

# Development of an *in Vitro* Novel Device that Simulates the Real Life of the Biofilm Formation on Catheters under both Static and Continuous Fluid Flow Systems

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**Abstract** Biofilm model systems are essential to explore the development and the nature of the microbial community within the biofilm as well as the mechanism of their resistance. The aim of this work is to develop a simple *in vitro* novel device which mimics the real life of the biofilm formation and could be modulated to contain most catheter and tubes and readily allows biofilm formation under different experimental conditions. Two clinical isolates, *Staphylococcus epidermidis* and *Candida albicans*, were used to validate the device. The viability of the microorganisms within the biofilm was demonstrated quantitatively by viable count and semi-quantitatively by using Scanning Electron Microscope and Confocal Scanning Laser Microscope. The shear stress on the inner and outer surfaces of the catheter was determined at different flow rates of the culture medium. The presented device supports biofilm formation of the tested microorganisms under static and dynamic fluid flow systems. The results are comparable to that of other biofilm models. The number of cells contained in the biofilm under static system was significantly higher than that of the biofilm which formed under dynamic system for both microorganisms. For *S. epidermidis*, the log value of the number of cells contained in the biofilm under static system was  $6.41 \pm 0.22$  compared to  $5.18 \pm 0.13$  of the biofilm which formed under continuous fluid flow system ( $p < 0.001$ ). For *C. albicans*, the log value of the number of adherent cells was  $6.44 \pm 38$  and  $5.47 \pm 0.05$  respectively ( $p = 0.012$ ). The presented well suited to study the real life of the biofilm formation by microorganisms. It enables the formation of a reproducible biofilm of bacteria and yeast on the catheter surface in both static and dynamic systems and its design permits low laminar flow system.

**Keywords:** *In vitro* biofilm model, Continuous fluid flow, Catheters, *Staphylococcus epidermidis*, *Candida albicans*, laminar flow

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## 1. Introduction

Biofilm formation commonly associated with many infections and health problems are usually a serious concern for clinicians because of their resistance to a wide range of antibiotics [1,2] and to clearance by humoral or cellular host defense mechanisms [3]. It has been estimated that 65-80% of all human infections are biofilm-related [4]. The problem of treating biofilm-associated infections is not only attributed to the difficulty of eradicating the biofilm focus but also to the lack of susceptibility of cells disrupted from the biofilm to antimicrobial agents [2]. Because of the evident relationship between various infectious diseases and the presence of the microorganisms in the biofilm, there has been an escalation in the interest in studying the microbial biofilm and its impact in human health.

*In Situ* and *in vivo* experimental studies of the biofilm in humans are difficult to approach due to limitation in accessing and sampling, the difficulties of standardization and control of assay conditions as well as the complicated ethical aspects [5]. Animal models, on the other hand, are useful in studying the pathogenicity and to search for compounds with therapeutic potential against biofilm [6,7,8].

Starting with the simple microtiter plate assay and scanning electron microscopy, tools for studying biofilm formation *in vitro* have since evolved to include specialized equipments such as flow cell systems and real-time analysis using reporter gene assays [9]. Several *in vitro* models have been described to study the formation of microbial biofilms and the factors that affect their behavior under static or dynamic conditions. The tube test as well as microtiter plate assay were among the earliest methods described for assessing microbial biofilm formation under static system [10,11]. Variations in data

between and within different laboratories when using the tube test and the microtiter plate assay have raised a lot of concerns [11]. In addition, a number of ELISAs (Enzyme-linked immunosorbent assays) for investigating factors influential in biofilm have been also developed [12].

Although they are routinely used when evaluating biofilm structure and susceptibility to antimicrobial agents, *in vitro* models that adopt the static system seem to be misleading if compared to the real dynamic condition. Static system does not take into account key factors such as the fluid shear stress which affects the adhesion and biofilm formation by microorganisms.

One of the most used dynamic models to study the biofilm mode of growth is the flow cell system [7]. This system was first developed as Robbins Device (RD), modified Robbins Device (MRD) [13] and recently as commercially available flow cells [14,15,16]. Most of the commercially available devices are expensive, have minimum configuration limits and are designed to test the biofilm on small segments of the catheter materials rather than to use the whole catheter. To simulate the real environment of the biofilm on catheters, it would be advantageous for the design of the model to allow for the insertion of the catheter in its original state. The physical configuration of the studied catheter such as the diameter and length are important especially when studying the effect of fluid flow on biofilm.

The aim of this work is to develop a simple *in vitro* novel device which mimics the real life of the biofilm formation and could be modulated to contain most catheter and tubes and readily allows biofilm formation under different experimental conditions.

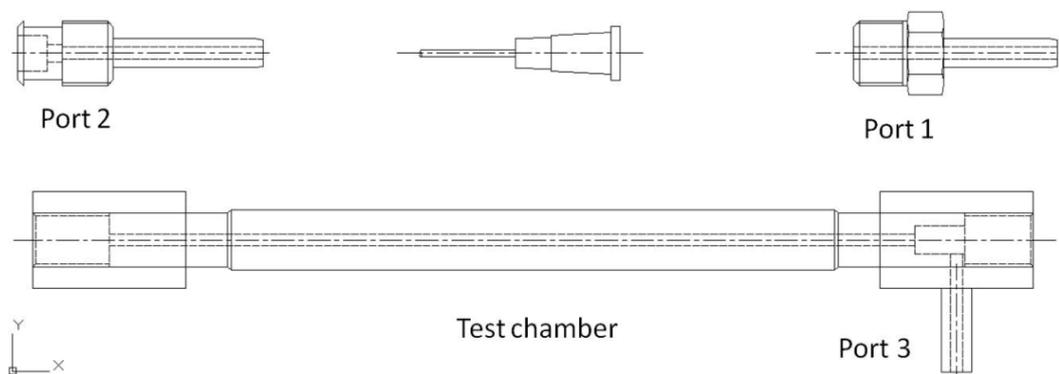


Figure 1. Design of the biofilm device

The various parts of the device may be fabricated from stainless steel, Pyrex glass or other materials which do not affect the formation of the biofilm.

### 2.3. Configuration of the Device

The device can be set up flexibly in different configurations to suit the studied conditions as follow:

#### 2.3.1. Static System

The device can be configured to test the biofilm under static condition by simply using it as a closed system (Figure 2A). Ports 2 and 3 are blocked while port 1 is used to inoculate the chamber and provide the system with the culture medium.

## 2. Materials and Methods

Unless otherwise indicated, all chemicals (analytical grade) were purchased from Sigma-Aldrich, USA.

### 2.1. Microorganisms

Two clinical isolates, *Staphylococcus epidermidis* and *Candida albicans*, were used to validate the ability of the device to support biofilm formation on intravenous (IV) catheter. The microorganisms were isolated from bloods of patients with central venous catheters. *S. epidermidis* was identified using conventional microbiological techniques and *C. albicans* was identified to species level by using API 20 C AUX for yeast (bioMérieux).

### 2.2. Design of the Biofilm Device

The device comprises a tubular body defining a test chamber (Figure 1 & supplemented video file). The size of the test chamber is of varied height within the range of about 10-50 cm, and the diameter is in the range of about 0.8-2.5 cm. The volume of the test chamber is in the range of about 5-20 ml. The body has upper and lower ends provided with closures. Each of the end has one port which can be used as an outlet or inlet based on the studied conditions. The body has a built in side port with the all three ports can be connected to a tubing system or blocked by the removable closures. The ports in the upper and lower ends of the device are designed to mount the tested catheter. The design allows the fluid to be pumped through the inner lumen of the implant tube before filling the inner chamber to allow biofilm formation on the inner and outer surfaces of the catheters.

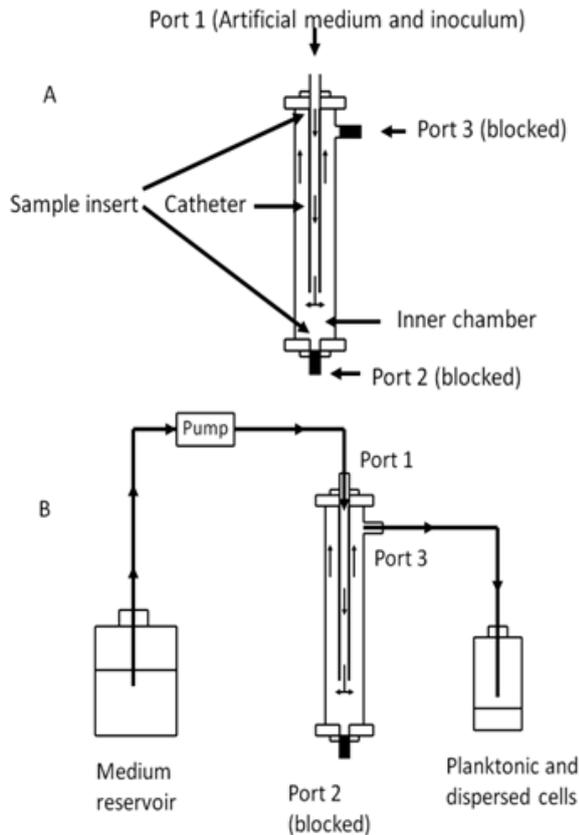
#### 2.3.2. Dynamic Cell Flow System

The biofilm can be kept under continuous flow of fresh medium provided from a reservoir via IV infusion pump and IV tubing set. The medium is allowed to flow through the lumen of the catheter before passing to the chamber. Port 1 is used as the inlet, port 2 is blocked while port 3 is used to collect the overflowed liquid which contain planktonic and cells dispersed from the biofilm (Figure 2B).

#### 2.3.3. Sterilization of the Device

The device and reservoir were sterilized by autoclaving before insertion of the catheter and connection to the infusion pump via sterile IV tubing set. During experiment,

the whole system was kept in laminar flow cabinet to avoid contamination.



**Figure 2.** Configuration of the biofilm device under static (A) and dynamic fluid flow systems (B)

## 2.4. Validation of the Device to Support Biofilm Formation

The device was configured to test biofilm formation of *S. epidermidis* and *C. albicans* under static and dynamic conditions as mentioned above. For biofilm formation under static condition, 24 hours old culture of *S. epidermidis* on Tryptic Soya Agar (TSA) or *C. albicans* on Sabouraud Dextrose (SAB) agar medium were used to inoculate Tryptic Soya Broth (TSB) or Yeast Nitrogen Base (YNB) media supplemented with 2% dextrose respectively. The initial inoculum size was standardized in the media to give  $1-5 \times 10^6$  CFU/ml of the tested microorganisms. Peripheral venous catheter BD Angiocath, reference number 382259 (Becton, Dickinson and Company, USA) was placed in the sample insert. The culture medium, with either microorganism, was pumped into the catheter to fill the chamber. The device was then incubated at 37°C for 48 hours after which the catheter was washed by passing sterile phosphate buffered saline (PBS) (pH 7.3) at 30 ml/hour for 2 hours to remove the planktonic (free-floating) cells. The catheter was aseptically removed using sterile forceps and cut to 1 cm segments in microbiological safety cabinet. The biofilms on the catheters were washed and dislodged by sonication in 1 ml ice-cooled PBS at 30% cycle, 3.5 output for 30 seconds followed by vortexing for another 30 seconds. The number of sessile cells was determined by viable counts on SAB agar or TSA medium and calculated as colony forming unit (CFU)/cm<sup>2</sup> of the catheter surface.

In another set of experiments, the device was configured to test the biofilm formation under dynamic condition. Briefly, the culture media, supplemented with  $1-5 \times 10^6$  CFU/ml of the tested isolate, was delivered to the catheter at 30 ml/h for 2 h. The planktonic cells were removed by delivering sterile media at the same rate for another 2 h. The biofilms on the catheter were then kept under continuous flow of fresh medium at 10 ml/h for 48 hours. The catheters were then cut to 1 cm segments, and the biofilms were dislodged and sessile cells were determined as previously mentioned.

The device was compared to biofilm devices which support biofilm formation under static condition, the disc model [17], and continuous fluid flow system, the MRD.

## 2.5. Scanning Electron Microscopy

The Scanning Electron Microscope (SEM) was used to visualize the biofilm architecture of *S. epidermidis* and *C. albicans* which formed on the vascular catheters under dynamic conditions. Catheters, from the experiment in which the biofilms were formed under continuous flow of the medium, were aseptically cut to 1cm segments and prepared for Scanning Electron Microscope examination as previously described [18]. Briefly, they were fixed in glutaraldehyde in 0.1 M cacodylate buffer containing 0.15 ruthenium red for 3 h at 4°C. The segments were then rinsed in fresh 0.1 M cacodylate buffer for 10 min (repeated three times) and post-fixed in 1.5% osmium tetroxide for 1 h. They were dehydrated in a series of aqueous ethanol solutions (30–100%) and dried by a critical point dryer (Autosamdri) with CO<sub>2</sub>. The specimens were mounted on aluminium stubs with silver paste, allowed to dry for 3 h and then coated with gold/palladium using a cool-sputter coater E5100 II (Polaron Instruments). The segments were then examined in a SEM (S-500; Hitachi) at 20 kV.

## 2.6. Confocal Scanning Laser Microscopy (CSLM)

The viability of *S. epidermidis* cells within the biofilm environment was demonstrated using Confocal Scanning Laser Microscopy (CSLM). Catheter segments on which the biofilms were formed under continuous flow of the medium were used. Briefly, the catheter segments were transferred to a tube containing 1 ml ice-cooled saline and the adherent cells were dislodged by sonication. Hundred microliter portion of the suspension was transferred to a cover slip, and the dislodged cells were stained with LIVE/DEAD BacLight bacterial viability stain (Molecular Probes, Eugene, OR, USA) following manufacturer's instructions. Finally, the sample was examined by Olympus Fluoview CSLM (model IX 70, Olympus America Inc. NY, USA).

## 2.7. Determination of the Shear Stress of Fluid Flow on the Catheter Surfaces

The shear stress on the inner and outer surfaces of the catheter was determined at different flow rates of the culture medium (1-100 ml/h). The flow type is either laminar or turbulent based on the value called Reynold's number ( $R_e$ ), where  $R_e$  is calculated using the following equation: [19].

$$R_e = \rho u D / \mu$$

Where  $\rho$  and  $\mu$  are the fluid density and viscosity,  $u$  is the velocity and  $D$  is the catheter diameter.

The shear stress can be calculated using the following equation if the flow is laminar:

$$\text{Shear stress (Newton, N m}^{-2}\text{)} = KQ$$

Where  $k$  is a constant which depends on the catheter diameter, fluid density and viscosity and  $Q$  is the volume flow rate (milliliter/hour).

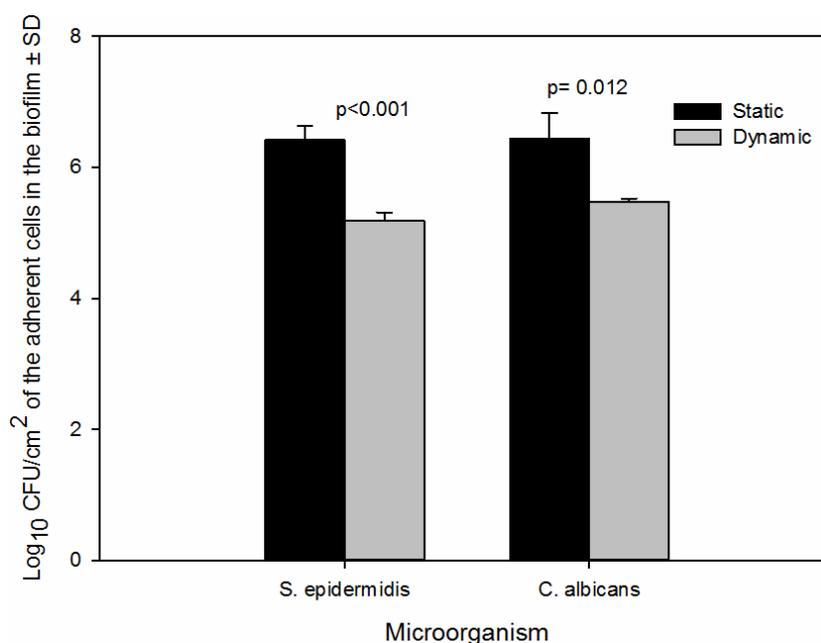
## 2.8. Statistical Analysis

Each experiment of the biofilm formation was performed in quadruplicate and the mean and the Standard Deviation

(S.D.) were calculated. One-way analysis of variance (ANOVA) was used to determine the differences between various treatments. Tukey's pair comparison test was used at the chosen level of probability ( $P < 0.05$ ) to determine significance difference between means.

## 3. Results

The biofilms of the tested microorganisms on the catheter were evaluated quantitatively by viable count technique and semi-quantitatively by visualizing the biofilm communities by SEM and CSLM. The device was found to support the biofilm formation of the tested bacteria and yeast under both static and continuous fluid flow systems (Figure 3).



**Figure 3.** Validation of the device to support biofilm formation

When comparing the means of log values of microorganisms contained in the biofilms, difference in the number of adherent cells was demonstrated in the two systems. For *S. epidermidis*, the log value of the number of cells contained in the biofilm under static system was  $6.41 \pm 0.22$ . This value was significantly higher ( $p < 0.001$ ) than that of the biofilm which formed under dynamic system ( $5.18 \pm 0.13$ ). Significant population variability ( $p = 0.012$ ) was also observed with the biofilm of *C. albicans*

where the log value of the number of cells was  $6.44 \pm 0.38$  and  $5.47 \pm 0.05$  under static and dynamic conditions respectively.

The device was compared to two biofilm models devices that adopt static or continuous fluid flow system under the same tested experimental condition, the disc and MRD respectively. The device showed better support to the biofilm at the tested conditions (Table 1).

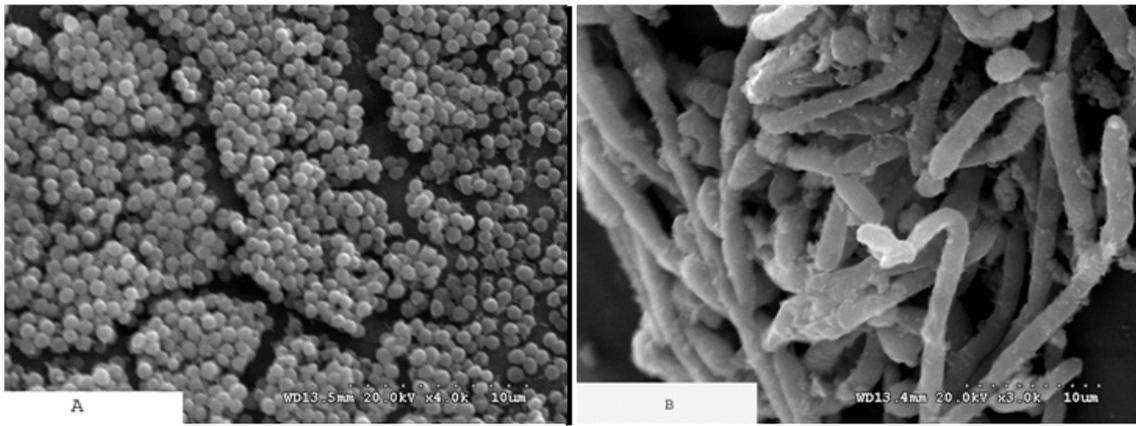
**Table 1. Comparison of the device to other biofilm-supporting devices**

Microorganism	Log Colony Forming Unit (CFU) of cells in the biofilm /cm <sup>2</sup> of catheter surface ± SD			
	Static System		Dynamic System Model	
	Disc Model	Presented model	Modified Robbins Device (MRD)	Presented model
<i>Staphylococcus epidermidis</i>	7.40±0.218	6.41±0.220	4.28±0.185	5.18±0.131
<i>Candida albicans</i>	5.12±0.461	6.44±0.386	4.35±0.150	5.47±0.0529

The device was compared to 2 biofilm models devices that adopt static or continuous fluid flow system under the same tested experimental condition, catheter materials, inoculum, incubation and biofilm assay. The polypropylene disc and the MRD models allows for testing the biofilm on catheter segments while the device allows for testing the whole catheter.

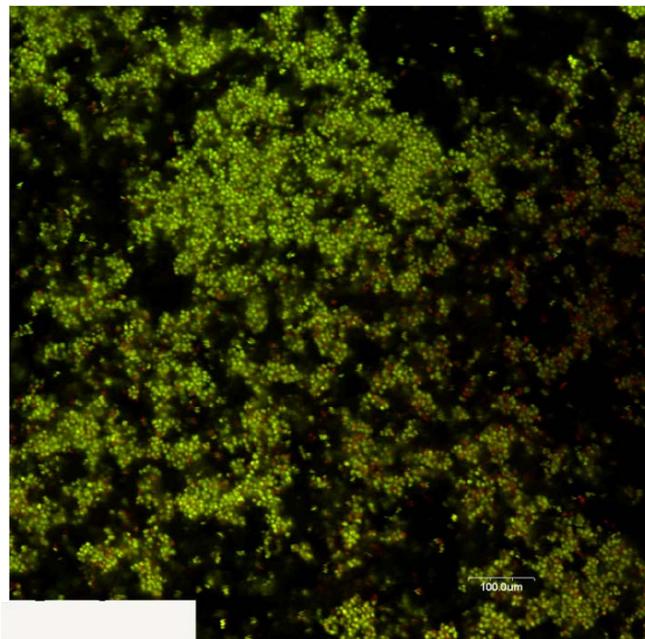
Further endorsing evident for the ability of the device to support biofilm formation was also concluded from SEM (Figure 4 A & B). The biofilm of *S. epidermidis* appeared as homogenous thick layers while the biofilm of *C. albicans* consisted predominantly from filamentous

growth with few yeast cells. Similarly, CLSM (Figure 5) demonstrated the viability of the majority of *S. epidermidis* cells, stained with green fluorescence, within the disrupted biofilm compared to low number of dead cells stained with red fluorescence.



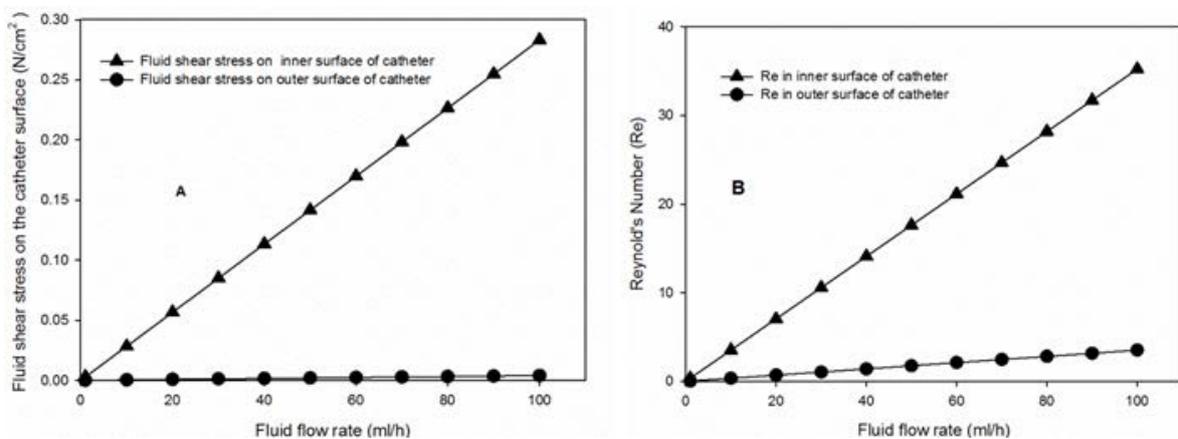
**Figure 4.** SEM showing the biofilm architecture of *Streptococcus epidermidis* (A) and *C. albicans* (B) on vascular catheter segment using the biofilm device

Note that the biofilm of *S. epidermidis* appears as homogenous thick layers of adherent cells while that of *C. albicans* appears as filamentous form with few yeast cells.



**Figure 5.** Visualization of the viability of *S. epidermidis* cells within the biofilm environment using Confocal Scanning Laser Microscopy (CSLM)

CSLM images of *S. epidermidis* disrupted biofilm on plastic cover slips. The bacterial cells were stained with LIVE/DEAD BacLight bacterial viability stain to directly visualize the biofilm on the catheter. The green fluorescence reflects processing of the dye by metabolically active cells while the red fluorescence is characteristic of dead cells. Note that the green fluorescence is predominant compared to very few red dead cells.



**Figure 6.** Determination of the fluid shear stress on the inner and outer surface of catheter at different flow rate of the media

The values were measured based on the Reynold's number ( $R_e$ ). Laminar flow was evident where the ( $R_e$ ) values were very low (less than 2100) (A). The shear stress was calculated using the equation: Shear stress (Newton)  $N m^{-2} = K Q$ , where k is a constant which depends on the catheter diameter and fluid density and viscosity and Q is the volume flow rate. Note that the values of shear stress on the catheter surfaces were remarkably low (B).

In order to measure the shear stress on the biofilm, Reynold's number ( $R_e$ ) was determined at different flow rates of the culture medium. At 1-100 ml/h, the recorded  $R_e$  values were in the range of 0.35 - 35.24 and 0.035- 3.52 on the inner and outer surfaces of the catheter respectively, which indicates a low laminar flow system. Based on the laminar flow system, the shear stress was determined on the inner and outer surfaces of the catheters where the values were in the range of 0.0028- 0.28 and  $4.01 \times 10^{-5}$ - 0.004 N/ m<sup>2</sup> respectively (Figure 6 A & B).

## 4. Discussion

### 4.1. Validation of the Device

Biofilm model systems are essential to understand the development, behavior and the nature of the microbial communities within the biofilm and the mechanism of their resistance to antimicrobial agents and host defense system. *In vitro* biofilm models are important tools in experimental medical science to better understand biofilm physiology and micro-ecology [5]. Such models may be used to simulate environmental conditions within the laboratory or to focus on selected variables such as growth rate or fluid flow [20].

The presented biofilm device is designed to mimic the real environment of the biofilm. Its design makes it easy to contain a full length catheter in the main chamber instead of using segments or piece of plastics as in other available devices. The device can also be adjusted to different configurations to fit the tested experimental conditions required to simulate and study microbial biofilm by controlling the fluid flow through the different ports. The two ends of the device are detachable and come with different sizes of sample insert to fit most types of catheters.

The use of venous catheters is well known as the leading cause responsible for health care-associated infections [21,22,23]. Therefore, IV Catheters were used in this study to test the ability of the device to support microbial adherence and biofilm formation. Catheter-related bloodstream infections affect more than 200,000 patients per year in the United States [24,25]. These infections are associated with an increased risk of death, with increases in morbidity, length of stay, and health care costs [22].

Two microorganisms, *S. epidermidis* and *C. albicans*, were selected to validate the device. Among the coagulase-negative staphylococci (CNS), *S. epidermidis* is the most frequently isolated species and the most common species responsible for infection [26]. Infections caused by *S. epidermidis* are often persistent and relapsing. *S. epidermidis* and other CNS are generally the causative organisms in the majority of device-related infections [27]. *C. albicans*, on the other hand, is the fourth leading cause of vascular catheter-related infections and the third leading cause of urinary catheter-related infections [28,29]. Among vascular catheter-related infections, those due to *Candida* spp. are associated with the highest rate of mortality [30].

The device was found to support the biofilm formation of the tested bacteria and yeast under both static and continuous fluid flow systems (Figure 3). The results were

comparable to what have been published before using other *in vitro* biofilm devices [2,31]. When compared to the disc model and the MRD, the device showed better support to the biofilm at the tested conditions (Table 1). The number of cells contained in the biofilm under static system was significantly higher than that of the biofilm which formed under dynamic system for both microorganisms. The difference could be attributed to the nature of the static system where factors such as longer contact time and sedimentation of the microorganism by gravity force could allow the microorganisms to better adhere and colonize the catheter surface. On the contrary, in continuous fluid flow system, factors such as fluid flow rate and wall shear stress may affect the adhesion, and detachment of microbial cells [5].

The ability of the device to support the biofilm formation was demonstrated using SEM (Figure 4 A & B) and CLSM (Figure 5). The micrographs showed the biofilm of *S. epidermidis* as homogenous thick layers while the biofilm of *C. albicans* as predominant filamentous growth with few yeast cells. *C. albicans* has both a budding and filamentous forms. Both forms are important for invasion and biofilm formation. The yeast form is important for dissemination in blood whereas the filamentous form is suited for invasion and propagation on tissues and surfaces. *C. albicans* biofilms incorporating both yeast and filamentous forms, with budding yeast attached to the surface and the filamentous form on top. The CLSM, on the other hand, demonstrated the viability of the majority of *S. epidermidis* cells within the disrupted biofilm compared to low number of dead cells.

### 4.2. Determination of the Shear Stress of Fluid Flow on the Catheter Surfaces

Based on recorded Reynold's number ( $R_e$ ), the design of the presented device permits low laminar flow system with very low shear stress on the inner and outer catheter surfaces (Figure 6 A & B). The wall share stress inside the flow cells is directly influencing the process of biofilm formation. It depends on the fluid flow rate (ml/h), fluid velocity (mm/s), and dimensions of the system, and acts as the main source of fluid flow forces on adhering organisms [32]. The shear stress on the inner and outer surfaces of the catheter was determined at different flow rates of the culture medium (1-100 ml/h). Various flow rate values have been mentioned in the literatures with regard to the different scientific applications of flow cell systems to simulate biofilm formation in different sites of the human body [33,34,35]. Liquid flow can be described as either laminar or turbulent based on the Reynold's number ( $R_e$ ), a function of the density, viscosity, linear distance traveled and velocity of a flowing fluid.  $R_e$  can be used to describe the hydrodynamics of a flowing system. In flowing systems with a low  $R_e$ , laminar flow may exist with little or no mixing. In turbulent flow, the liquid in the plug is rapidly mixed. Laminar flow is usually characterized by low fluid velocity ( $R_e < 2100$ ) while high fluid velocity generally leads to turbulent flow ( $R_e > 4000$ ) [19].

Increased fluid flow towards or parallel to a substratum surface results in faster adhesion of microorganisms due to higher mass transport [36]. A higher fluid shear, on the other hand, may lead to their detachment [37] and a very

high value would prevent the adherence of microbial cells and biofilm formation [38].

## 5. Conclusions

The presented device is simple and can be fabricated from cheap materials. It is not only simulating the real biofilm environments, but also could be modulated to contain most catheter and tubes and readily allows biofilm formation under different experimental conditions. The design permits low laminar flow system which is common in studying biofilms in flow cell systems. Accordingly, it is well suited to study the real life of the biofilm formation by bacteria and yeasts.

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## List of Abbreviations

ELISAs: Enzyme-linked immunosorbent assays  
 RD: Robbins Device  
 MRD: modified Robbins Device  
 IV: Intravenous  
 TSA: Tryptic Soya Agar  
 SAB: Sabouraud Dextrose  
 TSB: Tryptic Soya Broth  
 YNB: Yeast Nitrogen Base  
 CFU: Colony forming unit  
 SEM: Scanning Electron Microscope  
 CSLM: Confocal Scanning Laser Microscope  
 CNS: Coagulase-negative staphylococci.

## Competing Interest

The authors declare that he has no competing interests.

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