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METHODS AND PROTOCOL
FLOW CELLS: TECHNIQUE USED FOR STUDYING MICROBIAL BIOFILMS

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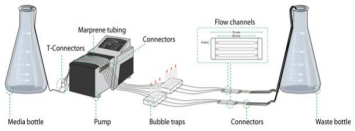

Abstract

Biofilms are sessile communities of microorganisms growing on material surfaces and embedded in self-accumulated extracellular polymers. A comprehensive analysis of physical, chemical and biological factors including hydrodynamic and nutrient conditions that regulate their formation is required to adequately gain insight to this complex multicellular microbial life style. Reproducible experimental models that consider all the conditions under which they grow and develop also remain a required tool for studying the biofilms. As a result of its ability to create hydrodynamic and nutrient conditions coupled with continuous and non-destructive ability to grow biofilms, flow cell technology has become one of the most recently patronised models used to study microbial biofilms. This article focuses on recent advancements, principles and practical application of flow cell technology to study microbial biofilms.

Keywords: Flow-cell, biofilm, staining

Abstrak

Biofilem merupakan komuniti sesil yang bertumbuh di atas permukaan dan dilitupi oleh polimer ekstrasel. Analisis yang komprehensif dari faktor fizikal, kimia dan biologi termasuk keadaan hidrodinamik dan nutrisi yang mempengaruhi pembentukannya diperlukan untuk melihat secara mendalam kehidupan kompleks mikrobial pelbagai selular. Model eksperimen yang mempunyai pelbagai keadaan untuk biofilem bertumbuh dan membesar menjadi suatu alat yang diperlukan untuk mengkaji biofilem. Sebagai alat yang berkebolehan untuk membentuk kondisi hidrodinamik dan nutrisi, digabungkan pula dengan kebolehan pembentukan biofilem berterusan dan tidak musnah; teknologi sel mengalir menjadi satu peralatan yang diperlukan kini untuk mengkaji mikrobial biofilem. Artikel ini memfokuskan kemajuan kini, prinsip- prinsip dan aplikasi praktikal teknologi sel aliran untuk mikrobial biofilem.

Kata kunci: Aliran-sel, biofilem, pewarnaan

1.0 INTRODUCTION

The existence of microorganisms in a complex structures made up of single or multiple bacterial population enclosed in self-accumulated polymers. The microbes often plunged on both biotic and abiotic surfaces as sessile community [1, 2]. Their formation is influenced by a number of factors ranging from the chemical composition and physiology of the surface, composition and concentration of the carbon source, flow rate (shear stress) of the surrounding mobile phase to the type and population of the microbial species [3].

Our basic understanding of this microbial multicellular life style depends on our ability to effectively study them with sophisticated models that will consider all the factors obtainable in their natural environment such as nutrient and hydrodynamic conditions.

A basic tool is required to monitor the biofilms grow continuously under a non-destructive conditions. For a particular method to be considered 'continuous' and 'non-destructive', it should be able to analyse biofilms under aqueous system with no sample removal and in real time [4, 5]. Until recently monitoring of the biofilms and their cellulolytic activities were made by subjecting the samples collected from the reactor to light microscopy, electron microscopy and sometimes fluorescence *in situ* hybridisation (FISH) [6, 7]. This approach only allows monitoring of different set of biofilms and different cellulolytic materials for each sampling. This makes it almost impossible to accurately compared biofilm events such as thickness, maturation and sloughs at different sampling times and hence the comparisons were mostly based on inferences [7]. Scientists have since developed different models to study and characterize microbial biofilms. However, as a result of complexity of biofilms and the surrounding conditions, these methods and techniques tend to varies from one research group to another, there by producing biofilms with different structures and physiology and hence difficulties in comparing results obtained by different research groups [8]. Some of this models include multi-well plates or petri dishes used for growing biofilms in static conditions. Drip-flow reactors also be used to represents air–liquid interface flow. Whereas disk reactors capable of growing biofilms under low shear forces and offers reasonable reproducibility. The details of internal flow structure of these models are also not well understood and may therefore ill-define the real biofilm conditions [9] The reproducibility of the models used to investigate the biofilms is a major consideration in biofilm research. The design and development of flow cells have make it a convenient technology for the analysis and investigation of microbial biofilms [7]. Several studies have applied flow cells to study biofilms ranging from evaluation of their formation on food processing equipment [10] and within the waste water and industrial pipe lines [11]. Additionally, flow cells capable to monitor the effectiveness of antimicrobials on both single and multi- species biofilms on various material surfaces [12-15] and also be able to determine the interaction of human cells with biofilms [16, 17].

2.0 THE FLOW CELL

Historically, the flow cell (originally called Robbins device) was invented about 30 years ago by Jim Robbins to enhanced the reproducibility of biofilm formation in a fluid flow which was later modified and named Modified Robbins device (MRD) by McCoy *et al.* [18, 19]. A biofilm flow cells is a device designed to enhance biofilm growth on substrata under controlled and wide range hydrodynamic and nutrient conditions [20]. The various commercially available flow cells and the in-house fabricated flow cells are all based on operating principles of the MRD however the design of flow cell itself tends to differ from one manufacturer or research group to another [21, 22]. The flow cells are composed of retractable specimen plugs that can accommodate coupons of different materials on which biofilms can be deposited [21, 23]. It is an open system that enables fresh nutrients to be continuously supplied to the system in the same manner obtainable in the medical, natural or industrial environments. The design of the flow cells is such that it can be mounted directly on different optical microscope stage for direct visualization of the biofilm without causing any destruction to the biofilm [2].

Flow cells has also been reported to allow application of video skills to capture the processes of microbial adhesion, accumulation of EPS and possible attachment of other materials to the biofilm [22, 24]. These advantages make it a suitable and one of the most recently patronized device employed for sequential imaging of the same biofilm under hydrodynamic conditions [7]. The flow cells is the most important component of a biofilm flow-cell system that provides chambers for biofilm growth. There are different designs of flow-cell, however a modified version originally developed by Wolfaardt *et al*. [25] applied by most biofilm researchers. It is made up of a polycarbonate base with different parallel channels of individual measurements and a conventionally designed with microscopic glass cover slip. Its multi-channel design make it suitable for running 2 to 4 biofilm experiments depending on the number of channels. Both end of each channel is made up of 1 mm port to serve as the media inlet and waste outlet [26]. The open side of the channels are normally covered with microscope glass cover slips by applying a thin string of silicone glue on the basement before placing the coverslip in order to ensure proof leaked channels [1]. Apart from ensuring a closed channel, the cover slip also serves to provide substratum for the biofilm formation and more over optically adapted to the microscopy of the biofilm [1, 26]. On the other hand, Bio-surface Technology is one of the companies that recently introduced some model of flow-cells that used silicone rubber gasket to provide proof leak flow cell and can be autoclaved and reused.

3.0 SETTING UP THE FLOW CELL

The in-house construction of flow cell system is considered to be less expensive and allows the researcher the much needed versatility. However, purchasing the commercial pre-assembled components saves the researcher's time [2]. The flow cell system is essentially made up of the flow cell, bubble trap, medium/effluent bottle and peristaltic pump. The bubble trap is normally placed between the peristaltic pump and the flow cell purposely to collect the bubbles that may disrupt the biofilm structure in the flow chamber [2]. The peristaltic pump on the other

hand, is used to pump a pulseless media at the required flow rate. It is important to use multi roller peristaltic pump for this purpose because the conventional peristaltic pump do not deliver a pulseless flow while the medium bottle/effluent bottle contains sterile medium and collect spent medium respectively [2].

In order to ensure continuous supply of nutrient to the biofilm cells, there is inevitable need to correctly connect the flow cell to other components that make up the flow cell system. These connections can be achieved with the aid of silicone or glass tubing and connectors as illustrated in Figure . Prior to connection of the medium bottle, all the contaminations should be removed from the system. This can be achieved by using autoclave to sterilize the autoclavable components to a temperature of 121 °C or by sterilizing with ethylene gas. The non autoclavable components such as the peristaltic pump, and syringe barrel and the valve of the bubble trap should be disinfected with strong disinfectants such as ethanol or sodium hypochloride [1]. The medium bottle containing sterile medium of interest is subsequently connected to the system by splitting the tubing from the medium bottle and connecting them to Marperene tubes of the peristaltic pump. The Marperene tubes are subsequently connected to the inlet of the bubble trap with the aid of silicone tubing and the outlet tubes of the trap can then be connected to the individual inlets of the flow chamber. The length of the tubing should be long enough to allow the flow cell to be moved to the stage of the microscope. The outlet tubing from the flow cell should also be connected to the effluent bottle using connectors.

It is however important to always placed the effluent bottle at the same level with the flow cell and never below flow-cell level. The tubing that goes to the waste bottle should be a bit above the level of waste liquid to prevent back flow that may occur as a result of siphon effect that may occur during the process of the experiment. There is also need to maintain the entire sequence order of the tubes to enhanced identification of faults that may occur in the course of connection [2].

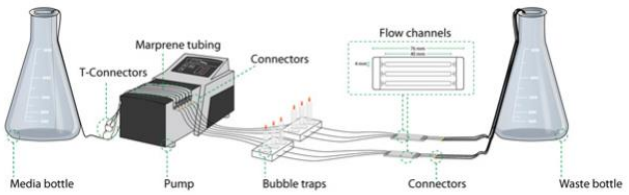

Figure 1 The components and set up of the flow cell system [27]

4.0 PREVENTION OF BUBBLE FORMATION IN THE FLOW CELL SYSTEM

The novices as well as the experienced biofilm researchers are frequently (one in every 3 biofilm experiments) faced with the challenge of the bubble formation within the flow cell system (Figure 1). As stated earlier, the bubble is capable of disrupting the growing biofilm architecture in the flow chambers and need to be adequately prevented. Bubbles originate from fluctuation in temperature of the medium, fluctuation in pressure as a result of change in tubing length and the action of the peristaltic pump [1, 9]. The following measures innovated by Crusz *et al*. [2] maybe useful to prevent bubble formation

i. Rendering the medium bottle airtight by using silicone glue around the port and fitting it with a non- collapsible tube connected to air filter
ii. Inverting and suspending the medium bottle above the flow cell will enhance gravitational flow of the medium thereby reducing the work of the peristaltic pump and the pressure differences created as a result of pumping action.
iii. Using a 50 mL syringe to drawn off the air in the inlet vessel above the medium
iv. Ensuring the medium does not cool below the ambient temperature of the experiment after autoclaving as the air bubbles tend to build up in the system as the temperature rises
v. Moving the pump downstream of the flow cell and subsequent removal of the bubble trap from the system would prevent introduction of the bubble by the peristaltic pump that frequently occurs despite the incorporation of bubble trap. This modification will also simplify the assembling of the system, minimizes contamination and allows the pump to pull out unattached biofilm materials that may interfere with microscopy of the biofilm.

5.0 STERILISATION OF THE FLOW SYSTEM

As a measure to prevent unexpected contamination, there is need to sterilize the system after it has been set up and prior to inoculation. This can be achieved with the use of 0.5 % (v/v) sodium hypochlorite that can eliminate any microorganisms that is accidentally found within the system. This disinfectant is normally pumped through the flow system for 3 to 4 hours at a flow rate of 3 mL/h/channel. Following that, the system should be washed 2-3 times with 1.5 L sterile distilled water to get rid of the sodium hypochlorite and ensuring the bubble trap is completely rendered empty after each wash. The bubble trap can be emptied by pumping in air at high speed until it is free of all the bubbles. The system can be subsequently filled with sterilized liquid medium of interest pre-warmed to the required temperature of the experiment and allowed to run over night in order to saturate the tubing before the inoculation [2, 27].

6.0 INOCULATION OF THE FLOW SYSTEM

Basically, the flow cell can be inoculated using two methods. Some researchers combine the two methods in a single biofilm experiment. It is important to consider an inoculation method that has high tendency to avoid contamination and introduction of bubble to the flow

system since these are two critical parameters in inoculating the flow system. In the first method, a medium of interest is pumped into the flow chamber. Subsequently, overnight culture that has been diluted to the required optical density (OD) is loaded into the flow chamber using 0.5 mL syringe or 1 mL insulin syringe and 27G or 21G needle [20, 27]. The peristaltic pump is stopped and the silicone tubing leading to the flow cell is clamp off to prevent back flow. This is followed by the sterilization of the inoculation site on the tubing with 70 % or 96 % ethanol. Subsequently, the needle is insert into the silicone tubing to ensure the tip of the needle get to the inlet of the flow cell before injecting the inoculum. Care must be taken while injecting to avoid bubbles formation. After the inoculation, the needle is removed and the injection site is wiped with 70 % or 96 % ethanol followed by immediate sealing of the hole with silicone glue. The flow cell should be turn over for 1-2 hours without any media flow to allow the microbes to attach to the surface [2, 9, 20]. The media flow can be subsequently started and the system can be incubated at temperature of 37 °C and 30 °C for the *Escherichia coli* and *Pseudomonas aeruginosa* respectively [27]. Incubation can also be done at room temperature depending on the temperature requirement of the organism used for the experiment.

The second method of inoculating the flow cell is by inoculate 500 µL of a starter culture into 200 mL of a suitable nutrient medium and shaken overnight. This culture can subsequently be inoculated into intermediate tank containing the same nutrient medium and incubated at 30 °C with agitation too. It is also important to aerate the intermediate tank using an air pump. The OD and glucose concentration will be monitored from the recirculating tank at the required density before feeding into the flow cell. The total volume in the recirculating tank should be kept constant through over flow [28]. This second method is more preferable to the earlier method since it does not involve the risk of contamination and introduction of bubble as is obtainable in the insertion of needle.

7.0 STAINING THE BIOFILM FOR CONFOCAL LASER SCANNING MICROSCOPY

Confocal laser scanning microscopy (CLSM) with the aid of fluorescent stains can be excited by light is most suitable for imaging of biofilms grown in flow system [29]. A dilution of appropriate stain such as propidium iodide (PI), SYTO9 and Calcofluor White are required for an excellent visualization of the biofilm grown in the flow cell. In order to apply the stain, the peristaltic pump should be stopped and the tubing leading to the flow chamber should also be clamp off followed by sterilization of the inoculation site by wiping it with 70 % ethanol. A 0.5 mL syringe and 27G needle should be subsequently carefully inject the stain into the flow chamber. Then, the injection site should be sealed back with silicone glue and the flow cell allowed to stay for 15 minutes before resuming the flow rate of 3 mL/h. The image(s) can be subsequently acquired with the appropriate microscope [27]. Figure 2 shows confocal laser scanning image of *P. aeruginosa* biofilm.

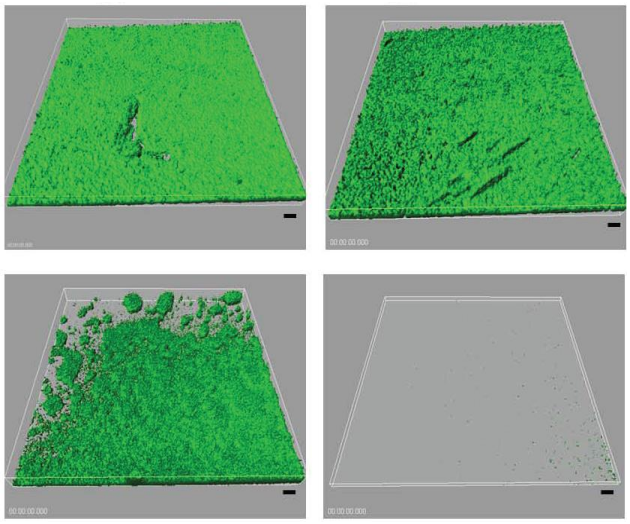

Figure 2 Confocal laser scanning images of *P. aeruginosa* biofilm [30]

8.0 CONCLUSIONS

Biofilm is the predominant natural microbial life style that exerts both negative and positive effects on man and the surrounding biotic and abiotic surfaces. Investigating these complex communities under adequately monitored conditions in laboratory will established a good understanding of their inherent ability to form multicellular communities. Though flow cell technology offer many advantages in biofilm studies, the novice biofilm researchers frequently faced series of challenges that ranges from bubble formation within the system and subsequent destruction of the biofilm structure to contamination of the flow system in their attempt to apply the flow cell technology to the their research. Detail description of the principles, operating procedures as well as recent advancement in this technology will make it more beneficial to both the novices and the experience scientist in the field of biofilm research. Future studies that will innovate useful sophisticated technique as well modifications capable of addressing the current challenges of the existing flow cell technology will increase our reliable insight in to hydrodynamic biofilms and their control.

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