Investigation of biofilm structure, flow patterns and detachment with magnetic resonance imaging

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Abstract Detachment of biomass from biofilms is still a nearly unknown process which has to be investigated in more detail. Magnetic resonance imaging (MRI) is a promising method which supplies information on the structural data of the biofilm, its surface and the hydrodynamic conditions at the bulk/biofilm interface. Both the structural data and the shear stress are key parameters for understanding biofilm detachment. In this paper a fast quantitative MRI technique was used to investigate the detachment from a heterotrophic biofilm which was grown in a tube reactor. The investigated biofilm was cultivated in a test segment (length 12 cm, diameter 7 mm) of a tube reactor at a constant Reynolds number of 3,000 and a substrate load from 6 g glucose/m^2 and day. For the MRI experiments, the test segment with a biofilm of 1,200 μm thickness was connected to the flow loop and placed inside the NMR magnet. During the experiment different hydrodynamic conditions were adjusted for two minutes (Reynolds number Re: 3,000, 4,000, 5,000, 6,000, 7,000, $>$9,000). Flow velocity and relaxation time were then measured at laminar flow conditions. The MR images show very impressively the increasing detachment of biomass from the biofilm surface with increasing Re. After the last step (Re $>$ 9,000) only a thin biofilm of about 200 μm thickness with a very homogeneous surface remained in the test tube.

Keywords Biofilm structure; detachment; flow velocity; magnetic resonance imaging

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Introduction

The confocal laser scanning microscopy (CLSM) has been the main method used for the investigation of biofilm structure and composition in the last decade (Caldwell *et al.*, 1992; Lawrence and Neu, 1999). Beside the analysis of distribution of bacteria or algae in the biofilm new techniques have been developed which allow the determination of volume distribution of EPS–glycoconjugates in biofilms (Staudt *et al.*, 2004). This technique is an important tool for the investigation of biofilm structure and stability under varying hydrodynamic growth conditions.

Nevertheless, in most cases the biofilm has to be removed from its cultivation environment for the CLSM measurement. For the investigation of relationships between biofilm structures, hydrodynamic conditions and biomass detachment a method which allows a quasi-online monitoring would be more suitable.

Lewandowski *et al.* (1993) introduced nuclear magnetic resonance (NMR) to investigate a single biofilm directly in the reactor. NMR is a non-invasive method which has been employed to study water distributions and transport in biological systems (Callaghan, 1991). In biofilms, the water molecules can be classified by their respective mobilities, corresponding to bulk water and bound water in the biofilm. This effect can be exploited to selectively image biofilms using relaxation time weighted NMR imaging (Hoskins *et al.*, 1999). The so-called NMR relaxation times T_1 and T_2 can be used to measure the mobility of molecules (Brownstein and Tarr, 1979). For the investigation of biofilms usually the T_2, which describes the so-called spin–spin interaction, is considered. Manz *et al.* (2003) used magnetic resonance imaging (MRI) to show that the surface

roughness of the biofilms can be determined in one experiment for the complete cross-section of biofilm test tubes under both flow and stagnant conditions. Furthermore, the local shear stress was calculated from the measured velocity profiles. In the biofilm systems investigated the local shear stress at the biofilm surface was up to three times higher compared to the mean wall shear stress calculated on the base of the mean flow velocity (Manz *et al.*, 2003).

Seymour *et al.* (2004) have presented spatially resolved data on the distribution of biomass by using T_2 mapping within a capillary bioreactor. Furthermore, they showed the flow pattern which was influenced by the biofilm structure within the bioreactor.

Regardless of these advantages of MRI, a main disadvantage of MRI has usually been the long measurement time for one image, which can be up to several hours. In a new study Manz (2004) presented a fast method called COMRADE for quantitative imaging of T_2 and displacement (flow and diffusion). The pulse sequence combines multi-PGSE NMR with multi-echo acquisition and compensates for flow effects in the read gradient and diffusion during multi-echo trains. The author showed that separate T_2 and displacement images with microscopic resolution can be obtained within minutes. Furthermore, the capability for 3D flow imaging was demonstrated.

The developed technique allows the investigation of detachment processes in biofilms in an appropriate time. Detachment in biofilm systems was usually investigated by measuring the biofilm thickness before and after detachment experiments (Horn *et al.*, 2003) or by measuring the suspended solids in the effluent of the biofilm reactor (Choi and Morgenroth, 2003). Both techniques represent more or less the average detachment behaviour of the biofilm. Local detachment events where biofilm structure and shear stress interact cannot be identified.

In the paper presented, a fast quantitative MRI technique has been used for the online monitoring of detachment in a heterotrophic biofilm. The method allows the interpretation of local detachment on the microscale.

Materials and methods

The heterotrophic biofilm was cultivated in a tube reactor. The technique has been described several times and is well established for the investigation of hydrodynamic conditions and their influence on biofilm systems (Horn and Hempel, 1997).

Rinsed activated sludge from a waste water treatment plant was used as the inoculum. The biofilm was cultivated at pH 7.0, an oxygen concentration in the bulk flow of 6.0 g/m^3, a Re number of 3,000 and a temperature of 20 °C. Test segments (inner diameter: 7 mm) of the tube reactor were used for the MRI experiments, which were carried out on a Bruker DMX 400 NMR spectrometer (Bruker, Rheinstetten, Germany) with standard Micro2.5 microimaging equipment.

The MRI techniques applied have been described elsewhere (Manz *et al.*, 2003; Manz, 2004). Quantitative T_2 images were calculated on a pixel-by-pixel basis from the signal attenuation of 16 individual images, which were acquired with different echo times T_E ranging from 10 ms to 310 ms. The flow image in Figure 1b was calculated from the signal phase of 16 individual images, which were acquired using a standard flow imaging sequence (Callaghan, 1991). The velocity encoding gradient was applied along the axial direction with strengths ranging from 0 to 0.6 T m^{-1}. The series of flow images shown in Figure 2b was obtained using the COMRADE pulse sequence (Manz, 2004) with eight echoes and a velocity encoding gradient strength of 0.15 T m^{-1} applied along the axial direction. All NMR images were recorded as 1 mm thin slices and with a repetition delay of 2 s.

The test segments with the biofilms were connected to the flow loop and placed inside the vertical bore NMR magnet (9.4 tesla). The velocity profiles were recorded at a

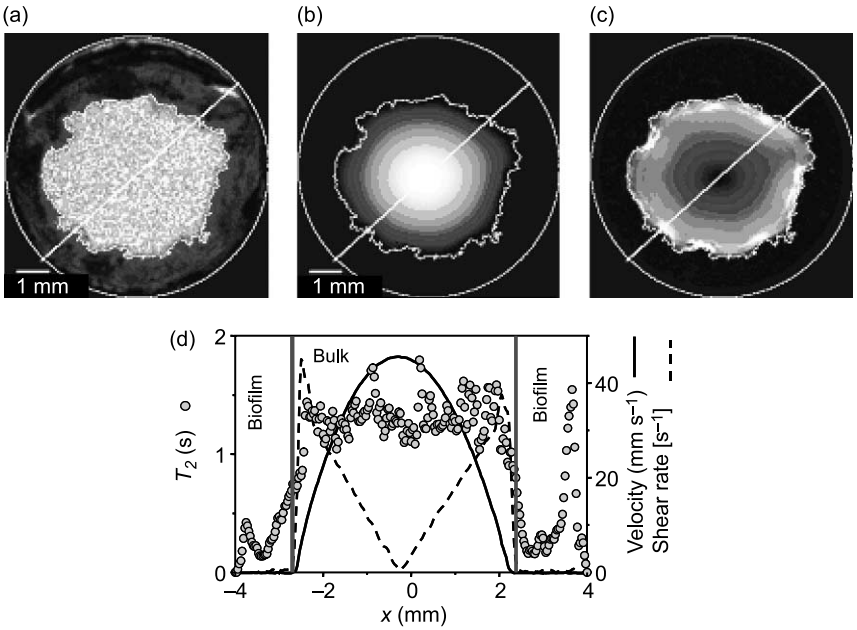

Figure 1 (a) T_2 image (white and grey is the bulk phase and black is the biofilm); (b) velocity image (white are the highest flow velocities); and (c) shear rate (again white are the areas with highest shear rates). The image matrix consists of 256 × 256 points and covers an 8 × 8 mm^2 field of view. (d) The graph shows the values for the cutting plane which is shown in all three images

constant volumetric flow rate at laminar flow conditions using a Pharmacia P-1 peristaltic pump. The recording of flow velocity in the bulk phase is restricted to laminar flow conditions. Nevertheless, the biofilm areas which were exposed to high shear stress can be identified even at low flow velocities.

The detachment experiments were carried out at different flow velocities which are equivalent to the following Reynolds numbers: 3,000, 4,000, 5,000, 6,000, 7,000, >9,000. The flow rate corresponding to these Reynolds numbers was applied for 2 minutes. Then the flow was adjusted to laminar flow conditions to perform the MRI experiment at a flow rate of 0.4 l/h. At each stage a T_2 and a velocity map were recorded. The T_2 maps yield information about the biofilm surface structure, while from the velocity maps the local shear stress, which is a measure for the hydrodynamic forces acting on the biofilm surface, can be calculated.

Results and discussion

Figure 1 displays three typical images from MRI experiments. Figure 1a shows the T_2 map, from which the surface structure of the biofilm can be extracted. Parts of the map with the highest T_2 value can be assigned to the bulk water, as these parts are located in the center of the tube. Because the ion content varies between different samples, the absolute T_2 values of the bulk water are slightly different for each sample.

Usually the components with mean T_2 values of approximately 800 ms or below can be assigned to the biofilm, as they are located near the tube walls, and it has been shown that the transverse relaxation time of bound water in the biofilm is reduced significantly compared to bulk water (Manz *et al.*, 2003; Hoskins *et al.*, 1999). The interface of the bulk/biofilm is indicated by a white contour line. It should be noted that the image in Figure 1a was not generated with the fast method COMRADE for quantitative imaging of T_2 (Manz, 2004). Figure 1b shows the flow velocity profile of the same biofilm and

finally Figure 1c shows the shear rate. The values of T_2, velocity and shear rate along the line drawn in all three images are shown in Figure 1d.

The images of the detachment experiment are shown in Figure 2. All velocity images were recorded with the same flow rate of 0.4 l/h. Due to the detachment between subsequent images a larger cross-section becomes available for the fixed flow. As a consequence, the average fluid flow velocity decreases with increasing void space in the image sequence from left to right. Therefore the scaling of the bars under the images is different.

Table 1 shows the maximum local shear stress τ_{local} which was obtained after each detachment experiment out of the images in Figure 2c. The values were in the same range after the first three steps 3,000, 4,000 and 5,000. Only when the Reynolds number was increased from 5,000 to 6,000 did the maximum local shear stress drop from 0.032 to 0.013 N/m^2. This was the step with the highest decrease in biofilm thickness from 890 to 460 μm.

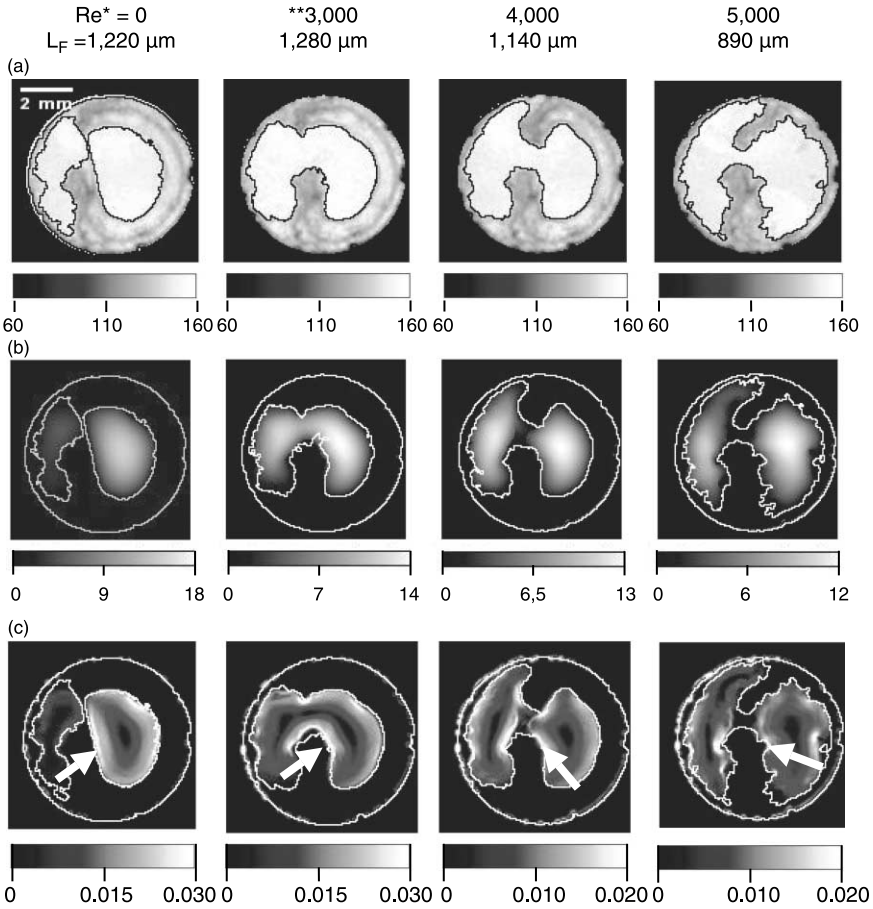

Figure 2 (a) The pictures show the relaxation times T_2 (ms) in the test tube for the investigated biofilm. (b) The flow patterns were measured after the detachment experiments and show the flow velocity profiles in the bulk phase (mm/s). On the basis of these profiles the shear stress at the bulk/biofilm interface has been calculated. (c) These pictures show the shear stress τ_{local} (N/m^2) at the biofilm surface. Lighter areas indicate higher shear stress and possible biofilm detachment. White arrows indicate the region with the maximum local shear stress (see Table 1). *Re = 0 is the original biofilm which was directly taken from the biofilm tube reactor. ** Re = 3,000 was the first detachment experiment, two minutes at the same Reynolds number as during the biofilm growth. All images consist of 128 × 128 data points and cover an 8 × 8 mm^2 field of view

Obviously not only the shear stress, i.e. the external forces, is decisive for the detachment but also the internal forces, i.e. the biofilm strength, contribute to the detachment. Otherwise the parts of the biofilm which were exposed to high shear stress at Re = 4,000 and 5,000 (marked with white arrows) would already have been detached. Similar results were obtained by Telgmann *et al.* (2004). The authors presented results where in one

Table 1 Maximum local shear stress after detachment experiment

Reynolds number	3,000	4,000	5,000	6,000	7,000	>9,000
Maximum local shear stress τ_{local} (N/m^2)	0.033	0.031	0.032	0.013	0.008	0.004

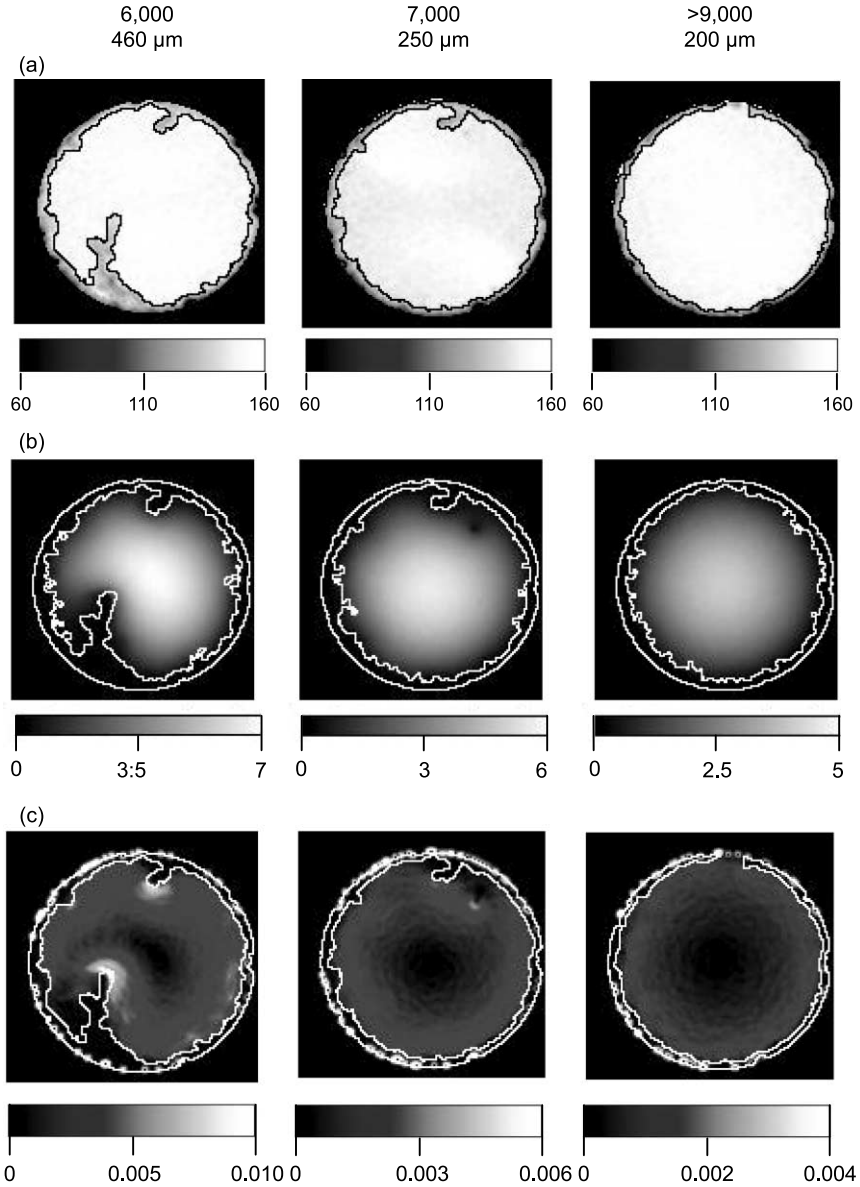

Figure 2 *(continued)*

case the entire biofilm detached from the substratum during the detachment experiments. In other experiments the biofilm detached in pieces. The available experimental results show that the interaction between internal forces of the biofilm and external forces at the biofilm surface is still not well understood. After the last step from 7,000 to >9,000 the maximum local shear stress reached 0.004 N/m^2. This value is very close to the mean shear stress of 0.0038 N/m^2 which was calculated by

$$\tau = (8/Re)\rho_{Water}\bar{w}^2$$

where $\bar{w}$ is the mean flow velocity in the tube. Both values indicate that the maximum local shear stress has been reduced to the mean shear stress by the detachment experiments. The time series of T_2 images, flow velocity and shear stress mapping show very impressively the usefulness of the MRI technique in biofilm research and especially for the investigation of detachment.

Conclusions

The results presented show the relevance of MRI in biofilm research to gain new insight into the structure and detachment properties of biofilms. The whole biofilm system can be investigated non-invasively and without any application of chemicals and/or fluorescent dyes. Local shear stress at the biofilm surface can be calculated from the local flow velocity. Biomass detachment can be monitored quasi-online by the method presented. MRI is an excellent tool to visualize and quantify detachment areas in biofilm systems.

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