of an analytical solution. Explicitly the differential equation is:

$$\frac{\partial C_A}{\partial t} = D\frac{\partial^2 C_A}{\partial z^2} \quad (1)$$

where C_A is the concentration of FQ, D is the diffusion coefficient, t refers to time, and z is the distance perpendicular to the interface. Boundary conditions constrain the FQ concentration to be zero within a region estimated as the diffusion boundary layer for the laminar flow conditions of the experiment (~100 μg). (As expected, the curve generated from the program reproduces the analytical solution.) On the same figure are plotted data points from an experiment in which a 30-μm-thick *P. aeruginosa* biofilm was exposed to 100 μg ml^{-1} of the FQ, ciprofloxacin. The D value of the theoretical (non-dimensional) abscissa values has been adjusted so that the slope of the linear section of the theoretical curve and the data curve are approximately equal. For a 30-μm-thick film this requires a diffusion coefficient of the order of 10^{-9} cm^2 s^{-1}, which is between two and three orders of magnitude lower than the diffusion coefficient of ciprofloxacin in water [69]. In other words the rate of appearance of the FQ at the biofilm/substratum interface can be modelled by assuming that the biofilm is a homogeneous sheet with a very small diffusion coefficient for the FQ. Obviously, the theoretical curve does not model an important aspect of the data curve: i.e. the model predicts an appreciable delay in actual initial appearance of the FQ at the interface which is not observed.

There is no evidence that FQs are degraded by, for example, bacterial exoenzymes. However, our experience

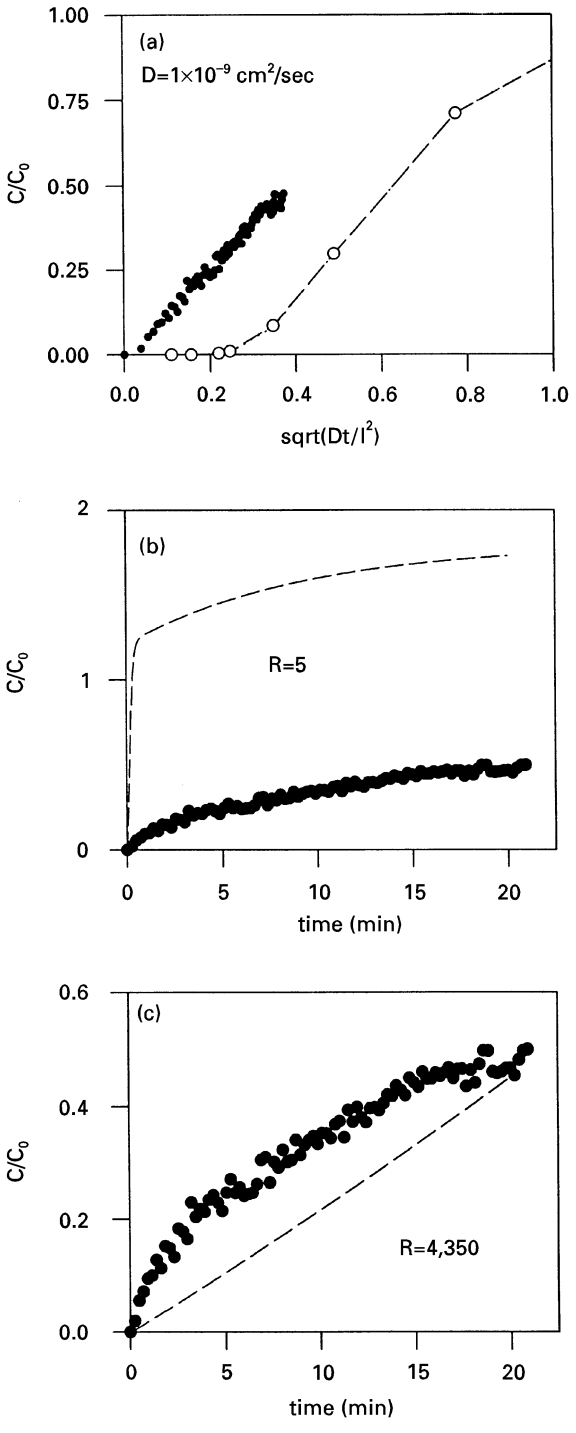

Fig. 8. (a–c) filled circles: data for appearance of fluoroquinolone at base of biofilm acquired by ATR/FTIR [69]; broken line: theoretical curve (AQUASIM model); (a) theoretical curve according to Eq. (1). Abscissa is in non-dimensional coordinates; $l = 30$ μm (3×10^{-3} cm); t, time (min); sqrt, square root; $D = 1 \times 10^{-9}$ cm^2 s^{-1} for best fit of slope of linear section; open circles are analytical solution [71]; b, c, theoretical curve according to Eq. (2); (b) $R = 5$; (c) $R = 4.350$ (see text for definition of R).

with thinner biofilms indicates that FQs can bind to biofilm components. Binding to biofilm components would reduce the amount of free FQ in the film and perhaps delay its rate of appearance at the interface [37]. In order to see whether this effect might model the data curve, two terms have been added to Fick's equation to account for the adsorption/desorption reaction of the FQ from biofilm components. The resulting differential equation is:

$$\frac{\partial C_A}{\partial t} = D\frac{\partial^2 C_A}{\partial z^2} - k_S C_S C_A + k_D C_{AS} \quad (2)$$

Two additional coupled equations are needed to complete the mathematical description:

$$\frac{\partial C_S}{\partial t} = -k_S C_S C_A + k_D C_{AS} \quad (3)$$

$$\frac{\partial C_{AS}}{\partial t} = -\frac{\partial C_S}{\partial t} \quad (4)$$

where C_A is the concentration of unbound FQ, C_S is the binding site density, C_{AS} is the concentration of the bound FQ, and k_S and k_D are rate constants for adsorption and desorption, respectively. For these fits D was fixed at its estimated value for the FQ in water [69].

With the addition of terms accounting for adsorption of FQ within the biofilm one can adjust the density of adsorption sites and the rates of adsorption and desorption so that the model captures certain aspects of the data curve (Fig. 8b and c). Fig. 8b shows that the model is capable of reproducing the shape of the data curve at later times, but only at the expense of an initial jump to a concentration exceeding the bulk fluid concentration. On the other hand, when the model is altered so that the observed and theoretical FQ levels correspond at 20 min, the shapes diverge. Obviously, because neither case provides a 'good fit', there are contributing factors which have not been included in the model.

Nichols et al. [37] have derived a method for calculating the density of sites for adsorption of an antimicrobial agent onto a biofilm. This 'R value' was computed on the basis of binding to EPS and to whole cells, the latter of which was the dominant contributing factor. For a nonmucoid *P. aeruginosa* exposed to tobramycin, R is 322. The delay in reaching 90% of the bulk concentration at the base of a 100 μm biofilm was 2.4 h. At final saturation of the biofilm, R is equivalent to the density of total adsorption sites divided by the bulk antimicrobial concentration. For curves presented in Fig. 8b and c, R is 5 and 4.350, respectively.

The biofilm has been modelled as a homogeneous, thin film. Although, the *P. aeruginosa* biofilm which colonized the surface of the IRE appears to be quite homogeneous according to thin sections [69], it may actually contain small channels through which antimicrobial agents can penetrate rapidly. Antimicrobial agents might reach more densely populated regions (clusters) via diffusion from these channels.

3.2. Multivariate analysis for decomposition of temporal changes in spectral components

As described above, FQ appearance at (and disappearance from) the interface can be followed by computing the area of a prominent absorption band of the FQ in a set of difference spectra. When one computes areas of specific bands to analyse the ATR/FTIR spectral data, the available information is not being used to its full potential: one focusses on small regions of the spectrum in which the desired bands are apparent, and essentially ignores the rest of the spectral information. One powerful set of mathematical techniques, which might allow utilization of the spectral data at its capacity, is known as multivariate analysis or factor analysis [73]. The potential of these techniques to analyse similar sets of IR spectra, specifically for classification of bacteria based on IR spectra, has been previously demonstrated [65].

Spectra are represented as vectors in a multidimensional space. The dimensional size is equivalent to the number of wavenumbers at which IR absorbance is measured, and the coordinates of the vectors are the absorbance values at each wavenumber. Using matrix algebra one can then compute a set of eigen vectors (factors) for this vector space. Each spectrum (represented as a vector in the multidimensional space) can be reproduced as a linear combination of the factors. The contribution of each of the factors to each spectrum is a number known as a score. In addition, each factor is ranked according to its relative importance in reproducing the entire set of spectra. The factors can be thought of as spectral trends (or patterns) in the set of spectra and the scores indicate the contribution of that trend to each of the component spectra.

For the present application, a set of spectra is acquired during exposure of the biofilm to an antimicrobial agent. Since the bands associated with the antimicrobial agent wax and then wane coordinately during the experiment, these should be described by one trend, which ideally will be represented by one significant factor. The corresponding scores, versus time, would indicate the kinetics of appearance and disappearance of the antimicrobial agent. If the bacterial spectrum was changing during this time period as well, this would ideally be represented by different factors and an associated set of scores. The potential of this technique of analysis would be to use data acquired at each wavenumber in each spectrum to decompose the set of spectra into trends occurring temporally. Some of the trends might indicate interactions, which would be difficult or impossible to identify from inspection of the original data set.

Thus far, our attempt to apply the principles described above to analysis of the data has been limited to the use of software commercially available for IR analysis (Gallactic Ind. Corp., Salem, NH, USA), and only marginally successful. In the simplest case in which one can see clearly the spectra of the FQ increase and then decrease during the time course of exposure and wash-out, the most significant factor is indeed closely related to the spectrum of the pure FQ. The associated scores, plotted with respect to time, indicate a time course which is nearly equivalent to that obtained from computing areas. Interestingly, for a set of difference spectra in which the overlying FQ spectrum was almost entirely obscured, five bands, corresponding with peak positions of bands of the FQ, appeared in one of the most significant factors. In other words, the analysis was capable of gleaning useful information from the data which could not be obtained by inspection. However, the scores associated with this factor, when plotted versus time, indicated no apparent temporal trend. There are techniques within factor analysis (e.g. target testing) which allow one to adjust (or rotate) the factors so that the most significant factors correspond to meaningful vector arrays (i.e. interpretable spectra). These are currently unavailable for the GRAMS software package, but are available elsewhere (Chemometrics Toolbox, University of Washington; Eigen Vector Technologies).

4. Characterization of antimicrobial interactions with biofilms on biomaterials

In order to investigate biofilms colonizing surfaces other than Ge or ZnSe using the ATR/FTIR technique, one must modify the surface of the IRE. In principle, any relevant surface modification which produces a coating which is physically and chemically stable, and thin enough to allow penetration of the evanescent field into the adjacent aqueous medium, will suffice. ATR/FTIR has been used extensively to investigate blood/materials interactions [74]. Much of the development of practical surface modifications for ATR/FTIR has originated from these types of studies.

Self-assembled monolayers (SAMs) refer to a surface modification by chemisorption of either substituted alkanethiols on metals (commonly gold) [75] or of organosilanes on dielectric materials (commonly silicon or silicon dioxide) [76, 77]. The latter method has been used to modify Ge IREs for an ATR/FTIR study of protein adsorption [78]. By modifying alkyltrichlorosilanes with various functional groups, the authors were able to investigate the effect of different chemistries on fibronectin adsorption. Thin films with presenting surfaces representative of the surfaces of bone or teeth have been produced on IREs for ATR/FTIR studies using ion beam sputtering [79, 80]. Thin polymer films have been coated onto IREs and protein adsorption on these films has been characterized using ATR/FTIR [81–84]. Thin metal films can be sputter deposited [85] or vapour deposited [86] onto IREs. However, it is difficult to obtain a film which is both continuous and transparent to the

evanescent wave. Ordered films of functionalized lipids have been used to modify the surface of IREs for ATR/FTIR studies using the Langmuir–Blodgett (LB) technique [87]. LB films are physisorbed to the surface and may not be robust enough for microbiological studies. Plasma polymerization has been used to produce thin films for biomedical studies [88] and could presumably be used for ATR/FTIR studies.

The problems encountered with using either SAMs or thin polymer coatings to modify IREs are related to the stability and characterization of the films. It is well established that chemisorption of alkylsilanes onto silicon produces a covalently bound, stable, ordered monolayer with a well-characterized structure [89]. Analogous monolayers fabricated from organosilanes bound to Ge or ZnSe surfaces have been less well characterized. However, it is clear that organized adsorption of the compounds onto Ge from organic solvent occurs [90] and that the films are stable enough to permit protein adsorption studies [78]. The chemical composition, as well as surface roughness, of thin polymer films can be more conveniently controlled than that of the bulk material [82]. However, even relatively thin films (50 μm) produce a large background IR signal which may be difficult to distinguish from the signal of the biological sample if it changes even slightly. Films of relatively hydrophobic polymers tend to peel off the hydrophilic Ge surface. This problem can be alleviated in some cases by pretreatment of the IRE with an organosilane coupling agent [82, 91]. Despite these caveats, based on the success of ATR/FTIR studies of interactions of biomolecules in aqueous medium with surface-modified IREs, studies of interactions of antimicrobial agents with biofilms colonizing biomedically relevant surfaces is feasible.

5. Summary and research direction

Infections associated with implanted biomaterials are often difficult to treat. An impaired immune response, acute inflammatory reactions and the presence of recalcitrant attached microorganisms are all contributing factors. The recalcitrance of sessile bacteria to antimicrobial agents has been demonstrated in controlled laboratory experiments, making this an accessible target for laboratory research aimed at improving treatment of biomaterial-centred infections. Two hypotheses have emerged from this research effort. They can be summarized as: (1) biofilm bacteria are protected from exposure to the bactericidal concentration of the antimicrobial agent; (2) biofilm bacteria have special physiological attributes which confer recalcitrance despite being subjected to the otherwise bactericidal concentration.

Distinction between hypotheses concerning the nature of biofilm recalcitrance towards antimicrobial agents has significant implications for the direction of applied research. For example, the effort to develop antimicrobial agents which are more efficacious against biofilm bacteria could focus on design and synthesis of compounds which penetrate EPS components more readily, or which more effectively eradicate slowly growing bacteria. Both these development strategies could involve a different laboratory abstraction of a biofilm. For example, in one case compounds could be tested for transport through isolated EPS components; alternatively, efficacy of compounds could be screened against continuous cultures of planktonic bacteria having slow growth rates.

ATR/FTIR methodology has the advantage that the antimicrobial agent can be measured within biofilms colonizing solid substrata in a flowing system in real time. The technique revealed that transport of the FQ, ciprofloxacin, to the base of a relatively thin (30 μm) *P. aeruginosa* biofilm was quite drastically altered [69]. Research efforts are aimed at exploiting the sensitivity of this technique to explore the contribution of transport limitation to biofilm recalcitrance. The strategy is to compare transport and efficacy of a number of FQ structural analogues. Modelling the transport kinetics will yield a number of representative (numerical) parameters. Correlation of one or more of these parameters with efficacy might indicate a structure/function relationship which could aid in development of antimicrobial agents, or permit more effective use of existing ones.

It seems plausible that the observed recalcitrance of biofilms will be explained, in most cases, by an interplay of regulated transport and dynamic physiological responses. The ATR/FTIR methodology allows measurement of not only the appearance of an antimicrobial at the base of a biofilm, but interactions between the antimicrobial agent and biofilm components. In order to exploit this powerful analytical capability, more sophisticated mathematical techniques, such as factor analysis, will have to be used to condense the temporal spectral data.

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