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**In situ** molecular imaging of hydrated biofilm in a microfluidic reactor by ToF-SIMS

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One Sentence Significance

A novel microfluidic reactor for biofilm growth was constructed to enable *in situ* chemical imaging of hydrated biofilms using ToF-SIMS.
COMMUNICATION

In situ molecular imaging of hydrated biofilm in a microfluidic reactor by ToF-SIMS


A novel microfluidic reactor for biofilm growth and in situ characterization using time-of-flight secondary ion mass spectrometry (ToF-SIMS) was constructed to enable two-dimensional chemical imaging of hydrated biofilms. We demonstrate the detection of characteristic fatty acid fragments from microfluidic reactor-grown biofilms and illustrate advantages of hydrated-state ToF-SIMS imaging.

This paper reports a unique approach of molecular imaging of biofilms in their native environments using time-of-flight secondary ion mass spectrometry (ToF-SIMS) to address potentially the grand challenge of complex interfacial dynamics in biogeochemistry. Biofilm is grown on a silicon nitride (SiN) membrane window in a recently developed microfluidic flow cell as a single channel flow reactor. Continuous imaging of complex liquid samples can be performed with high precision and sensitivity using this technique. Direct probing of the biofilm occurs in situ within a windowless detection area of 2 µm in diameter as soon as the hole is drilled through by the SIMS primary ion beam. One of the most important processes in nature involves bacteria forming surface attached microbial communities or biofilms. Biofilms possess a complex structure made of a highly-hydrated milieu containing bacterial cells and self-generated extracellular polymeric substances (EPS).

Flow cells have been developed to make microscopic observations of biofilm growth possible. Stain-based approaches, such as confocal laser scanning microscopy (CLSM), have been used to monitor hydrated biofilm development and physiology. Previous work has shown that biofilms develop metabolic and chemical heterogeneities that affect overall biological activities as biofilms respond to environmental microenvironments at the individual cell level. However, prerequisite chemical modifications required for vacuum based imaging of biofilm microenvironments, such as the removal of water, can cause drastic changes to EPS integrity and biofilm morphology. In situ molecular imaging tools are necessary in understanding how the spatial heterogeneity and structural difference affect the microbial community activities in an unperturbed, hydrated state.

ToF-SIMS is a unique surface analytical tool that provides molecular information (e.g., mass spectra) and image mapping with a lateral resolution of ~0.2 µm. It has been used increasingly in characterizing biological and soft materials. However, most known applications are limited to solid samples because of the challenge to detect liquids with high volatility in a vacuum chamber. We have recently developed a portable microfluidic interface to address this challenge and direct imaging of liquid surfaces using ToF-SIMS is now possible. This capability was demonstrated in the high vacuum mode in scanning electron microscopy (SEM) and comparison between this approach and environmental SEM (ESEM) was discussed elsewhere. Previous design consideration and validation demonstrated that temperature drop across the aperture is only a few K, thus freezing is not an issue. Beam damage is overcome by either renewing the detection surface and passing liquid continuously through the aperture via an electroosmotic pump or using the diffusivity of liquid itself within the microfluidic channel aggravated by evaporation at the aperture in vacuum. This liquid ToF-SIMS application enables direct probing of the anisotropic and heterogeneous interfacial region that is relevant to biofilm attachment and adhesion to surfaces.
We report here the first in situ hydrated, molecular imaging results of a biofilm grown in a single channel microfluidic flow reactor using ToF-SIMS. Comparisons of hydrated biofilms grown in the microfluidic reactor were directly made with dehydrated biofilm samples and an uninoculated liquid medium solution. ToF-SIMS m/z spectra and 2D images revealed differences in chemical compositions and spatial distributions. Furthermore, principal component analysis (PCA) results show clear distinctions among these samples. This approach provides a novel pathway to study in situ biofilm physiology at the molecular level and understand complex environmental processes as biofilms interact with surfaces across multiple domains (e.g., from molecular to mesoscale).

Detailed device fabrication and interface assembly were described in our earlier papers. Soft photolithography was used to make the microfluidic channel on a polydimethylsiloxane (PDMS) block. To reduce the possibility of clogging during the biofilm growth, the channel dimensions were chosen to be 100 µm wide by 500 µm deep. Two holes were punched at the two ends of the microchannel to form inlet and outlet for bacteria inoculation and delivery of growth medium. See the ESI† for more details.

ToF-SIMS imaging and detection was conducted using a ToF-SIMS V spectrometer (IONTOF GmbH, Münster, Germany). Detailed SIMS conditions were given in the ESI†. Principal component analysis (PCA) was achieved using PLS_Toolbox 6.7.1 (Eigenvector Research, Inc., Manson, WA, USA) in Matlab 2012(a) (MathWorks Inc., Natick, MA, USA). Data were preprocessed by autoscaling prior to PCA analysis.

The experimental setup for biofilm growth was depicted in Fig. 1. The microfluidic reactor was connected to a custom-made manifold that allows fresh medium delivery. To harvest the microfluidic reactor for ToF-SIMS imaging, the reactor was stopped and immediately sealed prior to attaching to the ToF-SIMS stage for analysis within 1 hr. of harvesting.

The microfluidic device was placed on a small platform and the PTFE tubing portion was taped to the platform to prevent it from moving around. A transparent plastic cover was used to keep dust off. A needle was inserted into the inlet tubing and this assembly was connected to a custom-made manifold that allows fresh medium delivery. To harvest the microfluidic reactor for ToF-SIMS imaging, the reactor was stopped and immediately sealed prior to attaching to the ToF-SIMS stage for analysis within 1 hr. of harvesting.

Our previous studies have demonstrated the feasibility of a microfluidic reactor as an analytical tool for SIMS analysis of aqueous samples. Here, we showed that our microfluidic reactor was robust enough to support SIMS drilling at multiple points across the channel dimensions without compromising the integrity of SiN membrane and underlying aqueous samples under vacuum. This facilitated obtaining biological replicates within a sample and provided the possibility to study biofilm spatial differences across the microfluidic channel.

ToF-SIMS provides molecular recognition for organic or biological molecules. Effort has been made to study biofilms using
ToF-SIMS recently using either dried or cryosection samples. [M-H] peaks (representing quasi molecular ions typically formed from loss of a hydrogen) are commonly observed molecular ion peaks in the negative mode of ToF-SIMS spectra. The ToF-SIMS negative m/z spectrum showing characteristic fragments of dried biofilms was depicted in Fig. 2(a).

2D images of characteristic fatty acids (FA) fragments obtained from the hydrated biofilms grown in a microfluidic reactor were depicted in Fig. 2(b). In the hydrated biofilms grown in the microfluidic in reactor, SIMS revealed several peaks between m/z 200-300 amu. No discernible SIMS peaks were found in the uninoculated medium (Fig. 2(a)), which demonstrated that the peaks observed in the hydrated biofilm samples were specific to biofilm growth. SIMS also revealed characteristic peaks of FA fragments such as m/z 199, 213, 227, 241 and 255 in the dried biofilm samples. These m/z FA fragments were similar to peaks in the hydrated biofilm and partially in accordance with a previous report for dried biofilms was depicted in Fig. 2(a).

Assignments of the dried biofilm peaks were displayed in Table S1†, in which m/z 199 was attributed to C14H27O2 (lauric acid), m/z 213 to C13H26O2 (tridecyl acid), m/z 227 to C14H25O2 (myristic acid), m/z 241 to C13H24O2 (pentadecyl acid) and m/z 255 to C14H23O2 (palmitic acid). Comparison between our work and the LIPID MAPS Structure Database (LMSD, http://www.lipidmaps.org/data/structure) showed slight shifts potentially due to sample uniformity and instrument responses in an experiment (see Table S1†).

The spectra of the hydrated biofilm sample have lower counts mainly due to (1) poor geometry around the aperture area reduces collection efficiency of secondary ions, and (2) the experimental settings might not be fully optimized. An interesting observation is that the spectra of dry film and hydrated film look similar, however, some peaks were shifted (e.g., in Fig 2(a), m/z 197, 209, 225, 257 in hydrated films vs. 199, 213, 227, 255 in dried samples). More than one biofilm samples were analysed and similar phenomena occurred. The spectrum of the uninoculated medium solution showed no characteristics peaks, unlike the hydrated biofilm sample. This confirms that the characteristic peaks indeed came from the hydrated biofilm. Currently, we do not have an explanation for the peak shift between dried and hydrated films. However, this observation suggests that the surface chemical components may change during the biofilm drying, and in situ characterization is necessary.

High spatial resolution is preferable to observe liquid surface over the windowless portion of the flow cell after the SiN membrane was drilled through by the primary ion beam. Secondary ion images with a 10×10 µm² area were obtained from microfluidic reactors containing hydrated biofilms or uninoculated medium using the imaging mode after punching through the SiN membrane (Fig. S3†).

The overlay of Si and PO₃ signals clearly demonstrated that the SiN membrane was drilled through and displayed the shape and size (i.e., ~2 µm in diameter) of the aperture (Fig. S3†).

False-colour SIMS maps of qualitative spatial distributions and concentration gradients for observed peaks are shown in Fig. 2(b). The 2D images show representative biofilm fragments in the liquid surface (m/z 227 and m/z 241) corresponding to C14 and C15 straight chain FA fragments. In both images, a concentration gradient across the hydrated region might be indicative of chemical spatial differences at the sample site. Furthermore, visual comparisons between the concentration gradients for hydrated biofilm and uninoculated medium samples illustrated that the characteristic SIMS fragments of biofilm were distinguishable. This demonstrated the feasibility of our approach for label free, in situ imaging of microbial biofilms.

The ToF-SIMS primary ion beam was used to drill the aperture and simultaneously excite the secondary ion beam of the fragments and consequently acquire real-time composition information of a sample in the depth profiling mode. A depth profile was obtained as shown in Fig. 3a, which provides time- and space-resolved depiction of the SiN substrate, biofilm (resolved from the substrate attached interface towards the fluid interface), and the biofilm suspended in medium solution past the biofilm/fluid interface (see shaded areas corresponding to different sputtering time in the depth profile and ESI†). Fig. 3a illustrated the signal changes of six ion fragments: H-, O-, Si-, Cl-, PO₂⁻ and PO₃⁻. Sharp intensity rises for H- and O- coupled with decrease of Si- at ~52 s indicated the beginning of SiN membrane punch-through. Another sudden increase of PO₂⁻ and PO₃⁻ at ~110 s suggested that the SiN membrane was thoroughly punched through and the liquid surface was being detected. Along the depth profiling temporal series, 52-110 s and 110-150 s are considered to be the transition period, where two small bumps were seen. After ~150 s, signals of H- and O- flattened out and the increase of PO₂⁻ and PO₃⁻ signals slowed down, indicating that a relatively stable and renewable liquid surface formed in the aperture.

Normally, the information depth for static ToF-SIMS is estimated to be a few nm according to reported SIMS emission depth measurements. However, the interface is very dynamic in this approach. As described in Fig. S4†, high current density was used (i.e., ~4×10¹⁶ ions/cm²), and
SiN/biofilm erosion occurred continuously. Also, in a liquid system, ion species of interest may diffuse from the adjacent biofilm/medium solution interface or the bulk to the aperture area (e.g., 10⁻⁹–10⁻⁶ m²). Furthermore, the aqueous solution surface is self-renewable due to evaporation and capillary effect within the microchannel. Therefore, the SIMS spectra shown here contain information not only from the SiN/biofilm interface (a few nm thick), but also from the medium solution (up to a few microns) and the aperture side wall (SiN/biofilm).

PCA was employed to evaluate the intrinsic similarities and discriminations among samples. Integrations of characteristic peaks from ToF-SIMS spectra during 40 s before and after the transition period were exported for PCA analysis, which were defined as “before SiN punch-through” and “biofilm in medium solution” and depicted in Fig. 3a. For the dried biofilm samples, high mass resolution spectra were collected within a scanning area of (100×100 μm²). The composition was homogeneous according to the signal intensity-time relationship shown in Fig. S2†. The spectra between various segments (e.g., 200-240 s) were pretreated by autoscaling instead of mean centring. Mean centring is useful to ensure the first principal component (PC1) describes the direction of maximum variance. It is sensitive to peaks with higher intensity (i.e., H, O) while contributions from peaks with lower intensity (i.e., m/z 227, 241) were almost omitted. Autoscaling results in data having a standard deviation of one; and the analysis is then based on correlations instead of covariances as in mean centring.

Fig. 3(b) shows the PCA score plot that revealed four distinct clusters of SIMS data representing: the SiN membrane, the hydrated biofilm after punching through the SiN membrane, the uninoculated medium solution after the aperture was drilled through, and dried biofilm sample on a Si wafer. These data clusters were compelling evidence that liquid biofilm was indeed observed by ToF-SIMS, showing differences in composition among hydrated biofilm samples, dried biofilm samples, and uninoculated controls. Two principal components (PC) seem to be sufficient, as the sum of PC1 and PC2 represent about 87% of all signals. The similarities observed between the hydrated and dried samples in the PC1 scores indicated that there was a common biological component between these samples. Likewise, the similarities between the hydrated biofilm and uninoculated medium solution in the PC2 scores exemplify the liquid nature of these samples. Clearly, PCA analysis of SIMS data confirmed that hydrated microfluidic reactor samples represent an important advance for in situ imaging of biofilms.

ToF-SIMS imaging results demonstrated molecular recognition for label-free organic and biological molecules enabled via a unique microfluidic reactor. Cluster ion sources have seen rapid development in the past decade resulting in Bi⁺, C60⁺, Ar⁺, and Ar₋, which may provide better detection limits compared to the 25 keV Bi⁺ ion beam used in this study.

We demonstrated that the innovated microfluidic device can be used for controlled biofilm cultivation and as a microreactor for in situ hydrated, molecular imaging by ToF-SIMS. Our microreactor was amiable to monitoring biofilm growth in real-time using CLSM; and ToF-SIMS was subsequently used to obtain images of the same biofilm using different modes. First, depth profiles of the biofilm were used to show drill-through of the SiN membrane and the membrane-attached biofilm. Once the SiN membrane was removed, high spatial resolution m/z spectra of the biofilm were acquired. Characteristic FA fragments of biofilm were observed in hydrated samples and compared with those from dried biofilm samples using PCA. These analyses confirmed the microfluidic reactor as a unique approach for correlated biofilm growth and in situ molecular imaging. Current research is being conducted to study soluble EPS using the approach described here and further ascertain the differences or similarities between EPS and intact hydrated biofilm.

Most importantly, this new technique provides the much sought opportunity to follow, in real-time and space, the hydrated state dynamics of biofilm attachment, growth, and dissociation dynamics with high resolution chemical mapping. This enables new opportunities to study biofilms in its native state without dehydration, cryogenic freezing or staining, which are currently needed for using vacuum-based characterization techniques.

Author contributions
The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes
The authors declare no competing financial interests.

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References

Key words

Hydrated biofilm, ToF-SIMS, molecular imaging, microfluidic reactor