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Microfluidics for Biofilm Studies

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Abstract

Biofilms are multicellular communities held together by a self-produced extracellular matrix and exhibit a set of properties that distinguish them from free-living bacteria. Biofilms are exposed to a variety of mechanical and chemical cues resulting from fluid motion and mass transport. Microfluidics provides the precise control of hydrodynamic and physicochemical microenvironments to study biofilms in general. In this review, we summarize the recent progress made in microfluidics-based biofilm research, including understanding the mechanism of bacterial adhesion and biofilm development, assessment of antifouling and antimicrobial properties, development of advanced in vitro infection models, and advancement in methods to characterize biofilms. Finally, we provide a perspective on the future direction of microfluidics-assisted biofilm research.

INTRODUCTION

General Overview of Biofilm

Microbial life exhibits incredible diversity in terms of habitat and metabolism that have enabled microorganisms to colonize our planet from the deepest ocean floor to the highest mountains. The ability to form communities in structures called biofilms is thought to be the most common lifestyle for many microorganisms such as bacteria, granting them selective advantages over a purely planktonic or free-floating lifestyle (1, 2). Biofilm can form either on solid surfaces or by self-aggregation, and this process takes place in a liquid or humid environment (3).

Biofilm represents a protected mode of growth that allows bacteria to survive in diverse environments and also disperse to colonize new niches. Biofilm formation usually involves the following stages: reversible attachment, irreversible attachment, maturation, and dispersion (4). During the reversible attachment, free-living planktonic bacteria approach the surface and loosely attach to the surface, readily able to detach from it (4). Later, some bacteria will enter the irreversible stage with close contact to the surface, resisting attempts to be physically dislodged (5). Following the irreversible attachment is the maturation I stage, when bacteria multiply and start producing a matrix of extracellular polymeric substances (EPS), forming aggregates. EPS are mostly composed of polysaccharides, extracellular DNA, and proteins (6). Growth of the biofilm proceeds as adherent cells divide and build up EPS around them. Given time, bacteria clusters enter the maturation II stage and can reach a thickness of a few hundred micrometers (7). Finally, biofilm evacuates portions of bacteria clusters and colonizes new surfaces. Furthermore, dispersion can occur when mechanical forces induced by, for example, liquid flow slough off parts of biofilm from a colonized surface.

Living within a biofilm offers bacteria many benefits: On the one hand, a biofilm shields individual cells from environmental stress. The secreted matrix confers mechanical resistance of the structure and prevents bacterial removal by mechanical stress. The matrix also impedes the penetration of toxic chemical into the biofilm. On the other hand, the proximity between members of a biofilm facilitate communication and enable them to share resources (8). Distinct species of microorganisms can form a multispecies biofilm, thus exhibiting a symbiotic relationship (9).

Challenges to Human Health Posed by Biofilm

In the medical context, bacterial biofilms offer many health benefits. For example, the large amount of bacteria living in the human gut forming our microbiome is now recognized as playing a critical role in the health of its host (10). However, biofilms are also frequently encountered in a disease context; 60–80% of all human infections involve biofilm (11). Pathogenic bacteria are able to form biofilm, resulting in health problems such as dental plaque leading to periodontitis, chronic infection of open wounds, or keratitis. Moreover, biofilm can colonize implantable medical devices (12). These medical device–associated biofilms are notoriously difficult to treat and bear high costs (13).

Two of the main factors behind the difficulty in treating medical device–associated biofilms are the enhanced tolerance to antibiotics and resistance to the immune system exhibited by bacteria growing in biofilms compared to their free-floating counterparts (14–16). Studies have shown that biofilm-associated bacteria can tolerate up to 1,000 times more antibiotic concentration than those in planktonic form (17). This is explained in large part by the sheltering effect of the biofilm (18). Furthermore, the microenvironment inside a biofilm characterized by low metabolic activity of the bacteria populating it and shielding it from antibiotic attack has been reported as an ideal breeding ground for bacteria to acquire new antibiotic resistance (19, 20).

Microfluidics

Flow is a ubiquitous phenomenon that enables the movement of nutrients and transportation of unicellular organisms such as bacteria in natural environments and within larger organisms. Inside the human body, flow is present in different compartments, ranging from saliva flow in the mouth cavity to chyme in the gut, blood in the circulatory system, or urine in the urinary tract. These compartments are colonized by bacteria (or at least susceptible to microbial colonization), and the flow characteristics inside these systems are known to influence bacterial life within. Consequently, flow chamber assays were developed to overcome the limitations posed by static assays. The adhesion and growth of bacteria under flow conditions more faithfully mimic their natural microenvironment while allowing some automation in the incubation process (21). More recently, the development of microfluidics enabled even more precise control of environmental conditions and has offered unprecedented advantages.

Microfluidics refers to the set of technologies and applications that deals with handling and controlling liquids on a micrometric scale. Since the second half of the twentieth century, microfluidics has enabled the development of many applications in scientific and technical domains. These range from inkjet printers that deliver microscopic ink droplets through a nozzle on paper during the printing process to miniaturized glucose sensors used by diabetic patients to monitor their blood sugar level. In biological research, microfluidics has benefited the study of biological systems, from cells to molecular biology, by allowing unprecedented temporal and spatial control over the experimental condition, multiplexing, and high throughput (22, 23).

Microfluidics is linked to the rise of the semiconductor and microelectronic industry with fabrication techniques such as photolithography borrowed from semiconductor manufacturing developed in the 1950s. George Whitesides and colleagues at Harvard University made a significant contribution to the field with their pioneering work on soft lithography in 1998 (24). Their widely adopted technique saw the prevalence of microfluidics in research laboratories expand globally, facilitated by the fast and inexpensive production of custom-made polydimethylsiloxane (PDMS) microfluidic chips. Today, several companies specializing in microfluidic component fabrication offer commercial solutions for research laboratories (25, 26).

Microfluidic chips are typically manufactured by soft lithography with PDMS, which allows the reproduction of channels with micrometric features. However, other techniques such as hot embossing and injection molding have been used to produce chips with other materials (27). Active pumping is commonly achieved with syringe pumps, positive pressure or vacuum controller pumps, or peristaltic pumps, among others, with each approach having its own benefits and drawbacks (28). Valves that are sometime directly integrated into a microfluidic chip allow an advanced level of flow control and the automation of complex flow operation of small volumes. For design of the microfluidics, we refer to a recent review by Pérez-Rodríguez et al. (29). **Figure 1** shows two representative designs for studying bacterial adhesion and biofilm development.

The use of transparent materials such as PDMS and glass enables the integration of microfluidic devices with optical methods for readout, such as high-resolution microscopy. Integration of microelectrodes within microfluidic chips has permitted multiplexed detection and analysis through different electrochemical methods (32). The behavior of the fluid within a microfluidic system is mostly laminar, meaning that in the absence of turbulence, mixing occurs almost exclusively via diffusion. Thanks to these flow characteristics, it is possible to establish concentration gradients of molecules of interest that are particularly relevant in cell biology and microbiology to study chemotaxis and toxicology (22).

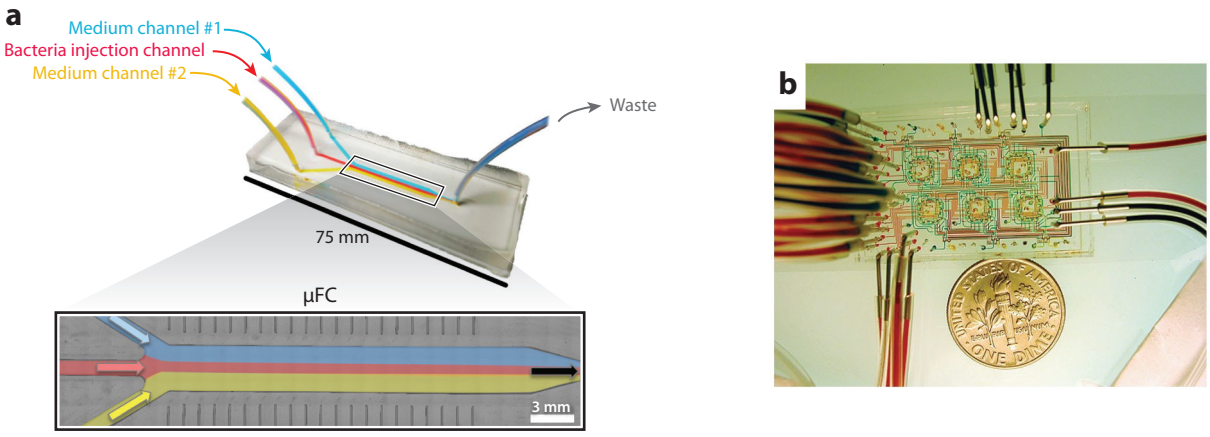


Figure 1

(*a*) Design of the microfluidic platform with a close-up view of the microfluidic chamber. Panel adapted from Reference 30 (CC BY 4.0). (*b*) Six integrated microchemostats on a single microfluidic chip designed to grow bacteria that can be potentially used in high-throughput experiments. Various inputs have been loaded with food dyes to visualize channels and subelements of the microchemostats. The coin is 18 mm in diameter. Panel adapted with permission from Reference 31; copyright 2005 AAAS.

Aim of the Review

This review provides an overview of recent advances in the development of microfluidics for biofilm study. Biofilm formation is a complex multifactorial phenomenon driven by the physicochemical properties of bacteria and the surfaces they adhere to, environmental parameters such as flow condition and medium, and the intrinsic biological response of the organisms. These aspects are intertwined and, thus, any attempt to study their individual contribution to the biofilm formation process represents a challenge and requires a multidisciplinary approach. Herein, we systematically summarize the applications of microfluidics in studying biofilm formation, biofilm eradication, and biofilm-associated infection models, as well as the approach to biofilm characterization. In addition, we elaborate the advantages and limitations of microfluidics. Finally, we discuss the future directions of this technology.

MICROFLUIDICS APPLICATIONS IN BIOFILM STUDIES

The possibilities offered by microfluidics have helped the development of their applications in biofilm research over time. Traditional static culture platforms such as the well plate, originally employed in studying biofilm formation, fail to provide the necessary hydrodynamic flow conditions. Flow chamber or other flow systems have also been applied, but they lack high-throughput properties and precise hydrodynamic control (33, 34). Therefore, microfluidics with precise control of the hydrodynamic and physicochemical microenvironment and better integration with analysis tools has contributed significantly to the improved understanding of biofilm development and biofilms' antimicrobial resistance (35). These applications of microfluidics are discussed below.

Biofilm Formation Under Flow

Flow is present in many environments where bacteria thrive. It is considered a vital factor that strongly influences biofilm formation and performance, including bacterial motility, bacterial adhesion, genetic behaviors of biofilm, the production of EPS, and bacterial communication, as well

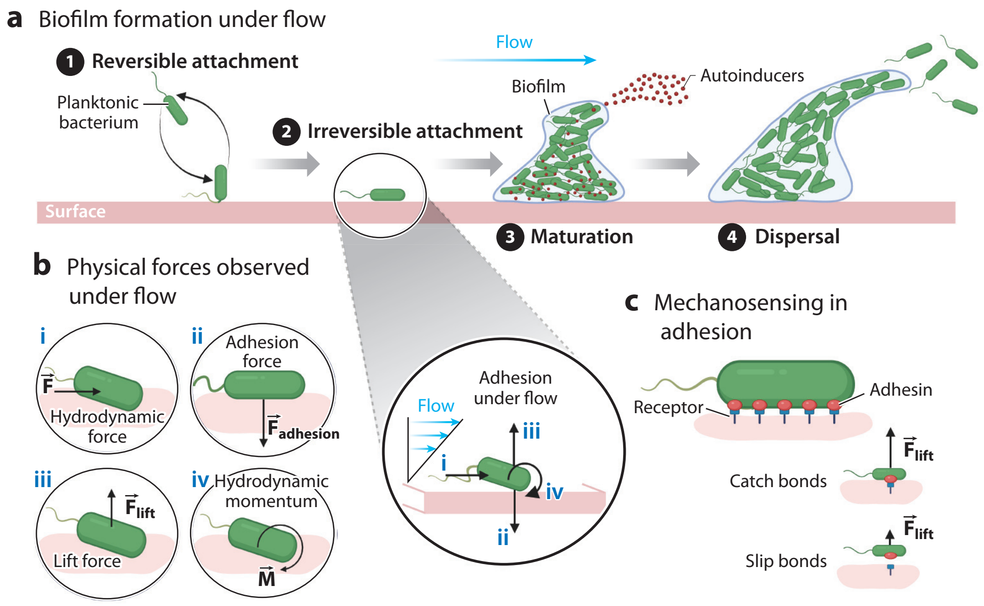


Figure 2

Schematic representing biofilm formation under flow and associated phenomena. (*a*) Biofilm formation usually comprises several stages, namely ❶ reversible attachment, ❷ irreversible attachment, ❸ maturation, and ❹ dispersal. The transition from reversible to irreversible attachment is realized for the planktonic bacteria when they closely approach a surface and resist the physical forces observed under flow. (*b*,*c*) Being a pivotal contributor to the irreversible attachment of planktonic cells to the surface, flow affects bacteria in different ways. The wall shear stress produces the hydrodynamic force (F) parallel to the direction of the flow that hinders the attachment of a planktonic cell to the surface (*b*, *i*). Thus, bacteria have developed various mechanisms allowing them to overcome the detachment induced by the hydrodynamic force. The most abundant one is accomplished by the so-called adhesins, i.e., molecules and appendages that attach to the surface through noncovalent bonds. At equilibrium, the adhesion force (*b*, *ii*) is balanced by the lift force (*b*, *iii*) (37). Flagella are found to move in a counterclockwise direction, while the net hydrodynamic momentum (M) (*b*, *iv*) resists their motion and has an opposite direction (41). Figure adapted from images created with BioRender.com.

as metabolite and nutrient transport. The impact of flow on biofilm formation is shown in **Figure 2** (36).

In the very beginning of their journey to approach the surface and form, bacteria exhibit dramatically different motility trajectories under static or various flow conditions (33, 37, 38). For example, *Escherichia coli* exhibit circular or random trajectories under static or low shear rate conditions (39, 40), direct upstream motility at a moderate shear rate (~6 s^{-1}), and sideways swimming at high shear rates (>30 s^{-1}) (40).

Once the bacteria are close to the surface, the shear stress generated by the flow on the surface can be overcome by the adhesion force anchoring bacteria onto the surface (37, 41) (see the sidebar titled Assessment of Bacterial Adhesion Force on the Surface). Bacteria evolved mechanisms in order to adhere to surfaces and resist detachment by shear stress. They use an array of molecules and appendages (such as pili and flagella) called adhesins that specifically bind to surfaces by noncovalent bonds (42). For instance, flagellar motility plays a crucial role in initial bacterial attachment, swimming in the bulk directly to the surface, while after attachment, the flagellar genes are downregulated to inhibit rotation or modulate reversal frequency (38, 43). Generally, these bonds between adhesins and surfaces are described as slip bonds or catch bonds, which are rather specific. Intuitively, slip bonds are characterized by a lifetime that decreases with

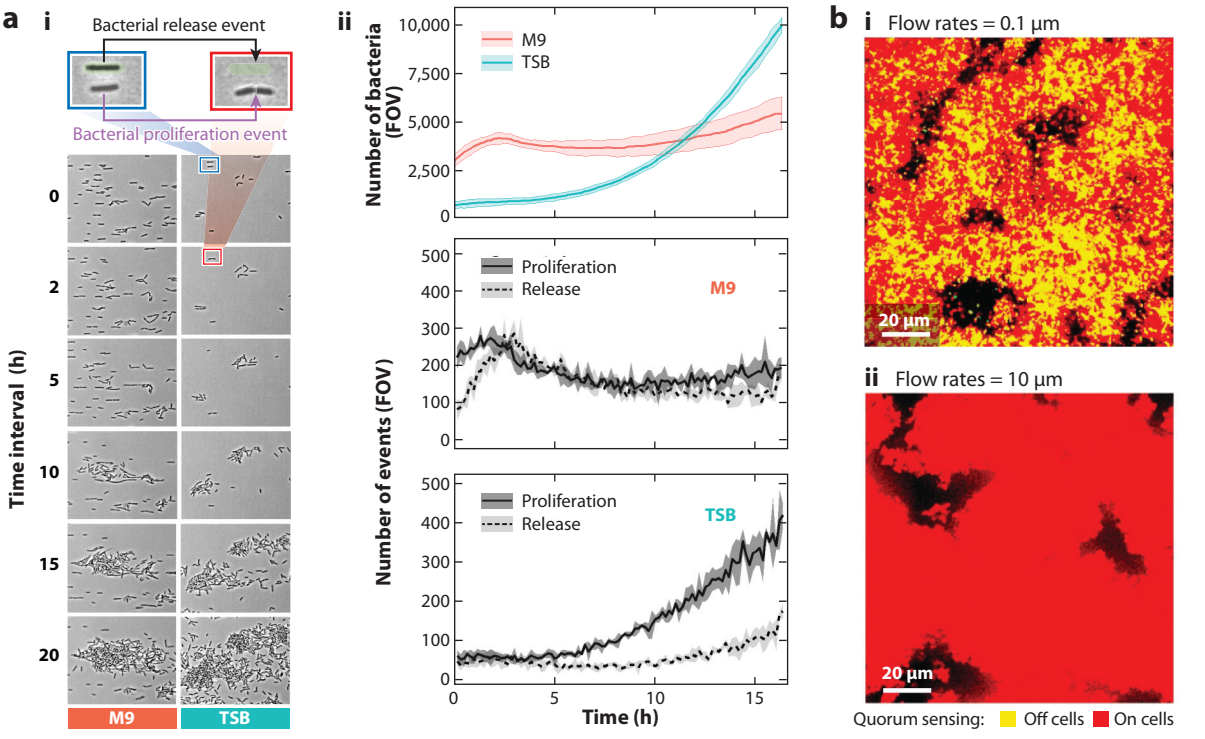
ASSESSMENT OF BACTERIAL ADHESION FORCE ON THE SURFACE

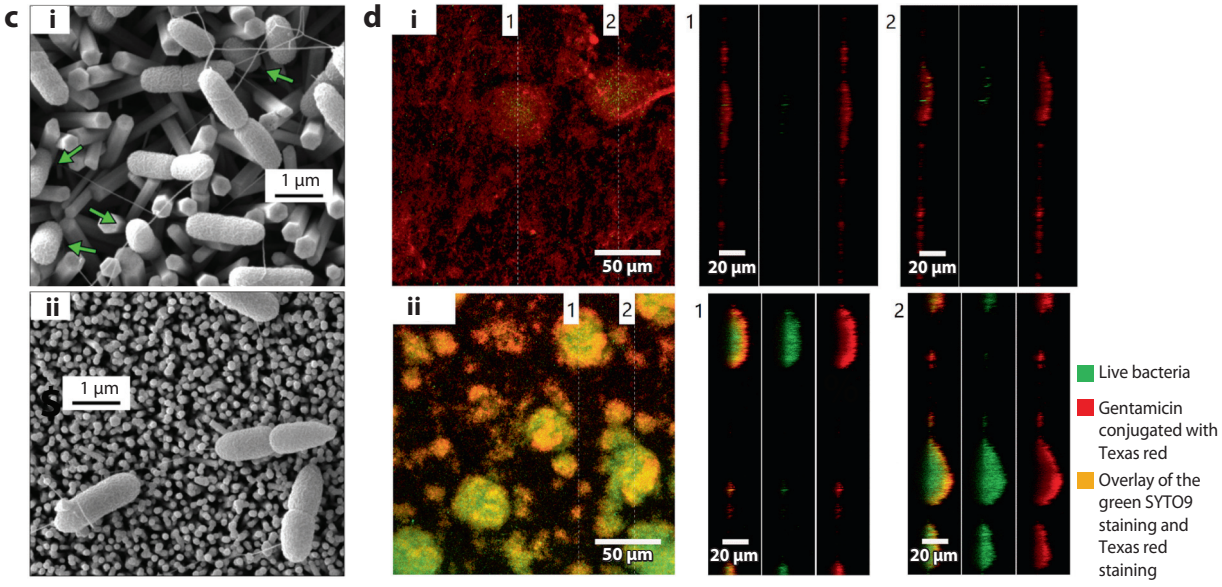
Shear stress, which on a solid surface (wall shear stress) generates a force parallel to the direction of the flow, plays a critical role in initial bacterial adhesion. Microfluidics has been used for noninvasively assessing bacterial adhesion force on the surface (51). Take the commonly used rectangular cross-sectioned microfluidics as an example. The wall shear stress is calculated by $\sigma_s = 6Q\mu/wh^2$, where Q is the flow rate, μ is the fluid viscosity, and w and h are the width and height of microchannels, respectively. In the flow, the drag force that a bacterium experiences is estimated as $F_{drag} = A\sigma_s$, where A is the bacterial cross-sectional area projected in the direction of motion (51).

tensile stress. Catch bonds exhibit an initial increase of lifetime at high tensile stress (41, 44, 45). A well-known example of a catch bond is between *E. coli* FimH adhesin and a surface mannose, a shear-enhanced adhesion on the surface at high shear stress, and a complex stick-and-roll adhesion that bacteria moves along with flow direction at low shear force (46). FimH enables *E. coli* to strongly adhere to the epithelial cells of the urinary tract by specifically binding to mannose residues decorating these cells. Consequently, the bacterial cells are able to resist washing by the urine flow inside the tract (47). Evidence also supports the fact that bacteria can sense the mechanical stress induced by flow (48). A central molecule determining the motile-sessile transition is the second messenger cyclic-di-guanosine monophosphate (c-di-GMP), which regulates the production rate and physicochemical properties of adhesins (49). In addition, shear stress can also alter the expression of other genes such as those involved in force-independent sensing (50).

In addition to the effects of shear forces on bacterial motility and adhesion, flow also influences transport of nutrient and signaling molecules during the biofilm formation process (41). On the one hand, flow benefits bacterial growth by providing fresh nutrients and carrying away waste products generated by the growing bacteria. For example, in microfluidics with a continuous perfusion of nutrients, the bacterial doubling time (∼2.4 h) is shorter than that without flow (∼3.4 h) (52). In addition, microfluidics with a laminar flow can be used to investigate the impact of culture medium or the molecules of interest on the biofilm formation, as shown in **Figure 3*a*** (30). On the other hand, flow can inhibit bacterial growth. Not only can flow shear off bacteria from a surface, but also it carries away common good molecules secreted by the bacteria and necessary for the capture of certain nutrients such as siderophores for the intake of iron (53). Furthermore, flow plays a crucial role in communication between bacterial individuals through quorum sensing, which is based on production and sensing of molecular autoinducers (9). Quorum sensing involves critical functions of microorganisms, including the development of biofilms, sporulation, acquisition of nutrients and production of EPS (8). Due to convection and advection of autoinducers under flow, the spatiotemporal distribution of autoinducers is dramatically affected, leading to a higher concentration of autoinducers near the substrate surface than near the interface of bacteria-liquid, and a higher concentration in the downstream than in the upstream, as shown in **Figure 2*b***. Consequently, quorum sensing in biofilm is repressed under flow despite a high number of bacteria (52). Notably, the local concentration of autoinducers determines the status of quorum sensing, as even one bacterium confined in a small volume is able to initiate quorum sensing and achieve quorum sensing–dependent growth (54).

In addition, flow stress influences biofilm deformation and dispersal, the late stage of biofilm formation, as commonly observed in microfluidics experiments (57, 58). Bacteria are held together by EPS networks to resist flow-induced deformation (57) and impair the dispersal to new locations (59). The activation of quorum sensing in biofilm can lead to the upregulation of the components that degrade the biofilm EPS to enable bacteria to escape, spread, and colonize new surfaces (52).





(*Caption appears on following page*)

Figure 3 (*Figure appears on preceding page*)

Selected examples of microfluidics applications in biofilm formation. (*a*) Nutrients have an impact on bacterial colonization in tryptic soy broth (TSB), a nutrient-rich culture medium, and modified M9 minimal medium (a nutrient-poor medium). (*i*) Time-relapse micrographs of adhered bacteria on the surface at different times, (*ii*) quantification of the number of adherent bacteria (*top*), and the generation of new ones and release from the surface in M9 (*middle*) and TSB (*bottom*). Panel adapted from Reference 30 (CC BY 4.0). (*b*) Merged images of biofilms grown under different flow rates: 0.1 μL min^{-1} (*i*) and 10 μL min^{-1} (*ii*). Panel adapted with permission from Reference 52; copyright 2016 Nature Publishing Group. (*c*) Influence of zinc oxide nanopillar density [low (*i*) and high (*ii*)] on bacterial adhesion. Arrows indicate cells trapped in void space. Panel adapted with permission from Reference 55; copyright 2022 American Chemical Society. (*d*) The vertical thickness of biofilm influences antibiotic penetration. Fluorescence confocal micrographs of biofilms formed by the wild-type *Pseudomonas aeruginosa* MPAO1 (*i*) and the flagella hook mutant *ΔflgE* (*ii*) treated with gentamicin and two cross-section views (*middle* and *right*; labeled as 1 and 2). Panel adapted from Reference 56 (CC BY 4.0). Abbreviation: FOV, field of view.

Influence of Surface Physicochemical Properties on Bacterial Adhesion

Bacterial biofilm formation on surfaces is influenced by physical conditions such as temperature, nutrients, and hydrodynamics, as well as characteristics of the surface on which the biofilm forms (60, 61) (see the sidebar titled DLVO Theory). The physicochemical properties of a material, including the charge, hydrophobicity, stiffness, roughness, and topography, can play a direct role in biofilm formation by favoring or inhibiting bacterial adhesion or an indirect role by inhibiting growth or killing adherent bacteria (55, 62–66). Efforts have been dedicated to developing surface modifications of biomedical devices to decrease the occurrence of biofilm formation. The modification aims to either render the surface antiadhesive to prevent bacterial adhesion as its name suggests or render it toxic to bacteria to kill them upon contact or when in close proximity. One limitation of the commonly used test for assessment of antiadhesive or bactericidal surfaces is the low in vivo relevance, which impedes their predictive power of antimicrobial performance under real-life circumstances. These tests are often designed to be performed under static conditions and resort to using nutrient-rich growth media that do not reflect the conditions under which bacteria encounter, adhere, and grow on these materials in vivo (67). Microfluidics has been applied to investigate the influence of these properties on biofilm formation by providing a precise physicochemical microenvironment.

Charge

Generally, bacteria are negatively charged due to negatively charged phosphoryl and carboxylate moieties on the bacterial cell envelope (69). Notably, the composition of the bacterial cell envelope varies between strains and is highly heterogeneous (43, 69). According to XDLVO theory,

DLVO THEORY

The projected interaction energy between bacteria and surfaces is based on the extended Derjaguin–Landau–Verwey–Overbeek (XDLVO) theory, including the Lifshitz–van der Waals, electrostatic, and acid-base interactions. The DLVO theory and the thermodynamic approach are the basis of the physicochemical aspects of bacterial adhesion. The modifications of physicochemical properties of material surfaces by incorporating specific chemical species or nanopatterning the surface have been applied to obtain antifouling by increasing the energy barrier of adhesion between bacteria and surface. Actual bacterial adhesion is a complex process and frequently deviates from the adhesion models. In particular, studies attempting to apply DLVO theories to describe bacterial attachment are limited, as the theories consider bacteria as ideally smooth and chemically homogeneous colloidal particles. For more extensive descriptions of bacterial adhesion theory, the reader is referred to another review (68).

positively charged surfaces favor bacterial adherence due to electrostatic attraction instead of negatively charged surfaces. Furthermore, positively charged surfaces have shown antimicrobial effects in subsequent bacterial growth due to the strong attractive electrostatic interactions that disrupt cell membrane integrity and impede cellular elongation and division (70).

Hydrophobicity

Hydrophobic properties of bacteria vary between strains. Generally, hydrophilic bacteria preferentially adhere to hydrophilic surfaces and vice versa (43). The hydrophobic property of PDMS, the most commonly used material in microfluidics, could be changed to hydrophilic with low-pressure plasma treatment or chemical modifications (64). When compared to a hydrophobic PDMS surface, hydrophilic PDMS shows an increased adherent number of *E. coli*, whose surface is hydrophilic (71), as well as a higher drag force to detach them from the surface (72).

Roughness

Surface roughness can increase or reduce the contact area between bacteria and surface, thus affecting the initial adhesion of bacteria onto the surface (73). Some studies have shown that rougher surfaces lead to a larger number of bacterial adhesions under flow (74, 75), but there are numerous contrary reports on the effects of substrate roughness on bacterial adhesion (76, 77). The discrepancies can be caused by different experimental conditions such as the used bacterial strains, chemistry of surfaces, incubation time, and flow rate. The different methods applied for readout can also affect the experimental outcome.

Nano-Topography

To obtain antifouling and bactericidal functions, the physicochemical properties of material surfaces can be modified by nanopatterning to increase the energy barrier of adhesion between bacteria and surface or to incorporate antimicrobial agents for killing bacteria upon adhesion. Nanopillars, inspired by the wings of cicadas, have shown a promising bactericidal property, as they might cause mechanical damage such as the deformation and rupture of the membrane of the attached bacteria; the property is called mechano-bactericidal (78–80). For example, under flow, a high density of self-assembled zinc oxide nanopillars has shown a reduced number of bacterial adherence compared to the low density of nanopillars (**Figure 3*c***). Furthermore, 30–80% reductions of bacterial adhesion force on the nanopatterned surface lead to a decreased drag force to detach the bacteria from the surface (55, 81). The efficiency of nanopillars also varies between different bacteria strains, possibly because the shape and composition of the cell envelope influence the deformability of the cell (78).

Material Stiffness

Recently, the mechanical properties of materials have attracted attention owing to their influence on biofilm formation. Materials are described in terms of their stiffness, referring to either Young's modulus or shear modulus. So far, microfluidics has not been often applied to studying the impact of material stiffness on bacterial adhesion and biofilm development, even though it offers more in vivo–relevant settings than does the static condition. One interesting example of a stiffness-tunable PDMS substrate prepared with different curing agents showed that under different flow dynamic conditions, bacterial adhesion can differ considerably: A similar number of adhered bacteria were found under static and low shear conditions, which were much higher than those under high shear flow under otherwise similar conditions (82). The large surface contact area between bacteria and soft PDMS greatly influences the adhesion force and retention force under flow (82, 83). Overall, these results illustrate the importance of flow conditions on the outcome of the assessment.

Antimicrobial Assessment

Antibiotic resistance is one of the biggest threats to public health and leads to increased medical costs and mortality. Bacteria residing in biofilm, protected by EPS, are expected to be up to 1,000 times more antibiotic resistant than planktonic bacteria (15, 20). In this section, we review the microfluidics applied as a platform to study antibiotic resistance, the antibiotic susceptibility test, and development of novel antimicrobial agents.

The mechanism of antibiotic resistance in bacterial biofilm is largely due to the poor penetration of an antibiotic through a biofilm, where the antibiotic has been found predominantly in the superficial layers of biofilms, especially in thick biofilm (56) (**Figure 3*d***). The uneven spatial distribution of antibiotics in biofilm may accelerate the evolution of antibiotic resistance. This hypothesis is supported by a recent microfluidics-based study showing that biofilm formation on the region of low antibiotic concentration expanded into the high-concentration region at longer culture times (84). Also, bacteria in biofilm may differentiate into resistant phenotypes in response to the altered microenvironment (85). In addition, the resistance genes can be shared in biofilm vertically via cell division and horizontally between species via cell surface pili (86, 87). Furthermore, some bacterial cells in biofilm exhibit dormant states owing to nutrient or oxygen limitations or time-dependent growth arrest (88). Such dormant bacteria can survive antibiotic exposure because their antibiotic target sites are deactivated. Microfluidics allows direct analysis of the interaction between antibiotics and biofilm, even at the single-cell level (30).

Indeed, the antimicrobial susceptibility test using microfluidics has attracted increased attention with the advance of this technology. As the occurrence of bacterial resistance to common antibiotics increases, rapid phenotypic and genotypic analysis of said resistance of microorganisms isolated from patients is crucial to tailoring antibiotic therapy. Microfluidics offers speed and accuracy unmatched by current methods (89). The optimum dosage of antibiotics is a critical factor in treating biofilm-associated infection, as low concentrations of antibiotic levels can impose selective pressure for the evolution of antibiotic resistance. However, the conventional treatment decision is based on the minimal inhibition concentration (MIC) result from planktonic bacteria or bacteria isolated from the biofilm, which can be remarkably different from those in biofilms. Antibiotic susceptibility testing is another area of high interest in biofilm research, where microfluidics has showed promising results (89). Microfluidic systems with a laminar flow, generating a gradient of antibiotic concentration, have been used to determine the minimal biofilm eradication concentration (MBEC) of antibiotics (84, 90, 91). Compared with MIC values obtained from planktonic bacteria using conventional methods, the microfluidic MBEC reveals that a much higher antibiotic concentration is needed. In addition, microfluidics has been applied as a platform to monitor polymicrobial biofilm formation and eradication in real time because, in some cases, polymicrobial biofilm may more accurately reflect the clinical infection (92–94). Moreover, combined treatments of different antimicrobial agents can be evaluated in microfluidic devices (95, 96).

Bacterial Infection Models Based on Organs-on-Chip

In addition to the abovementioned application of microfluidics in analyzing antimicrobial efficacy against biofilm, microfluidics has the potential to offer platforms mimicking in vivo situations at tissue and organ levels, which are called organs-on-chip. As animal experiments are costly, time consuming, ethically controversial, and sometimes inaccurate representations of human responses, there is an urgent need for drug test models at the preclinical stage (97). Given the ability of organs-on-chip to reproduce bacterial infections in human tissues from both healthy individuals and ill patients, similar approaches may be used to discover antimicrobials for emerging antibiotic-resistant organisms. For instance, gut-on-chip microfluidic systems contain two parallel hollow

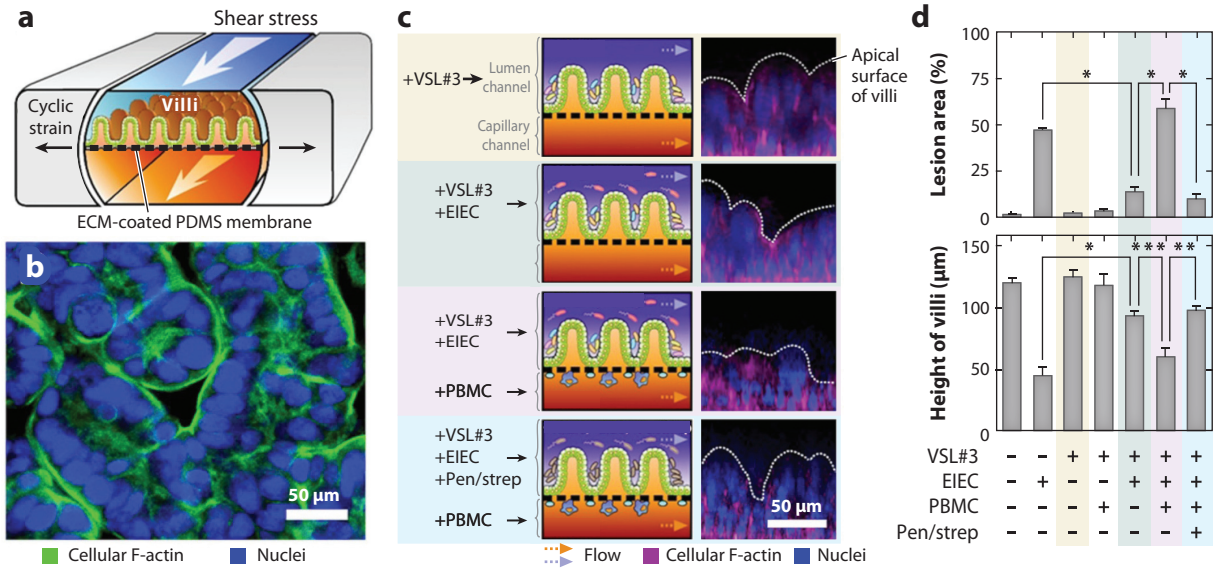


Figure 4

Bacterial infection model based on organs-on-chip. (*a*) Schematic drawing of a 3D cross-section of a gut-on-chip showing how repeated suction to side channels (*black arrows*) exerts peristalsis-like cyclic mechanical strain and fluid flow (*thick white arrows*) generates shear stress in the perpendicular direction. (*b*) A confocal immunofluorescence image showing a horizontal cross-section of intestinal villi stained for F-actin (*green*) that labels the apical brush border of these polarized intestinal epithelial cells (nuclei in *blue*). (*c*) Morphological analysis of intestinal villus damage. Schematics (*left*) and fluorescence confocal micrographs (*right*) of vertical cross-sectional views of villi after staining for F-actin (*magenta*) and nuclei (*blue*). Coculture infection models were treated with probiotics and/or antibiotics. (*d*) Quantification of intestinal injury evaluated by measuring changes in lesion area (*top*; $n = 30$) and the height of the villi (*bottom*; $n = 50$) in the absence or the presence of VSL#3, EIEC, PBMCs, or antibiotics, as indicated. * denotes $P < 0.001$, ** $P < 0.01$, and *** $P < 0.05$. Abbreviations: EIEC, enteroinvasive *E. coli*; PBMC, peripheral blood mononuclear cell; Pen/strep, penicillin/streptomycin; VSL#3, a registered trade name of a probiotics mixture. Figure adapted with permission from Reference 98; copyright 2016 National Academy of Science.

channels separated by a porous extracellular matrix–coated membrane, with intestinal epithelial cells on one side and vascular endothelial cells on the other. Furthermore, immune components such as mononuclear cells can be introduced through the vascular endothelium channel mimicking the intestinal inflammatory diseases. Other elements of interest such as probiotics, pathogens, or antibiotics can flow through epithelial or endothelial channels to study the interplay between host microbes and probiotic pathogens and antibiotic susceptibility (98, 99) (**Figure 4**).

One challenge encountered by a conventional coculture platform such as Transwell is the relatively short coculture time due to bacterial overgrowth and the loss of tissue cell viability. Using an organ-on-chip microfluidic device, the coculture time can be potentially extended to weeks. For instance, when intestinal epithelial cells cocultured with probiotic *Lactobacillus rhamnosus* GG under a static culture condition, intestinal barrier integrity failed within 24 h, whereas it was maintained for over 100 h in a microfluidic device with a cyclic strain (100). With the advantage of the elongation of coculture time, gut-on-chip has been used as an intestinal infection model to investigate the interplay between host, probiotics, and pathogens (87).

Another intriguing application of organs-on-chip is to engineer personalized chips with tissue cells or stem cells isolated from patients or healthy individuals for disease models or drug tests. This drug screening platform can recapitulate an individual's physiology much closer than can animal models. Recently, personalized lung-on-chip used primary human bronchial epithelial cells

isolated from cystic fibrosis patients or healthy individuals, grown under an air-liquid interface, and pulmonary microvascular endothelial cells exposed to flow. The pathological cystic fibrosis condition provides a more favorable environment for *Pseudomonas aeruginosa* growth, secretion of inflammatory cytokines, and recruitment of immune cells compared to the healthy personalized chip. This personalized chip with key features of the human cystic fibrosis airway can be further used for drug screening (101). Importantly, recently developed body-on-chip or human-on-chip with integration of multiple organs on a single chip, which replicates a systemic level of human biology in vitro, can be used as a valuable preclinical tool to advance the development of personalized medicine (102, 103). Furthermore, microfluidics infection models have also been used to investigate the tissue-biofilm-biomaterials interface because biomaterial-associated infection has emerged as a major cause of medical implant failure (104, 105).

CHARACTERIZATION OF BIOFILMS

Assessing bacterial adhesion and biofilm formation on materials is of paramount importance in the design of novel antimicrobial surfaces and treatments. Generally, techniques used in static platforms for biofilm characterization can also be applied in microfluidics. Conventional methods such as colony-forming units (CFUs), flow cytometry, and microscopy used in the laboratory for decades, as well as advanced techniques such as biosensors integrated with microfluidic devices, have been used to monitor and evaluate biofilm (106). These conventional and novel methods for the characterization of biofilm in microfluidics are discussed below (**Figure 5**).

Colony-Forming Units

CFUs are widely applied to estimate the viable number of bacteria in biofilm, and they represent an end-point destructive method that first requires bacteria to be released from the tested surface. Bacteria in microfluidics can be collected after detaching the bacteria from the surface with sonication or enzymes, followed by standard plate counting (107). Theoretically, CFUs can detect an unlimited number of bacteria, but the main disadvantage of the CFU method is that it is time-consuming. The method usually takes days from sample collection until the bacterial colonies grow on agar plates (108).

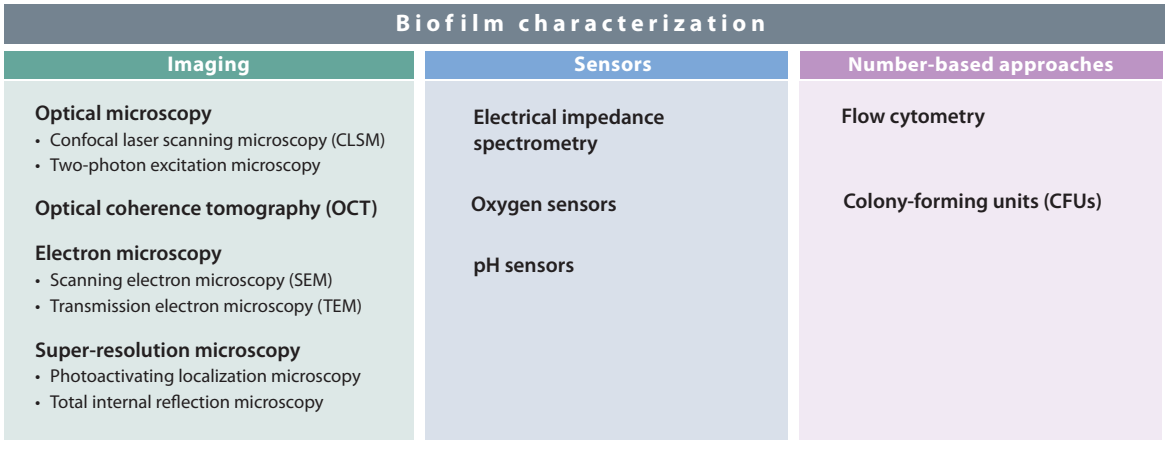


Figure 5

Techniques applied for monitoring and characterizing biofilm in microfluidic devices. The methods are discussed in the article and divided into three categories: imaging, sensors, and number-based approaches according to their action principle.

Flow Cytometry

Flow cytometry is an ideal method for quantitative analysis of the subpopulation of interest (109). For example, flow cytometry is used to study the transfer of antibiotic-resistant genes in biofilms, counting the number of donor and transconjugant bacteria at the end point of an experiment (86, 87). It is also possible to stain bacteria with fluorescent dyes and then apply the bacteria to flow cytometry, such as using live-dead staining probes to quantify the number or percentage of live or dead bacteria in the antibiotic susceptibility test (110). Similar to the CFU method, flow cytometry is an end-point destructive measurement, which requires collecting bacteria from microfluidics and preparing them in suspension. Another limitation of flow cytometry is the sensitivity of signals due to the small size of bacteria, approximately one micron, which can make it difficult to distinguish these low-intensity signals from the noisy background (109, 110). Furthermore, flow cytometry is not suitable for detection of a small number of bacteria, as at least a few thousand are required in the sample (109, 110).

Imaging

Conventional and advanced imaging techniques, including (fluorescence) microscopy, optical coherence tomography (OCT), and electron microscopy, have been used to evaluate biofilm grown in microfluidic conditions.

Due to the excellent optically transparent property of PDMS, light microscopy has been widely used in studies of biofilms formed on PDMS, especially bacterial adhesion with single-cell tracking (30) (**Figure 3*a***). Importantly, high-resolution time-lapse imaging provides a noninvasive possibility to study microbial motility, adhesion, and growth in response to their microenvironments at a single-cell level over time (30, 33, 40, 111, 112).

As the thickness of biofilm usually increases in the late stage of formation, it is tricky to analyze the biofilm with light microscopy due to blurry images caused by the light scatter (113). In this case, imaging methods such as confocal laser scanning microscopy (CLSM), two-photon excitation microscopy, and OCT are widely applied to analyze the surface coverage, thickness, and volume of the biofilm of interest (114). CLSM can be used as either an in situ nondestructive measurement or an end-point destructive measurement, as fluorescence signals could have originated from fluorescent-tagged bacteria or staining probes. However, confocal microscopy has several limitations with these thick biofilms, namely, limited diffusion of fluorescent probes into the deeper region of biofilm and light scatter limiting the depth of observation (99). As an alternative for imaging thick biofilm, two-photon excitation microscopy can be used. OCT is a nondestructive and real-time monitoring technique often used to analyze the thickness and biomass of biofilm. The image depth measured by OCT can reach from several millimeters up to 20 mm at the expense of lateral resolution. The main disadvantage of OCT is the low axial resolution, around several microns, making it almost impossible to identify an individual bacterium and limiting applications in the early stage of biofilm formation.

Generally, the resolution of conventional fluorescence microscopy including CLSM is limited by light diffraction, which makes it difficult to image bacterial substructures at the single-molecule level. Advances in super-resolution microscopy (SRM), which push its limit toward lateral resolution by sacrificing temporal resolution and imaging depth, allow observation of bacterial substructure at the single-molecule level (115). SRM methods such as photoactivated localization microscopy and total internal reflection microscopy have been used to study the composition and dynamics of single proteins of live bacteria on a microfluidic platform (115, 116). However, the acquisition of super-resolution images is slow because it requires time-resolved localization of fluorophores with sequential photoactivations. Furthermore, only a limited number of

compatible fluorophores and labeling options fulfill the strict criteria for high-quality single-molecule microscopy (117, 118).

The details of biofilms can be studied with electron microscopy, which has been used to analyze microbial substructures such as pili and their interactions with the microenvironment (55, 119, 120) (**Figure 3*c***). Although electron microscopy provides more details of bacteria–surface and bacteria–bacteria interactions, it is a time-consuming and destructive technique.

Sensors

Many conventional techniques such as CFUs are destructive end-point measurements that first necessitate the removal of biofilm from the microfluidic device. It is critical to develop nondestructive real-time detection methods to understand the dynamics of biofilm development and its interaction with microenvironments. Important advancements in biofilm assay have included the integration of electrical or optical biosensors with microfluidic devices to ensure continuous assessment of biofilm-related parameters (e.g., biomass quantification from optical absorbance reading) and environment-related parameters (e.g., pH and oxygen) in real time.

Electrical Impedance Spectrometry

The electrical impedance spectrum is based on sensing impedance changes due to direct deposition of bacteria on or across an electrode array (121, 122) or measuring the electrical signals of the growth medium because bacteria metabolism transforms the uncharged (or weakly charged) compounds into highly charged compounds, leading to a change of electrical properties (95). In general, measurements at low frequencies are dominated by charge transfer and mass diffusion of electroactive compounds (93). Electrical impedance spectrometry is a highly sensitive and label-free technique, with small alternating voltages applied and resulting alternating current flow from a working electrode across the sample to an opposite count electrode. The current is then measured and the impedance calculated as the ratio of the applied voltage to the current for given frequencies (123). The impedance spectrum can also be effective to distinguish biofilms with different structures, detect spatial diversity, and track the life cycle of biofilm attachment and maturation (124).

pH Sensors

Acidic waste products from many microbes can cause a change in local pH, which can influence bacterial metabolic activity and the efficiency of some antibiotics (125). Although pH values of biofilm grown in microfluidic conditions can theoretically be measured by checking the medium from the outlet with pH indicator paper, it may not accurately represent the status of biofilms. There are two types of pH sensors; one is based on pH-sensitive fluorophores and the other on electrochemistry. For example, pH-sensitive fluorophores embedded in nanoparticles capable of sensitive metal-enhanced fluorescence have been proven to allow an improved fluorescence intensity, real-time and rapid detection, and photobleaching resistance over one week of exposure (126). Some bacteria, such as *Geobacter sulfurreducens*, are capable of collecting electrons from soluble substrates and transferring them to electrodes, resulting in a change in formal potential that can be transformed into a pH indicator (127).

Oxygen Sensors

Oxygen plays an important role in generating energy for aerobic bacteria and their biofilm formation. The most widely used biosensors for oxygen concentration measurements are based on optical methods (128, 129). Oxygen-sensitive probes, in which luminescence can be quenched by

oxygen, are immobilized on a sensor and integrated with a microdevice. The oxygen distribution in biofilms can be mapped by excitation of the oxygen-dependent dye (130).

Furthermore, multiple sensors, such as pH and oxygen sensors, can be integrated with a microfluidic device for monitoring multiple indicators of biofilm (129). In addition, a dual optical sensor such as a pH and oxygen sensor has been proposed to monitor bacteria growth (130). Other sensors, including immunology-based and thermal-based sensors, have also been integrated with microfluidic devices for real-time detection and monitoring (131, 132). The authors recommend a recent review of biosensors integrated with microfluidic devices for biofilm study (133).

CHALLENGES AND OUTLOOK

While static and flow chamber assays are vital tools to assess bacterial adhesion and biofilm formation on materials of interest in order to develop strategies to reduce biomaterial-associated infection, microfluidics platforms offer long-term assays and permit dynamic real-time analysis and more precise control of relevant parameters. Besides all of the practical aspects of assays performed under flow, a microfluidics approach also adds in vivo relevance because flow is a ubiquitous phenomenon in natural environments such as the clinical setting of pathogenic bacteria (e.g., blood circulation, urinary tract, mucus flow in the airway). The movement of nutrients, waste products, and signaling molecules directly and indirectly influences biofilm physiology. Continuous improvement of the platform will hopefully incentivize microbiologists to adopt similar approaches for both fundamental research in biofilm formation mechanisms and the development of antimicrobial solutions. In addition to the applications mentioned above, such as the assessment of antibiofilm activity, the microfluidic system is particularly well suited to studying biofilm development under starving conditions and a nonconstant medium flow supply.

One challenge faced by microfluidics is the high risk of contamination during long-term incubation, especially for coculture of mammalian and bacterial cells that needs a nutrient-rich medium prone to contamination. Thus, care is needed to thoroughly sterilize the tubing, microfluidic channel, and medium. Other measures can also facilitate the reduction of contamination, for example, connecting a 0.22-μm sterile filter to the inlet tubing to prevent contamination from the surrounding environment during the infusion process (65).

The conventional microfluidic systems often encounter clogging problems because bacteria adhere not only to the microchannel floor but also to each other, leading to the formation of large clumps of cells in the area of low flow rates. This clogging phenomenon can be explained by the narrowing of the flow channel due to biofilm formation resulting in a local increase of the flow speed, which in turn increases the shear force (30). To overcome this problem, various designs have been attempted, for example, one with three inlet channels that merge into a single chamber followed by an outlet channel (**Figure 1*a***). This flow-focusing arrangement enables control of the flow rate from three different liquid reservoirs and thereby allows spatially separated flows of different media in the same chamber because of the laminar flow regime (30).

The automation of high-resolution microscopy image acquisition can quickly lead to the generation of a large amount of data that pose a challenge for manual and semiautomated image analysis. These processes are extremely time consuming and labor intensive and, without a suitable solution, there is a high risk of being overwhelmed by data and simply not being able to analyze them. Thus, the potential of artificial intelligence can be exploited for image acquisition and analysis. Development of deep learning algorithms for image segmentation and classification is needed now more than ever. Several aspects of the analysis that can result in bottlenecks could benefit tremendously from such algorithms, such as detection and flagging of artifacts, segmentation, object detection, denoising, object tracking, and plotting. Large data set handling is also an aspect that deserves attention.

Microfluidic systems have facilitated our improved understanding of the underlying mechanisms of bacterial adhesion and biofilm development. They provide a useful tool for investigating the dynamic interaction of biofilm and antimicrobial agents or surfaces in situ, thus supporting future developments of antimicrobial solutions to fight against bacterial infections.

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