BioChip J. (2011) 5(3): 236-241
DOI 10.1007/s13206-011-5307-9

Original Research

Effect of shear stress on the formation of bacterial biofilm in a microfluidic channel

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Received: 3 May 2011 / Accepted: 9 July 2011 / Published online: 20 September 2011

Abstract Biofilms form an irregular network matrix that is surrounded by extracellular polymeric substrate (EPS). The architecture of biofilm plays an important role in protecting bacteria under physical, chemical, and biological stress. The shear stress is one of the major factors to construct stable bioflim. The experimental observation of biofilm formation on large-scale water flow has been limited because most of fluid pipe are water and sewer lines. This study presents the biofilm formation in a PDMS-based microfluidic channel which is able to simulate fluid pipes at small scale. We could characterize the hydrodynamics of the growth of single-species bacteria between biofilm formation and the external environmental factors. Particularly, the dynamics of biofilm formation confirms that biofilm, under the optimum shear stress, efficiently form a stable EPS structure which provides a mechanical shield against high-pressure fluidic flow.

Keywords: Biofilm, Microfluidic channel, Hydrodynamics, Extracellular polymeric substrate, Shear stress

Introduction

Bacteria can adhere to a variety of surfaces including plastics, metal, medical implant materials, and human tissue. Biofilms surrounded by a self developed extracellular polymeric substrate (EPS) provide a mechanical shield[1]. EPS is a polymeric conglomeration composed of extracellular DNA, polysaccharides, glycoproteins, nucleic acids, and lipids. Self developed EPS provides the shield of biofilm with mechanical stability such as strong adhesive strength and viscoelasticity, which makes difficult to remove biofilm from the surface[2]. The EPS has important role in strong protection barrier from predators and physical or chemical shocks under the ecological environment[1-3].

Biofilm can be also grown in water-limited environment such as the surface of medical devices, which result in nosocomical infections[4,5]. *Pseudomonas aeruginosa* is one of the major opportunistic pathogens in nosocomial infection and an excellent model microorganism to study biofilms. In particular, lung infection of *P. aeruginosa* is the leading serious cause of death in patients with cystic fibrosis (CF), which strongly involves the biofilm formation of *P. aeruginosa* in airway[6,7]. Indeed, most CF patients suffer from chronic bacterial airway infections. It is difficult to prevent these infections because of the multi-drug resistant nature of biofilms. This resistance is attributed from delayed penetration of antimicrobial agents through the biofilm matrix, reduced growth rate of biofilm cells, and expression of multidrug efflux pumps[8,9]. The formation of bacterial biofilms can trigger expression of virulence genes[10] and prevents host defense mechanisms by blocking phagocytic pathways of immune cells[11]. *P. aeruginosa* is very ubiquitous microorganism because they can also proliferate in anaerobic or facultative conditions. In a mature of *P. aeruginosa* biofilm, towers or mushroom-shaped three-dimensional structure is observed[11]. The formation of biofilm is affected by various factors such as surface roughness, materi-

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als, temperature, and flow rate[12,13].

Quorum sensing in the formation of biofilms is another important biological event to allow communication between the microorganisms[14]. Because the bacterial population produces a small amount of the inducer leading population responses to activate adaptation systems, biofilm enable to response quickly to environmental change such as temperature, nutrients, antibiotics, or physical stress. In the case of Gram-positive microorganisms, autoinducing peptides (AIPs) and the signal molecules induce bacterial communication. Gram-negative microorganisms use acyl homoserine lactone (AHL) as a major signal molecules[15]. There are several technical problems to identify the cross-talk mechanism between distinct species in biofilm. Recently, biofilm experiments using microfluidic technology is successfully implemented[16]. The conventional bulk pipe system and the effect of industrially relevant materials on the formation of biofilm can be simulated by simple microfluidic system with high-throughput manner. Microfluidic systems provide well-defined cellular environment, which enables to investigate the influence of biochemical molecules and other pathogens on the formation of biofilms[17]. Moreover, small amounts of reagents are used and the reaction time is dramatically shortened while being examine a variety of conditions[18]. In this study, we have investigated biofilm formation and growth under the various shear stress. The information of shear stress could be physical cue to eradicate bacterial biofilms and fundamental dynamic study of bacterial biofilm formation and growth.

Results and Discussion

Fabrication of microfluidic device

The microfluidic device was fabricated using soft-lithography technique (Figure 1). First, the mold masters were fabricated using SU-8 photoresist on a silicon wafer using conventional photolithography. A mixture of PDMS prepolymer and curing agent (10 : 1 ratio) was thoroughly stirred, and then degassed in a vacuum oven. The degassed PDMS mixture was then poured onto the silicon master and cured at 65°C. After curing, the PDMS replica containing the microchannel pattern was lifted off from the silicon master. After cooling the replica, holes were punched to the replica to supplement the reagent lines. The PDMS replica was then exposed to oxygen plasma for 20 s, and then bonded to a glass slide. To fabricate microchip has downward outlet for increasing the UV irradiation time, the glass slide was punched with hand drill before bonding process. The dimensions of the final assembled microfluidic channels were 300 μm × 40 μm (width × height) as main microchannel.

Biofilm formation in microfluidic devices

A mature biofilm with homogenous cells coexists peacefully and interchange genetic information to gain

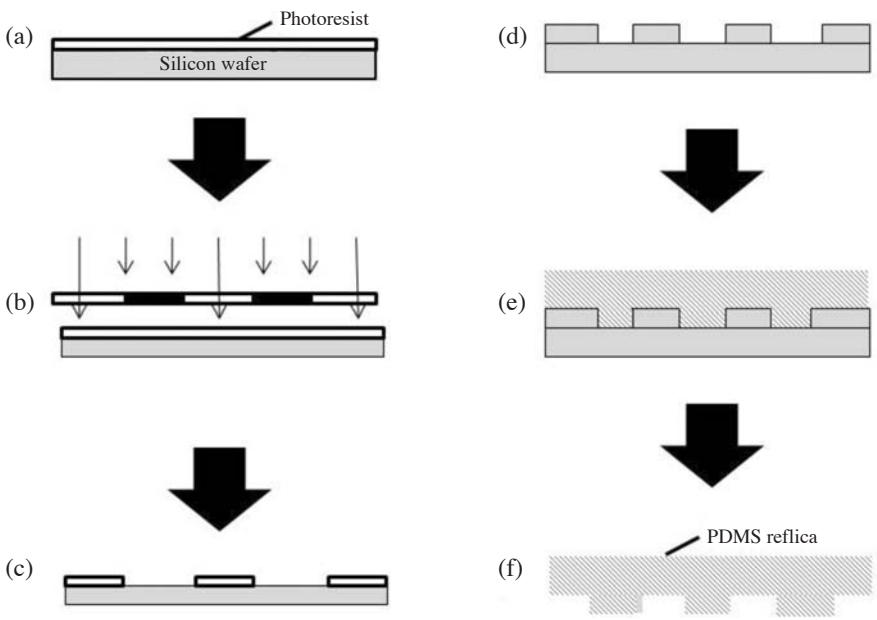

Figure 1. The microfluidic device fabricated by using conventional technique of photolithography (a-c) and soft lithography (d-f). (a) spin-coating of photoresist, (b) photolithography, (c) removing unexposed photoresist, (d) treatment with aquapel, (e) pouring the PDMS prepolymer and curing, (f) peel off the PDMS replica.

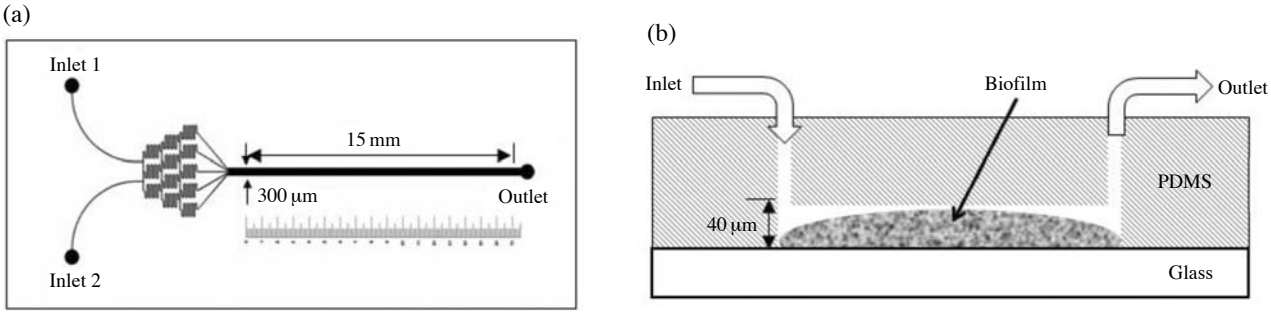

Figure 2. The microfluidic device for the formation of bacterial biofilm. (a) top and (b) side view. The dimension is 300 μm (width) and 40 μm (height), respectively. Bacteria were introduced from outlet to inlet and LB media was introduced from inlet 1 and 2.

new abilities such as strong resistance to antibiotics and chemical stress and enhances its viability under a nutrient-limited condition. Although many researches have been executed for the understanding of mechanism of biofilm development, there is few basic understanding of strategies for the control of biofilm growth or prevention[20,21].

The microfluidic system integrated with living cells is a promising tool because it can control the spatial and temporal condition of cell growth and environmental stimuli *in vitro*. We have applied a microfluidic system to monitor the formation of biofilms under the various shear stress conditions (Figure 2). As a consequence, we have found that the biofilm formation strongly depends on flow rate in microfluidic device. During initial attachment, most of planktonic bacteria contact the glass substratum and have transiently adhered onto it. We have removed nonadhered and weakly bound bacteria from the microfluidic channel using high flow rate (5 μL/mL) of washing buffer[2].

Finally, we find that stable formation of biofilm is developed at 0.1 μL/min flow rate. High flow rate (above 0.1 μL/min) prevents stable formation of biofilm because high shear stress induces easy cell detachment and blocks cell-cell interaction. The biofilm growth of *P. aeruginosa* PAO1 is monitored for 24 hrs (Figure 3). Transitional progress in biofilm development is detected at each stage. Time lapse fluorescence microscopic images clearly visualize growth of *P. aeruginosa* PAO1 and biofilm formation. The mature biofilm is shown as a flat-mat architecture because the fluorescent signal resulted from the biofilm is highly uniform (Figure 3). The bacterial in the biofilm are relatively accumulated in the channel wall compare to those of the central area. The microchannel wall under laminar flow has low velocity than that of center region, which give rise to small change of population. Thus, the channel wall relatively is more advantageous to form stable biofilm. Our results clearly confirms that the development of biofilms is multistage process: i) initial adhesion of bacteria to the surface, ii) formation of bacterial clusters, and iii) progress to mature biofilm structure.

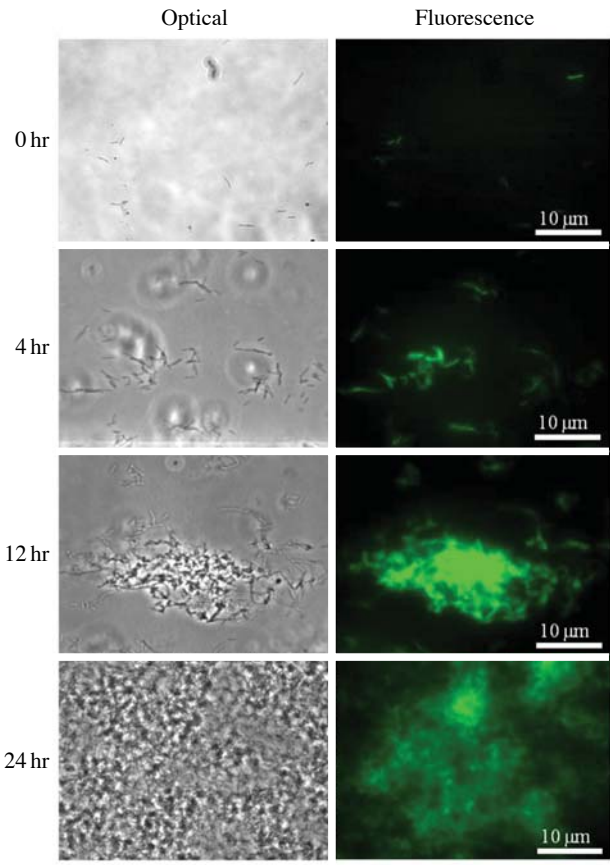

Figure 3. The formation of biofilm in the microfluidic device. Bacteria initially adhered and grew for 4 hrs and bacterial cluster was formed for 12 hrs. Finally, mature biofilm structure is developed and fully covered in a microfluidic channel for 24 hrs. Scale bars indicate 10 μm.

Effect of shear stress on the formation of biofilm

Because flow rate affects the dynamics of biofilm for-

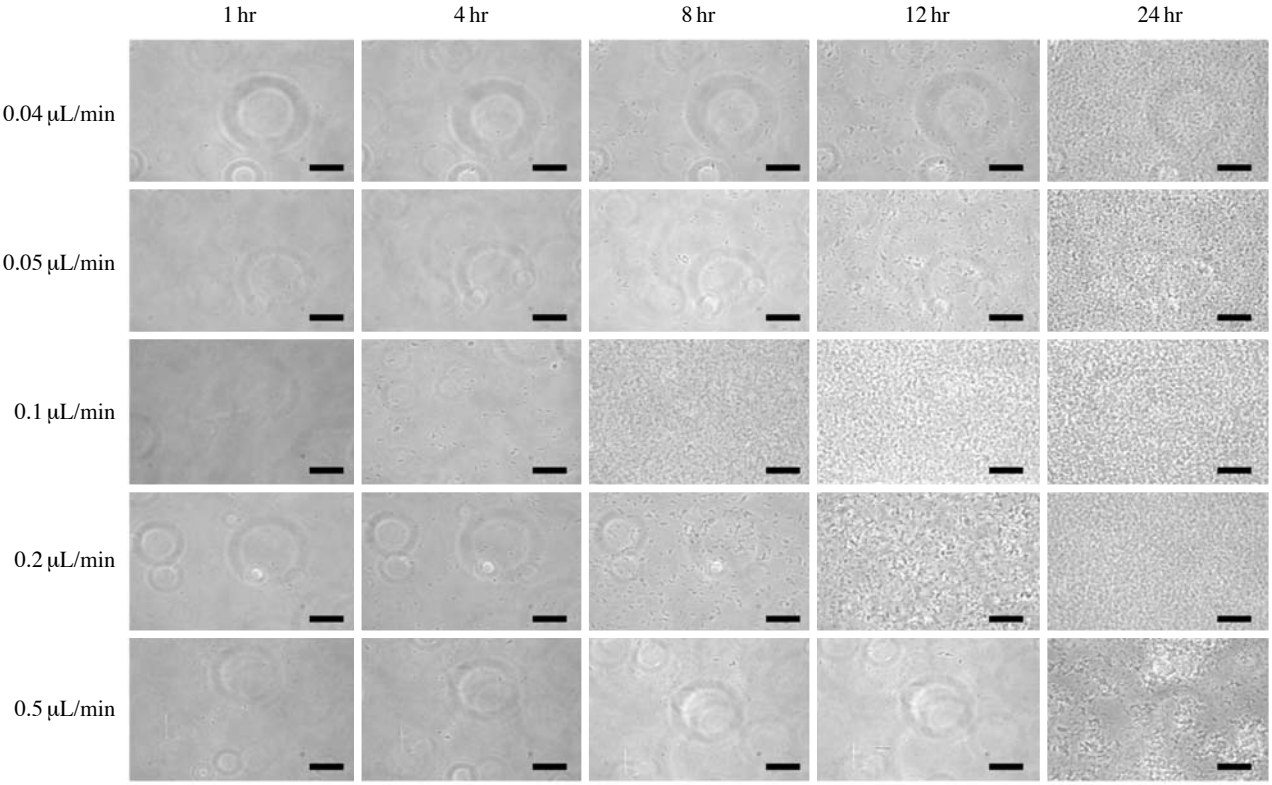

Figure 4. Optical images of biofilm growth in microchannel at each flow rate. Flow rates are 0.04 μL/min, 0.05 μL/min, 0.1 μL/min, 0.2 μL/min and 0.5 μL/min, respectively. Scale bars indicate 10 μm.

mation in a microfluidic channel, the rate of biofilm formation is aberrantly decreased and stable formation of biofilm is prevented. Further, shear stress interrupts cell-to-cell communication between bacteria and opposites the direction of fluid flow in microfluidic channel.

Adhesion of PAO1 strain at various flow rates in microfluidic channels is evaluated and the impact of shear stress reflect the change of morphology of biofilms (Figure 4). Since shear stress affects the dynamics of biofilm formation in a microfluidic channel[19,22], various flow rates (0.04, 0.05, 0.1, 0.2, and 0.5 μL/min), are applied for the evaluation of initial attachment of bacteria. Under the high flow rate (above 0.1 μL/min), some cells are detached from the surface because initial attachment is reversible or relatively weak adhesion. After the removal of floating and weakly bound bacteria, major bacteria are strongly adhering one. The entire surface is completely covered and stably uniformed at 0.1 μL/min. However, the coverage of bacterial biofilm in microchannel is dramatically reduced at low flow rate such as 0.04 and 0.05 μL/min, which implys that low flow rate could not provide relevant environment. In addition, low flow rate induces delivery problem of nutrient and oxygen, which leads to deficiency in both nutrient and oxygen although they are essential

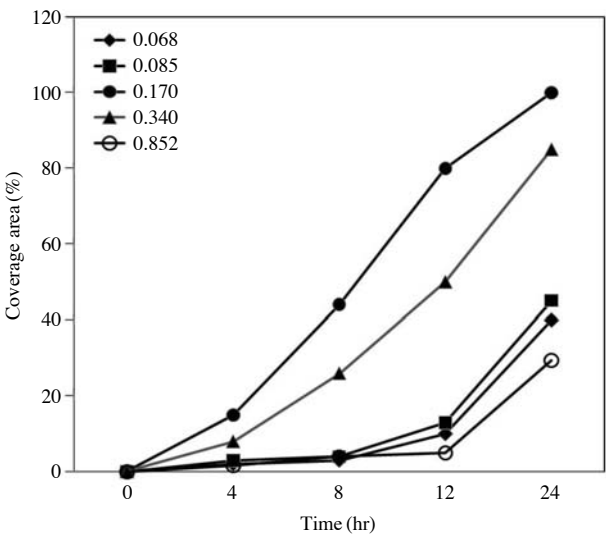

Figure 5. The effect of shear stress on the formation of *Pseudomonas aerugenosa* PAO1 biofilm. The area of biofilm developed in the microfluidic channel is evaluated by coverage ratio at each shear stress for 24 hrs.

to bacterial growth and biofilm formation. The coverage of biofilm at 0.2 μL/min and 0.5 μL/min is about

80% and 30% of entire area, respectively. These results confirms that the high shear stress strongly affect the formation and growth of biofilm.

From these results, the coverage of biofilm area calculated plots against the change of shear stress (Figure 5). We found that optimm shear stress for the formation of biofilm is 0.170 dyn s/cm^2. This result directly proves that biofilm formation is retarded with low and high shear stress. In addition, we can postulate the proper physiological environments is prerequisite for formation of bacterial biofilms and their metabolism. In other hands, the fundamental study of shear stress allow us to obtain the useful information for the prevention of biofilm and removal of adhered bacteria on certain surface.

Conclusions

The current strategy provides new insights into microfluidic systems and enables us to gain multiparametric information. To extend cellular biology approach, we carried out the effect of shear stress on the dynamics of bacterial biofilm formation in a microfluidic channel. This study shows significant differences in change of their morphology and the dynamics of biofilm formation. The attachment and growth of biofilm can be determined by the environmental condition. From our experimental results, the shear stress of 0.170 dyn s/cm^2 is the optimum condition to form stable biofilm. Indeed, the results suggest that relationship between biofilm formation and shear stress is strongly linked and physical stress activate or deactivate their corresponding metabolism and relevant response. The simple approach provides critical information about design of the fluid pipes or sewer lines. It would also be expanded to examine the effects of other environmental factors including pH, oxidative stress, and temperature on biofilm formation.

Materials and Methods

Experimental strains and reagents

The bacterial strain used in this study was *P. aeruginosa* PAO1 wild-type strain. The bacteria were transformed by electroporation with plasmid which stably expresses green fluorescent protein (GFP) at high levels. Single colony of *P. aeruginosa* PA01 was cultured in Luria-Bertani (LB) media at 37°C. After overnight culture, bacteria were centrifuged at 3,000 g for 5 min, and washed for three times with phosphate-buffered saline (PBS) (Sigma, USA). Finally, the bacterial concentration (1×10^9 cells/mL) is adjusted with fresh LB media. All chemicals were obtained from Sigma Chemicals (MO, USA).

Fabrication of microfluidic devices

As shown in Figure 1, the microfluidic channel was fabricated by using the lithography technology. The master was fabricated from SU-8 photoresist (MicroChem, Inc) on a 3-inch silicon wafer (Silicone Sense Corp.). On the SU-8 master, poly(dimethylsiloxane) (PDMS) (Dow Corning Sylgard 184, 10 : 1) was poured onto this master and partially cured in an oven at 60°C for 3 hour. After cooling, PDMS replica and a slide glass were exposed to oxygen plasma (PDC-002, Harrick, USA) for 30 s and boned to each other. The dimension of microfluidic devices is 300 μm (width) × 40 μm (height), respectively (Figure 2).

Influence of hydrodynamics on biofilm formation

To cultivate biofilm, bacteria were introduced into microfluidic channel at a flow rate (0.05 μL/min). During initial attachment, most of planktonic bacteria adhered onto glass substratum. Free-floating bacteria in the microchannel are discarded with high flow rate (5 μL/min). After the removal of floating and weakly bound bacteria, only strongly-attached bacteria remained. The effect of shear stress on biofilm formation is evaluated at various flow rates. Shear stress is calculated by the following equation.

$$\tau = 6\,\mu Q / w h^2$$

The shear stress τ (dyn s/cm^2) is calculated according the above formula[19], where μ is the fluid viscosity of the solution, Q is the volumetric flow rate (cm^3/s), w is the channel width, and h is the channel height. In this experimental, the viscosity of liquid medium is 0.0089 dyn s/cm^2.

Image analysis

Green fluorescence images of biofilm are characterized by the effects of shear stress on local formation of biofilm. The dynamics of biofilm *in situ* can be visualized with a fluorescence microscopy (NIKON TE2000, Japan). The surface coverage of biofilm is calculated using Image pro software.

Acknowledgements This study was supported by the Converging Research Center Program, through the Ministry of Education, Science and Technology of Korea (2010K001055), Polar Academic Program (PAP), KOPRI, and a grant from the Industry Academic Cooperation Foundation funded by the CHA University (CHAIACF-2011-S002).

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