NMR methods for in-situ biofilm metabolism studies: spatial and temporal resolved measurements

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Abstract We are developing novel nuclear magnetic resonance (NMR) microscopy, spectroscopy and combined NMR/optical techniques for the study of biofilms under known, controlled growth conditions. Objectives include: time and depth-resolved metabolite concentrations with isotropic spatial resolution on the order of 10 microns, metabolic pathways and flux rates, mass transport and ultimately their correlation with gene expression by optical microscopy in biofilms. We describe the implementation of ex-situ grown biofilms to improve growth environment control and NMR analysis. In-situ NMR depth resolved metabolite profiling techniques are introduced and demonstrated for a *Shewanella oneidensis* strain MR-1 biofilm. Finally, initial combined confocal fluorescence and magnetic resonance images are shown for a GFP-labeled *Shewanella* biofilm. These methods are equally applicable to other biofilm systems of interest; thus they may provide a significant contribution toward the understanding of adherent cell metabolism.

Keywords Biofilm; confocal scanning fluorescence microscopy; metabolite profile; multimodal imaging; nuclear magnetic resonance; *Shewanella oneidensis* MR-1

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Introduction

Biofilms are spatially heterogeneous in both physical and biological properties because of the influence of their environment and cellular activities. Lateral heterogeneity relative to water flow is very high in terms of water channels and the distribution of discrete colonies on surfaces and is a function of hydrodynamics and cell physiology. Vertical heterogeneity is generally thought to be a result of single cells developing into multi-cellular structures and establishing gradients of pH, dissolved oxygen and metabolites (Lewandowski and Beyenal, 2001) by the cellular respiratory activities and by the diffusional barrier of the extracellular polymeric substances (Bryers and Drummond, 1998). Biofilm cells, in turn, respond metabolically and genetically to these changing depth conditions (Stewart and Costerton, 2001). Novel technologies are needed to meet the demanding requirements to non-invasively measure changes that occur with depth in a biofilm.

Two successful methods developed for the study of live biofilms are confocal optical microscopy (Stoodley *et al.*, 1999) and nuclear magnetic resonance (NMR) techniques (Majors *et al.*, 2005; Seymour *et al.*, 2004). The past decade has seen much in the way of improvements and innovations in optical confocal scanning microscopy and real-time monitoring of cells by employing fluorescent reporter constructs. NMR, while of comparatively lower resolution, provides a wealth of non-invasive information for living samples. For live cells, NMR provides metabolite content, metabolic pathway and flux information, convective and diffusive mass transport, water compartmentation and does not suffer opacity losses and scattering effects. ^{1}H NMR spectroscopy (MRS) has been demonstrated to rapidly identify bacterial species with high selectivity, and has been used to probe microbe–metal interactions (Beveridge *et al.*, 1997). MRS methods allow for

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the identification and quantification of low-molecular-weight molecules (substrate, byproducts and xenobiotics) for in-situ biofilms without sample extraction. Further, NMR imaging techniques can be implemented with temporal and/or spatial resolution (Hoskins *et al.*, 1999; Paterson-Beedle *et al.*, 2001).

We are developing NMR techniques and combined NMR/optical (confocal fluorescence) microscopy techniques (Wind *et al.*, 2002) to correlate metabolism and transport with gene expression in live bacterial films. Time- and depth-resolved NMR "spectroscopic micro-imaging" methods are being developed to map metabolite concentrations in biofilms. Fluorescent labels for gene expression and biofilm architecture can be used in combination with confocal fluorescence microscopy to observe phenotypic and structural changes within a biofilm. Finally, the dual-modality NMR/optical microscope can be used to correlate these depth-dependent metabolic and phenotypic trends. Possible applications include characterizing the response to imposed changes in growth environment, exposure to antimicrobial agents, and metabolic or transport changes as a function of depth in a developing biofilm.

In a recent article (Majors *et al.*, 2005) we describe initial bulk (volume-averaged), time-resolved NMR metabolite measurements for in-situ grown *Shewanella oneidensis* strain MR-1 biofilms in a recycling flow reactor. This procedure yielded satisfactory bulk NMR results, but the biofilm distribution was non-uniform and thus inadequate for useful depth-resolved NMR measurements. Further, upstream biofilm growth partially depleted substrates and introduced by-products to the influent media at the NMR measurement section, yielding degraded environmental control.

This paper discusses the implementation of ex-situ biofilms to improve growth environment control and NMR analysis. In-situ NMR depth resolved metabolite profiling techniques and first successful depth-resolved biofilm metabolism measurements are introduced. Further, initial combined confocal fluorescence and magnetic resonance images are shown for a GFP-labeled *Shewanella* biofilm.

Methods and measurements

Strains and growth conditions

The facultatively aerobic gram-negative dissimilatory metal-reducing bacterium *Shewanella oneidensis* strain MR-1 was used for all experiments. A PIPES buffered (pH 7) minimal medium (modified from Zachara *et al.*, 1998) containing 35 mM fumarate as an anaerobic electron acceptor and 18 mM lactate as the carbon and energy source (MM1) was employed as the perfusate for most of the experiments. *Shewanella* biofilms were initially grown in a constant depth film fermenter (CDFF) using 5 mm diameter glass cover slips as growth platforms. Subsequently, the 5 mm samples were transferred to the perfused sample chamber of the combined NMR/optical microscope (Wind *et al.*, 2002) incorporated into a one-pass flow system. The flow system was operated with controlled flow rates (typically 0.080 ml/min) and sample temperature maintained at $25 \pm 1\,^{\circ}C$. Oxygen concentrations were neither controlled nor measured. The sample chamber was installed in the test section of a 11.7 T magnet during experiments, which ran for 1–3 days.

Measurements

All measurements were performed at 500.45 MHz for proton (^{1}H), using a Bruker Avance spectrometer with home-built combined NMR/optical microscope inserts (Wind *et al.*, 2002). The combined microscope (Figure 1 left) consists of an actively shielded vertical 89 mm clear-bore superconducting magnet, a top-loading NMR insert, a bottom-loading confocal fluorescence microscope insert, and a perfusable sample chamber. The sample is

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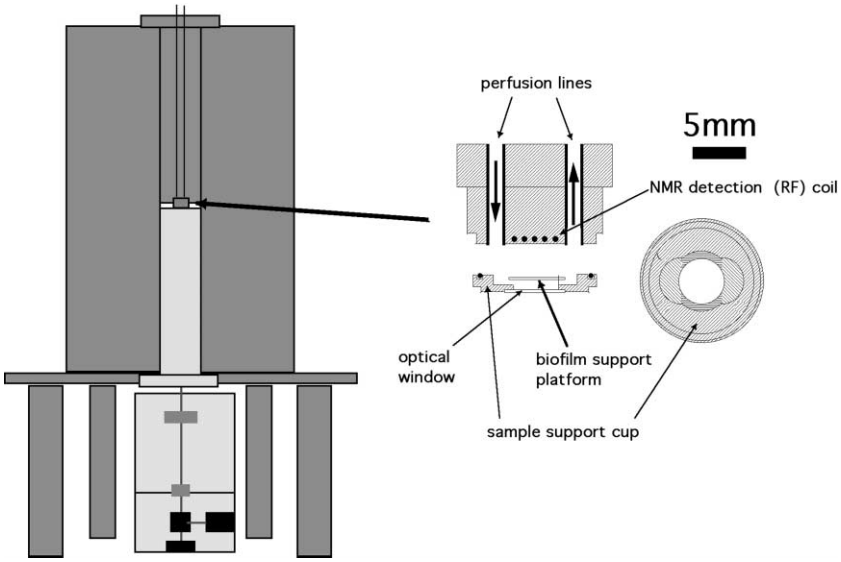

Figure 1 Left: diagram for the combined optical and magnetic resonance microscope, consisting of an actively shielded 11.7 T wide-bore magnet with top-loading NMR and bottom-loading optical components with the perfused sample chamber at their interface. Right: expanded and exploded side and top-down view of the sample chamber containing the glass cover slip sample support

installed by placing the 5 mm cover slip sample into the bottom sample support cup (Figure 1 right) with the biofilm facing downward, resealing the O-ring compression joint and inserting it into the magnet.

Measurements included rapid multidirectional NMR imaging to assess sample conditions (sample positioning and the absence of gas bubbles which adversely affect NMR spectra); 1D depth-resolved spectroscopic micro-imaging to measure metabolite spatial distributions; microscopic 3D NMR imaging (3D-MRI) to map the biofilm distribution; and (at early times) confocal microscopy to map biofilm growth. 3D-MRI measurements employed two, 0.5 second repetitions for each of 128 × 128 phase-encoding steps with an echo time of 4.7 milliseconds, for a total acquisition time of 4.5 hours and 40 × 40 × 20 μm spatial resolution (highest resolution in the biofilm depth direction). 1-D spectroscopic micro-imaging spectra were collected over a 2-mm-thick image plane carefully aligned orthogonal to the cover slip. WET water suppression (Ogg *et al.*, 1994) was employed to reduce the water signal over the sample chamber height (1 mm). Sixteen, 4 second averages were collected for each of 64 phase encoding steps over a 1.5 mm field of view (depth direction) with an echo time of 5.5 milliseconds, yielding a spatial resolution of 23 μm in the depth direction in 68 minutes total measurement time. Confocal images employed 488 nm excitation with pinhole detection of the GFP-labeled *Shewanella*, yielding 1–2 micron spatial resolution over a 1.25 mm by 1.25 mm focal plane.

Results and discussion

Figure 2 shows a composite of NMR and confocal images for a *Shewanella* CDFF biofilm soon after insertion into the microscope. Whereas MRI maps the contents of the entire sample chamber (Figure 2 top left), confocal images are restricted to a 1.25 mm diameter circular area of the cover slip (top left inset and top right), albeit with 20-fold higher spatial resolution. Biofilm-selective 3D-MRI (bottom right) showed an initially sparse biofilm with a maximum thickness of 40–60 μm that rapidly grew to fill the sample chamber after three days.

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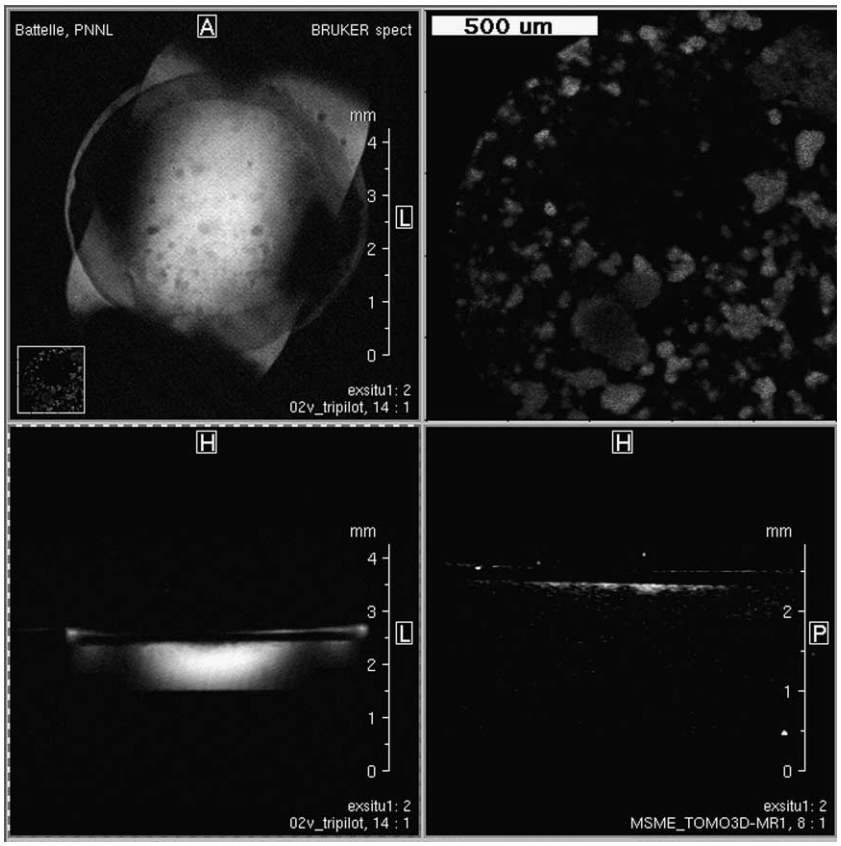

Figure 2 Sequential MRI and confocal images acquired for a sparse, early biofilm. Top left: water-selective top-down MRI of the coverslip with corresponding confocal image in the inset; top right: expanded view of the confocal image (same as inset); bottom left: water-selective side-view MRI (the dark rectangle is the cross-section of the cover slip); bottom right: biofilm-selective MRI (40 μm thick side-view plane from 3D-MRI measurement)

Figure 3 shows the depth-resolved metabolite map (Figure 3 right) and a corresponding biofilm-selective 3D-MRI plane (left; stretched vertically so that the depth-resolved axis matches that for the metabolite map) for a CDFF biofilm after a week of growth and aseptically transferred to the NMR chamber followed by perfusion with MM1 for 2 days. The vertical direction of the metabolite map corresponds with spatial depth, and the horizontal direction is the spectral (chemical resolution) dimension. A horizontal trace through the metabolite map (bottom right) shows the chemical spectrum for the depth corresponding to the approximate continuous media–biofilm interface (dotted line). [The signal appearing at the height of the cover slip (the dark zone above the biofilm in Figure 3 left) is for water at the periphery of the cover slip that is shifted and broadened by magnetic susceptibility-induced magnetic field distortions in that location, causing it to evade water suppression. A method for improved localization at the cover slip center is under development to eliminate this artifact.] This method takes advantage of the planar sample support geometry to enhance depth resolution by sacrificing in-plane resolution, i.e. the metabolite measurements are averaged over several square millimetres of cover slip surface.

Initial results (Figure 3) indicate that MR-1 is utilizing fumarate as an electron acceptor and lactate as a carbon and energy source producing succinate and acetate, respectively. These metabolites are spatially separated and indicate that the reduction of

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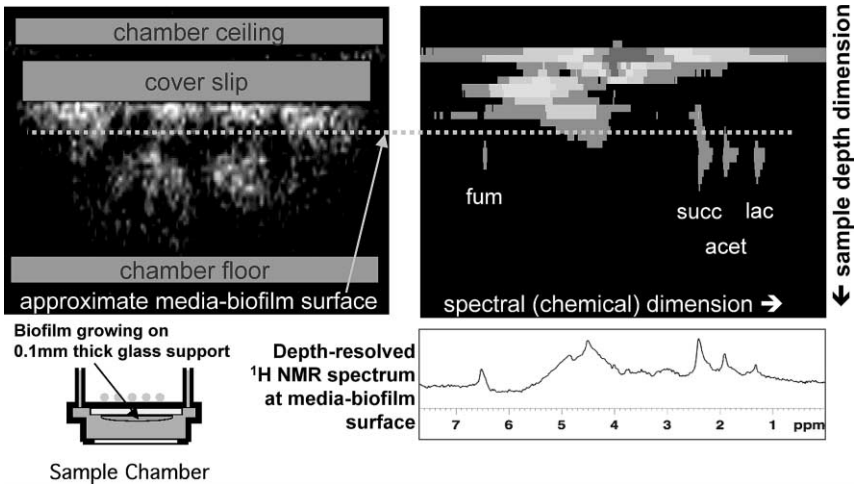

Figure 3 Depth-resolved metabolite measurements for a *Shewanella* biofilm in anaerobic growth media. Top left: biofilm-selective MRI with rectangular overlays showing the location of the sample chamber ceiling, floor and cover slip location, and a dotted line showing the approximate continuous media–biofilm interface. The cover slip overlay dimensions are approximately 0.1 mm tall and 5 mm wide. Top right: depth-resolved metabolite map showing the depth-dependent fumarate (electron acceptor), lactate (substrate) and succinate and acetate (byproducts) concentrations. Bottom right: horizontal trace through the metabolite map showing the NMR spectrum obtained at the approximate continuous media–biofilm interface

fumarate to succinate occurs throughout the depth of the biofilm to the glass coverslip substrate, in comparison to acetate, which shows a more shallow profile. These novel preliminary data will be used for detailed modeling of the system to fully interpret the complex spatial patterns that develop within a biofilm.

Ex-situ measurements of CDFF biofilms provided markedly improved results as compared with previous in-situ biofilm grown experiments (Majors *et al.*, 2005). The CDFF biofilms were invariably more firmly attached to the coverslip and more uniformly distributed, which is important since the depth-resolved metabolite measurements are averaged over several square millimetres of cover slip surface. Insertion of the ex-situ biofilm into the sample chamber immediately before analysis avoids upstream migration/growth that reduces environment control. Further, it avoids biofilm growth at the inner surface of the optical window of the sample cup (Figure 1) that can occlude optical measurements. Finally, sample throughput is significantly enhanced by growing multiple biofilm samples offline.

Conclusions

These initial experiments demonstrate the utility of NMR methods for the repeated, non-invasive spatial and temporal metabolic analysis of live biofilms. NMR measurements provide temporally and spatially resolved metabolite (substrate, electron acceptor and byproduct) concentrations. The resulting depth-resolved NMR-derived velocities, diffusion rates, metabolite concentrations and biomass distributions will be integrated using a biofilm process model. Initial efforts will involve the integration of experimental data to derive transport and reaction rates. Hardware and procedural improvements are planned to enhance NMR sensitivity (thus lowering the detectable metabolite concentration threshold), to improve growth environment control, to relate biofilm metabolite spectra to bulk free cell measurements obtained under controlled growth and to correlate NMR with confocal optical microscopy measurements. Thus, NMR of biofilms is expected to contribute significantly to the understanding of adherent cell metabolism.

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