Imaging biofilm architecture within porous media using synchrotron-based X-ray computed microtomography

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[1] A new method to resolve biofilms in three dimensions in porous media using high-resolution synchrotron-based X-ray computed microtomography (CMT) has been developed. Imaging biofilms in porous media without disturbing the natural spatial arrangement of the porous medium and associated biofilm has been a challenging task, primarily because porous media generally preclude conventional imaging via optical microscopy; X-ray tomography offers a potential alternative. Using silver-coated microspheres for contrast, we were able to differentiate between the biomass and fluid-filled pore spaces. The method was validated using a two-dimensional micromodel flow cell where both light microscopy and CMT imaging were used to image the biofilm.

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1. Introduction

[2] Biofilms are observed in both natural and engineered systems and are believed to be the primary habitat for most microorganisms [*Costerton et al.*, 1995]. In porous media, biofilm growth and development occur over a continuum of scales ranging from nanometers to millimeters or more and have been a topic of interest in applications and industries, including mining, filtration, water and wastewater treatment, and bioremediation [e.g., *Fitch et al.*, 1998; *Rawlings and Johnson*, 2007; *Rodriquez and Bishop*, 2008]. Because of the prevalence of biofilm in both natural and engineered porous media systems, a significant effort has been undertaken to understand biofilm growth and development in porous media, yet much of the information about the three-dimensional architecture of biofilms in porous media is based on the results of mathematical models rather than on direct experimental data. In order to verify and validate existing and new models, experimental data quantitatively detailing the structural arrangement and distribution of biofilm in porous media are required.

[3] Visualization of the biological phase in porous media provides a useful means for increasing our understanding of microbial-soil structure interactions and for developing quantitative experimental data sets for mathematical model validation. Conventional techniques for imaging biofilm include light microscopy [e.g., *Paulsen et al.*, 1997; *Sharp et al.*, 2005; *Yang et al.*, 2000], environmental scanning electron microscopy (ESEM) [e.g., *Davis et al.*, 2009], and confocal laser scanning microscopy (CLSM) [e.g., *Leis et al.*, 2005; *Rodriguez and Bishop*, 2007], all of which are useful for examining biofilm on surfaces or in two-dimensional or quasi-two-dimensional porous systems. While the aforementioned imaging techniques are capable of providing significant insight regarding microbial interaction and biofilm structural formation, imaging porous-media-associated biofilm using these techniques requires that model porous media systems be constrained to a few particle diameters, that the porous medium and fluid be index matched, or that samples be extracted and prepared, thereby disrupting the pore-scale structure. Thus, new techniques that allow for direct visualization of biofilm in situ are required in order to characterize biofilm growth, surface architecture, and three-dimensional spatial distribution within porous media to provide relevant experimental data for the verification and validation of porous-media-associated biofilm growth models. One such technique utilized by a number of groups to measure the three-dimensional features of biofilms is magnetic resonance microscopy (MRM) [*Manz et al.*, 2003; *Seymour et al.*, 2004, 2007]. In terms of gross structural characterization, these techniques generally have been successful. However, the method thus far has been limited in resolution (50–100 μm), and the acquisition time is very long, which could severely limit the method for some applications.

[4] A new methodology using synchrotron-based X-ray computed microtomography (CMT) to render high-resolution measurements of the spatial distribution of biofilms in porous media is presented in this work. This methodology has the advantages that (1) a variety of solid substrates can be used and (2) the method is nondestructive to the porous medium, allowing for three-dimensional in situ visualization of biofilm. CMT has been available for more than three decades and has been a powerful tool for studying a wide array of processes in porous media systems [e.g., *Nunan et al.*, 2006; *Werth et al.*, 2010; *Wildenschild et al.*, 2002]. However, synchrotron-based tomographic imaging of biofilms has yet to be accomplished, primarily

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because obtaining X-ray contrast between the biomass and water has posed a significant challenge because conventional contrast agents, such as potassium iodide, diffuse readily into biofilm when present in the fluid phase. To address the contrast issue, we used a particle-based fluid phase contrast agent that provides X-ray absorption contrast between the fluid and biofilm phases by adsorbing to the surface of the biofilm and thereby delineating the biofilm–aqueous phase interface. To our knowledge, we report the first use of synchrotron-based CMT to image biofilm within porous media as well as method validation using a separate imaging technique.

2. Methods

[5] Two-dimensional micromodel flow cells were constructed from polydimethyl siloxane (PDMS). The inner dimensions of the flow chamber were 9.8 × 30.0 × 3.4 mm, with 1 mm diameter cylindrical columns spaced 1 mm apart (see auxiliary material Figure S1 for details).[1] *Shewanella oneidensis* MR-1 was used in all experiments. *S. oneidensis* MR-1 is a gram-negative, facultatively anaerobic, polarly flagellated bacteria capable of dissimilatory metal reduction and biofilm formation [*Majors et al.*, 2005; *Venkateswaran et al.,* 1999]. Cultures were grown for 24 h in a 10% tryptic soy broth (TSB) solution, concentrated at 6000 rpm for 10 min, resuspended in 5 mL of 10% TSB followed by inoculation of the micromodel flow cell. The injected culture sat stagnant for 24 h, allowing for microbial attachment to the surfaces, followed by flow of 10% TSB solution at 0.01 mL/min for 12 days. Neutrally buoyant, hollow silver-coated microspheres with an average diameter of 10 μm (particle range of 5–15 μm based on Coulter counter measurements, Microsphere Technology Ltd, Pentlands Science Park, Edinburgh, United Kingdom) were deposited at the biofilm surface by flowing a concentrated solution (approximately 4 mg microspheres/mL) through the micromodel, followed by subsequent rinsing with 10% TSB to remove spheres not attached to the biomass. Imaging commenced following the micromodel rinse in order to limit the potential for biofilm rearrangement due to the antimicrobial nature of the silver microspheres.

[6] A Leica Z16 APO light microscope was used to acquire light microscopy images of the micromodel at a resolution of 1.4 μm/pixel. Regions of the micromodel containing unique, identifiable, biofilm features were observed using digital microscopy; the locations of the biofilm features were measured with a stage micrometer using the micromodel sidewall as a datum. Biofilm features were mapped in all three principal dimensions to enable alignment with the CMT data. The entire micromodel flow cell was imaged using CMT, and regions of interest were located in the reconstructed volume using the microscope coordinate measurements (see Figure S1).

[7] Microtomographic imaging was performed at Beamline 8.3.2 at the Advanced Light Source (ALS), Lawrence Berkeley National Laboratory. Preliminary experiments were performed at Beamline 13-BMD, GeoSoilEnviroCARS (GSECARS) at the Advanced Photon Source (APS), Argonne National Laboratory. The data collected at the ALS and APS were acquired at resolutions of 4.5 and 11.8 μm/pixel, respectively. An energy level slightly above the *K* shell absorption edge for silver (25.5 keV) was used for imaging to optimize X-ray absorption and thus contrast.

[8] Image reconstruction, postprocessing, and segmentation were done with scripts written in Interactive Data Language (IDL®) and using the commercially available visualization software Avizo®. The gray scale light microscopy images were coarsened to a 4.5 μm/pixel resolution corresponding to the resolution of the CMT data. Raw CMT data were preprocessed by integrating (or collapsing) volume data corresponding to the light microscopy image region of interest into a single slice for direct comparison of representative images. All images were then processed using a standard Sobel edge detection algorithm followed by segmentation using a simple histogram threshold. For additional information on the Sobel edge detection algorithm, we refer to the IDL software documentation as well as to *Duda and Hart* [1973]. The PointWrap surface-generating algorithm in Avizo® was then applied to the resulting binary images to generate surfaces for quantification of the spatial distribution of biofilm. The PointWrap algorithm generates a surface reconstruction from a cloud of unorganized points by simulating the path of a sphere through a cloud of points or along a surface. A triangulated mesh is generated by assuming that a surface exists every time a set number of points are in contact with the simulated sphere body simultaneously. This algorithm allows for the association and interconnection of a cloud of points, which correspond to the individual silver-coated microspheres adhering to the surface of the biofilm. For additional information on the PointWrap algorithm, we refer to the Avizo software documentation as well as to *Sander and Runge* [2000]. The PointWrap analysis was also verified using a standard marching cube surface-generating algorithm. Differences in surface area generated using the two methods was less than 1%.

[9] Volume rendering of the CMT data for three-dimensional evaluation of the biofilm was also done using the Avizo software package. The volume files were segmented to differentiate between the solid (PDMS) and silver particles. A Sobel edge detection filter was applied in order to segment the silver particles. Volumetric quantitative analysis using both the PDMS (solid phase) as well as the silver microsphere (biofilm) surfaces as indices was performed using the triangulated mesh created using the PointWrap algorithm in Avizo.

3. Results

[10] Preliminary experimentation using the new imaging approach was conducted at the APS and provided compelling evidence that our choice of contrast agent and CMT imaging method produces realistic three-dimensional representations of biofilm present in the experimental packed bead column system. A visualization of the preliminary results is provided in Figure 1 as well as in Animation 1.[2] Figure 1 indicates that biofilm (green) forms both on the

[1] Auxiliary materials are available in the HTML. doi:10.1029/2010WR009410.

[2] Animation 1 is available in the HTML.

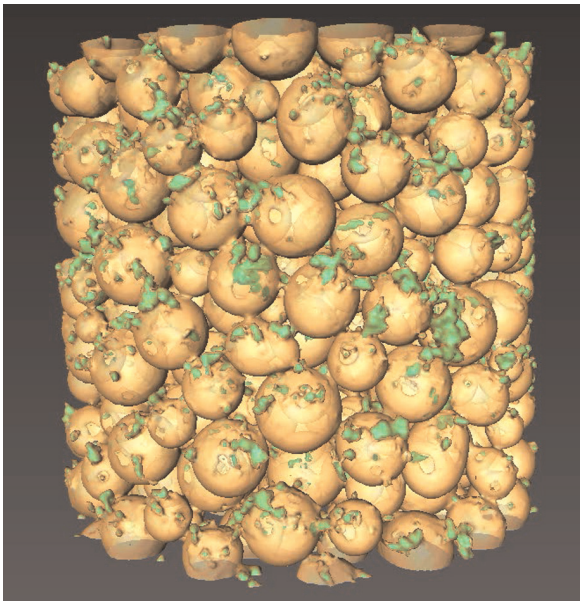

Figure 1. Preliminary three-dimensional X-ray computed microtomography (CMT) biofilm imaging results of biofilm (green) grown in a glass bead pack (gold). The visualization experiment was performed at the Advanced Photon Source (APS), Argonne National Laboratory, using neutrally buoyant, silver-coated hollow glass microspheres (10 μm diameter) as an X-ray contrast agent. The spatial arrangement of the silver particles is interpreted as being attached to the biomass grown within the bead pack.

surfaces of the glass beads within the column (gold) as well as within the column pore space bridging multiple beads together. In order to validate our preliminary findings, we developed the previously mentioned system in which the distribution of biofilm could be visualized using both digital microscopy and CMT. This validation of the CMT imaging technique for visualizing biofilm in situ in porous media is the focus of this work.

[11] In the two-dimensional micromodel used for validation, two distinct regions of the micromodel flow cell containing *S. oneidensis* biofilm were selected for analysis (features A and B). Visualizations of the light microscopy and composite CMT images of both features are depicted in Figure 2 along with the processed binary and PointWrap biofilm representations. The flow cell column posts have been superimposed as gray circles in the gray scale images for ease of interpretation. Visual comparison of the light microscopy images to the CMT composite images indicates very good agreement between the imaging methods and suggests that the silver particles detected using CMT do, in fact, allow for the delineation of biofilm within the pore space. The correlation between the two imaging techniques for features A and B was quantified, via surface area, using the PointWrap surface-generating algorithm on the binary images, resulting in a percent error for the CMT images, normalized to the light microscopy images, of between 1% and 5%. Results of the comparison are detailed in Table 1.

[12] Three-dimensional renderings that illustrate the spatial distribution of biofilm within the porous medium for features A and B are provided in the auxiliary material (Figure S2). Through the use of the PointWrap algorithm, the volume change and change in macroporosity associated with biofilm growth were quantified for both features. The volume of biofilm associated with features A and B were calculated to be 1.67 and 2.44 mm^3, respectively, which corresponds to decreases in macroporosity from 63% to 48% for feature A and from 68% to 50% for feature B. This three-dimensional analysis is included to illustrate the potential for quantification that the technique allows for.

4. Discussion

[13] Validation of CMT imaging of biofilm in porous media using silver-coated glass microspheres as a contrast agent to outline a biofilm surface was successful. The microspheres adhere to the *S. oneidensis* biofilm aqueous phase interface, providing the necessary contrast needed for CMT imaging. Light microscopy images showed good correlation with representative CMT images, and biofilm volume renderings could be created from a cloud of points generated by the distribution of silver particles across the biofilm interface with the aqueous phase. Currently, the technique facilitates three-dimensional imaging of biofilm within porous media and accurately represents the solid-biofilm-aqueous phase spatial arrangement. This method is particularly suited for pore-scale investigations where a triangulated mesh can be generated, which provides a convenient platform for additional analysis (for example, fluid and solute transport within the biofilm via a finite element or finite volume numerical simulator). The proposed CMT-based method can image biofilm within porous media with high fidelity (see Animation 1) and also allows measurement in situ to a biologically relevant environment. Second, CMT can image at the micron scale and still provide spatial information at the centimeter scale. The beam line setup (beam width) ultimately determines the maximum sample diameter that can be imaged. The maximum specimen diameter at the beam lines utilized in this manuscript is approximately 4–5 cm. However, the image resolution is dependent on the magnification of the lens attached to the detector, which decreases the applicable sample diameter. In general, the resulting image resolution is 1/1000 of the specimen diameter. The sample length (axial length) is effectively limited by the acceptable data acquisition time for the collection of multiple scans at different heights. Thus, CMT can provide biofilm spatial distribution over many centimeters in the sample axial direction and resolve interfacial architecture at the micron resolution. A key benefit to using CMT to image biofilm in porous media is that no limitation to direct surface access or visualization depth exists, as in the case of CLSM. Additionally, refractive index matching of the porous medium is not required since attenuation and optical transparency are not significant issues associated with the presented method. As a result, biofilms within their natural environments (e.g., soil and rock) can potentially be imaged in situ.

[14] The key limitations to the method include the following: (1) X-ray exposure associated with CMT imaging is expected to kill or severely retard microbial growth; (2) silver is a biocide and, for this reason, must be added to sample specimens immediately prior to imaging; (3) the ability to visualize biofilm is dependent on the extent of

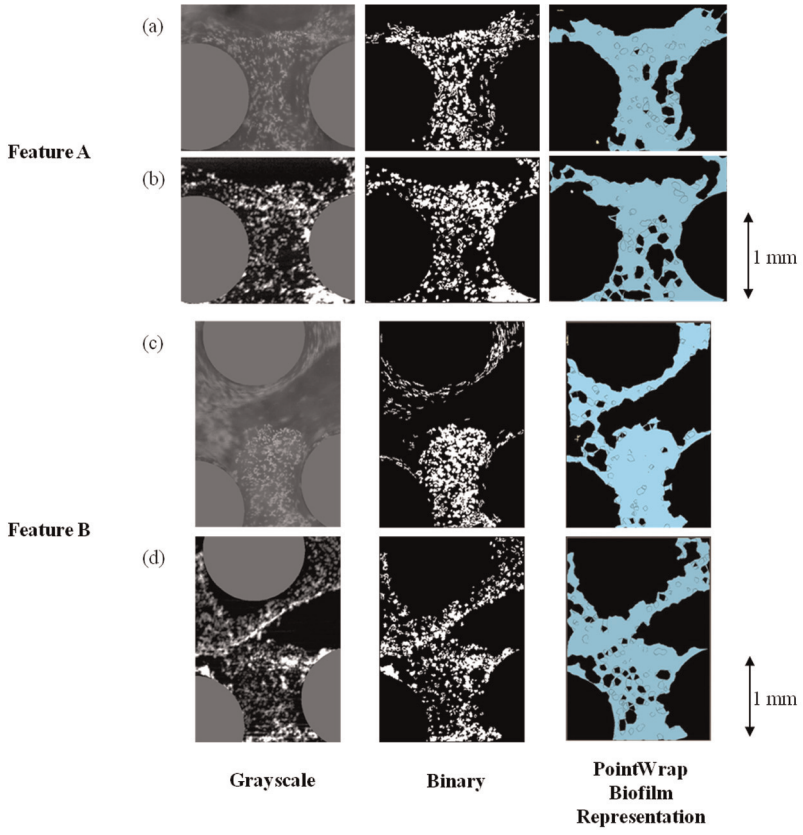

Figure 2. Comparison of light microscopy images to representative CMT images for two unique biofilm features at the gray scale, binary, and PointWrap stages of image processing. Dark areas correspond to fluid, and light gray areas show silver particles attached to biofilm. The light blue regions in the PointWrap images correspond to the representative region containing biofilm. (a) Light microscopy images of feature A, (b) CMT images of feature A, (c) light microscopy images of feature B, and (d) CMT images of feature B.

silver coverage on the biofilm surface; (4) the presented method, at this point, is limited in utility to imaging changes to the macropore structure of the porous medium. As a result, the internal porosity of the biofilm is not imaged using this method. The effect of X-ray exposure, as well as the introduction of silver microspheres, is expected to have a negative impact on biofilm growth. As a result, the imaging of biofilm using the presented method should be considered the terminal step for particular specimens. The quality of coverage by silver particles on the surface of the biofilm is key to successful use of the presented method. Since the silver addition occurs immediately prior to imaging, surface attachment will be limited to biofilm surfaces exposed to flow channels through the micromodel or porous medium column. Dead-end pores containing biofilm have the potential to be interpreted as fluid space if silver particles do not come into contact with the biofilm. Regions that are occluded from flow also have the potential to be attributed to biofilm if the region is surrounded by silver-coated biofilm. Since the method relies on the deposition of silver-coated microspheres on the biofilm surface, it is limited to providing insight into changes in macropore morphology external to the biofilm. However, the ability to acquire quantitative experimental data detailing the changes in macropore morphology can potentially provide insight into biofilm-associated changes in porous media hydrodynamics as well as solute transport.

[15] To our knowledge, this is the first successful attempt using high-resolution CMT to image three-dimensional biofilms in situ within intact porous media. Both spatial distribution of biofilm and change in porosity are important parameters for investigating the impact of biofilm on porous media hydrodynamics and on the mass transport and reaction processes that occur during bioremediation. Admittedly, we present one working approach for applying a contrast agent to biofilm for imaging using CMT. The

Table 1. Quantitative Comparison of the Light Microscopy and Representative Two-Dimensional CMT Images for Two Unique Biofilm Features Using the Avizo PointWrap Algorithm

	Surface Area (mm^2)	Absolute Error Normalized to Light Microscopy Image (%)
Feature A light microscopy	1.595	-
Feature A computed microtomography	1.529	4.13
Feature B light microscopy	1.998	-
Feature B computed microtomography	2.034	1.82

method is applicable using microspheres coated with a variety of elements. However, additional research into the efficacy of other contrast agents, as well as the effect of varying particle size and density, is ongoing.

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