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Low cost PDMS based model system for biofilm studies

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Abstract
Bacterial biofilms are aggregations of bacterial cells embedded into self-produced matrix of extracellular polymeric substrates (EPS). Biofilms develop on or within indwelling medical devices which eventually lead to nosocomial infections. Here we describe the development of a PDMS-based flow cell device which mimics catheters used in hospitals. The device was fabricated using soft lithography technique. *Staphylococcus aureus* and *Acinetobacter baumanii* which are known to form biofilm and are associated with urinary tract infections were used as representative organism's to demonstrate biofilm formation in the device. Quantitative assay of biofilm formed was done using Microscopy (Z-stacking). This device can be successfully used for semi-quantitative detection of bacterial biofilms and is ideally suited for use in screening of anti-biofilm compounds.

Keywords: biofilms, flow system, Z-stacking, PDMS

1. Introduction

Biofilms are defined as microbial-derived sessile communities which consist of cells that are attached to a substratum, interface, or to each other with altered phenotypes embedded in a self-produced matrix of exo-polymer saccharides [5]. Biofilms pose serious problems in a wide range of areas, especially in the food, marine, environmental, and biomedical fields. Many outbreaks of pathogens have been attributed to biofilms, and it is estimated that biofilms account for up to 80% of microbial infections [9]. They are relevant to hospital clinicians because colonization of indwelling medical devices plays a key role in spread of nosocomial infections. According to NIH, more than 80% of nosocomial UTI infections involve catheter associated biofilms. Urinary catheters are tubular silicone devices, which when inserted may readily acquire biofilms on the inner or outer surfaces causing their blockage and nosocomial UTI. The organisms commonly contaminating these devices and developing biofilms are *S. aureus*, *S. epidermidis, Enterococcus faecalis, Acinetobacter baumannii, E. coli, Proteus mirabilis, P. aeruginosa, K. pneumoniae*, and other gram-negative organisms. Silicon is one of the most common choices for construction of catheters because of its inherent chemical and thermal stability making it inert and unreactive to body fluids and a range of medical fluids with which it might come into contact. The increasing incidences of Catheter-Associated Urinary Tract Infections (CAUTI) raises the demand for fabrication of flow devices and system setup which will mimic *in vivo* conditions and thus help in studying biofilm formation in indwelling catheters. The present indigenously fabricated device helps to address this need.

Different techniques are currently used to grow and study biofilms. Recently, with advances in microfluidic technologies, Polydimethylsiloxane (PDMS) based devices have been developed for monitoring biofilm growth. They are simple, easy-to-use and inexpensive devices for quantitation of biofilm forming-bacteria. They can be easily used to address particular experimental needs. These microfluidic devices are fabricated via photolithography and soft lithography [1, 3]. According to studies, In comparison to 96-well microtitre plates (MTP), PDMS chips are found to yield considerably higher biofilm content. An increase in the yield of biofilm is observed with both incubation time and cell density added to PDMS chip as compared to MTP type assay using either biofilm colony forming units (CFU) or crystal violet (CV) measurements at 595 nm. This may be due to the large surface area available for biofilm-formation in PDMS or potentially with the hydrophobic characteristic of the PDMS external surfaces. PDMS -silicone based polymer is optically clear which makes it a superior choice for fabrication of flow devices thus allowing microscopic examination of the films. Hydrophobicity, low surface tension and inherent chemical and thermal stability make it an excellent substrate for formation of biofilms.

The present work focuses on development of recirculating model flow system with PDMS-based device, which mimic catheters, to grow and evaluate biofilms under hydrodynamic flow conditions. Hospital isolates capable of forming biofilms were used to test this system. Also, effect of plant extracts as anti-Quorum Sensing (QS) molecules and their effect on biofilm formation was studied as a proof of concept with this model. Fabrication of this device is less expensive as compared with currently available methods and assembly of the system is easy. It is non-destructive and offers “real-time” monitoring of biofilm development.

2. Materials and methods

2.1 Fabrication of Flow Cell Device

The PDMS-based flow cell device was fabricated in-house using soft lithography technique. Thick plastic straws (7mm × 3mm) stuck to the base of glass chamber (Cuboidal glass chamber of dimensions 3 ×1.5 ×1 cm) was used as mould. 25

mL of Polydimethylsiloxane (Sylgard 184, Dow Corning, Midland, MI, USA) base and curing agent were mixed thoroughly in a ratio of 10:1, poured into the mould and degassed for 20 minutes followed by curing at 80°C for 30 minutes. It was kept overnight and then peeled off carefully from the mould. PDMS channels so formed were then bonded to PDMS coated glass slide using plasma cleaner (PCD-001 Harrick Plasma, Ithaca, NY, USA).
Irreversible bonding was achieved by exposing bonding surfaces to air plasma for 4 minutes. Thus, two parallel channels of dimension of 65 × 3 mm (length × lumen diameter) were fabricated. The device was cleaned with acetone prior to use.

2.2 Assembly of flow system.

Silicone tubings with the dimensions 1 mm inner diameter (ID) and 3 mm outer diameter (OD) were connected to each end of the flow channel. They were fixed with superglue to avoid any leakage. The system was set up in recirculating pattern. The inlet tubing from medium vessel was connected to one end of flow cell and the outlet tubing at the other end was directed back to the medium vessel. The entire system was sterilized by autoclaving at 121˚C for 15 min. The peristaltic pump (Neolab Instruments, Mumbai) was positioned in the upstream of device to drive the flow of liquid from medium vessel. Multichannel peristaltic pump was used to allow operation of two channels simultaneously.

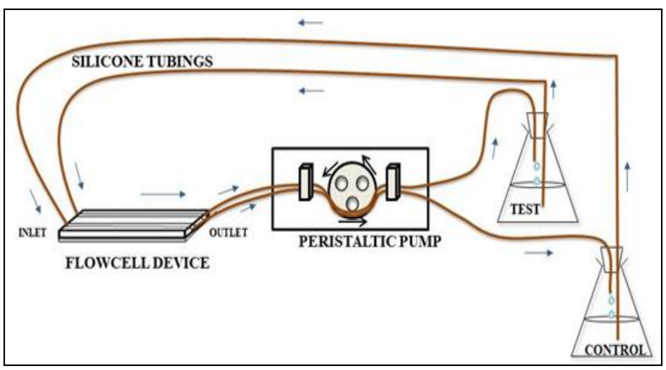

Fig 1: Assembly of recirculating flow system with PDMS based flow-cell device

2.3 Cultures Used

Two hospital isolates known to be capable of forming biofilms, *Staphylococcus aureus* K1 (SaK1) [7, 8] and *Acinetobacter baumannii* 8s (Ac8s) [3, 6] were used as representative Gram positive and Gram negative cultures respectively.

2.4 Experimental setup

The assembly was set up as depicted in Figure 1. One channel of the flow cell device was labelled as 'Test' while the other was labelled as 'Control'. Both the channels were connected to respective medium vessels containing 100ml of St. Tryptic Soy Broth supplemented with 1% glucose. The flask labelled 'Test' was inoculated with 1ml of test culture suspension of 0.1A. The flask labelled as 'Control' was kept uninoculated. The flow rate of medium through channels was kept 300µL/min and the entire system was incubated at 37°C. After 48 hours of incubation, the planktonic cell population, if any was removed from the channels by pumping sterile saline at a higher flow rate.

2.5 Assay of Biofilm Formation

2.5.1 Quantitative Assay

A. **Microscopic (Z-Stacking) Assay** Biofilm formed was visualised using The EVOS® FL Auto Imaging Microscope by mounting the device directly on the microscope stage to view the channels without staining under 10X objective. For each channel, Z stacking (Focus stacking) of 5 representative fields was done to calculate average height of biofilm formed within a channel and was repeated thrice to check its reproducibility.
B. **Crystal Violet Assay** The channels were stained with 1000µL of 0.1% Crystal violet for 15mins followed by washing with sterile saline (800µL, 8 times). The channels were then photographed for qualitative assays. For destaining, 1000µL of Alcohol: Acetone mixture (80:20) was introduced in each channel and kept for 15mins. This step was repeated three times for efficient destaining. The solubilized CV was then collected and absorbance of the same was taken at 630 nm using destaining solution as blank.

2.5.2 Qualitative Assay

A. **Observation under Fluorescence Microscope** Entire channel was viewed using 'Scan' mode of the Fluorescence microscope which gives stitched images of the full channel. The experiment was repeated three times for each culture and the entire channel was scanned each time. This helped in getting a complete picture of the entire channel and enabled comparison of biofilms produced by different organisms. It also helped in qualitatively checking the effect of plant extract on biofilm formation in comparison with the control. Initially the biofilm formed was observed in Real, Monochrome and Pseudo colour mode to check which mode was best suited for clear viewing.
B. **XTT Assay.** The channels were first washed with sterile saline. The channels were then flooded with 800µL of XTT reagent in each channel and were incubated in dark for 15-30 minutes. This assay was done only for experiments with plant extract.
C. **Live/Dead Staining.** LIVE/DEAD® BacLight™ stain was prepared as per manufacturer's instructions. 500 µL of stain was added to both the channels. Incubated for 15 minutes in dark and observed with fluorescence microscope, EVOS® FL Auto using green and red filter. Observations were done under 10X. This assay was done for effect of plant extract on biofilm formation.

2.6 Effect of Plant Extract on Biofilm Formation

Sub inhibitory concentration of plant extract was used for this experiment. According to literature, MIC of plant extract of *Terminalia chebula* (*T. chebula*) against the *S. aureus* K1 was 0.83mg/ml and *A. baumannii* 8s was 4.00 mg/ml [10]. Thus sub-inhibitory concentration MIC/4 was used for *S.aureus* K1 and MIC/8 was used for *A. baumannii* 8s to check antibiofilm activity of plant extract.

2.6.1 Experimental Setup
Four labelled flasks containing 100 mL of St. TSB were used in this experiment. To the flask labelled 'Test' plant extract of appropriate concentration and 1 ml test culture of 0.1 A was added. The other three flasks were used as controls i.e. Positive control (TSB + 1 ml of test culture), Medium control (only TSB) and Plant extract control (TSB + plant extract). All flasks were connected to their respective labelled channels. The flow rate of medium through channels was kept 300µL/min and the entire system was incubated at 37°C. Results were observed after 48 hours. Biofilm formed was quantitated using Z-stacking and values obtained were compared for control and the test with plant extract. Crystal violet assay was not done with plant extract as there is non-specific binding of crystal violet to plant extract giving erroneous results.

3. Results and discussion
3.1 Assay of biofilm formation
3.1.1 Microscopic Assay (Z- Stacking)
a) **For *A. baumannii* 8s -** The average height of the biofilm formed for *A. baumanii* 8s was 17.1 µm.
b) **For *S. aureus* K1 -** Average height of the biofilm formed for *S. aureus* K1 was 19.13 µm.

Table 1: Results of Z-stacking

	Average Height (µm)	
Strain	**Test**	**Control**
SaK1	19.13	0.0
Ac8s	17.1	0.0

This experiment shows that the device constructed in the laboratory was capable of allowing biofilm formation and quantitation. This device can be used for studying effects of various parameters on biofilm formation. Effect of plant extract on biofilm formation is shown as an example of one such application.

3.1.2 Crystal Violet Assay

Table 2: Results of crystal violet assay for quantitative assessment of biofilm

Sr. no.	Absorbance at 530 nm	
	***S. aureus* K1**	***A. baumannii* 8s**
1	0.232	0.172
2	0.189	0.160
3	0.201	0.193
Average	0.207	0.175

According to CV assay it was observed that *S. aureus* K1 biofilm was thicker than *A. baumannii* 8s biofilm which is in accordance with the results of Z stacking.

3.2 Qualitative Assay
Observation under Fluorescence Microscope in different Modes
Biofilms formed by both the organisms i.e. *S. aureus* K1 and *A. baumanii* 8s were observed and compared in different modes of EVOS FL Auto. *S. aureus* K1 biofilm was thicker and denser than *A. baumanii* 8s biofilm. Thus it was better visualised in Pseudo and monochrome mode as it enables the addition of hues and enhancement of contrast. *A. baumanii* 8s biofilm could be seen properly in all the three modes.
S. aureus K1 formed continuous biofilm with larger amounts of polysaccharide almost over entire stretch of the channel as seen in the channel scan. The biofilm formed by *A. baumanii* 8s was discontinuous and displayed a tubular network throughout the channel.
No film was observed in the control channels.
The biofilm was also seen using ordinary microscope with 10X objective and was seen very clearly. This shows that the device is suitable for studying biofilms even in laboratories with limited facilities.

3.3 Effect of plant extract on Biofilm
3.3.1 Qualitative assay
A. Live/Dead Staining
After observation under fluorescence microscope (10X), it was found that the Test (Culture + plant extract) had relatively less viable cells, less dense and more discontinuous as compared to that of control having only the culture. This indicates that the plant extract is effective in controlling the biofilm formation.

B. XTT Assay
Staining with XTT dye gave a visually detectable orange coloured formazan product within 15 minutes of incubation in dark indicating viable cells in the film formed. No colour change was observed in the control channels**.**

C. Crystal Violet assay
It was observed that crystal violet was non-specifically binding to Plant extract, therefore variable results were obtained with CV assay. There was no reproducibility in the results. Therefore CV assay was not used for experiments with plant extract.

3.3.2 Quantitative Assay
A. For *S. aureus* K1
Control which had just the culture, showed height or thickness of 20.39 µm, while the biofilm in the channel with Test (culture + plant extract), was just 5.46 µm in thickness, which is 73.22% reduction. Results of the *t*- Test show that the reduction is significant and hence it can be concluded that the plant extract of *T. chebula* is effective as an anti-biofilm forming agent against *S.aureus* K1 biofilm. (Table 3)

B. For *A. baumanii 8s*
Biofilm in the channel labelled Control showed height or thickness of 24.13 µm while the biofilm in the channel with Test (culture + plant extract) was 9.30 µm in thickness, which is 61.42% reduction. Results of *t*- test show that the reduction is significant and hence it can be concluded that the plant extract of *T. chebula* is effective as an anti-biofilm forming agent against *A. baumanii* biofilm (Table 3). This experiment also shows that the device constructed in the laboratory is capable of being used as a model flow system for biofilm

studies for various applications.

Table 3: Effect of Plant Extract on biofilm of *S.aureus* K1 and *A. baumanii* 8s

Strain	Average Height (µm)			
	Positive control	Medium control	Test	Percentage reduction
SaK1	20.39	0.0	5.47	73.17
Ac8s	24.125	0.0	9.3	61.45

3.3.2 Statistical Analysis

Table 4: *t*-Test (Two-Sample Assuming Equal Variances) for *S. aureus* K1 and *A. baumanii* 8s

Strain	Average Height (µm)			
	Positive Control	Medium control	Test	Percentage reduction
Sak1	20.39	0.0	5.47	73.17
Ac8s	24.125	0.0	9.3	61.45

Table 5: *t*-Test (Two-Sample Assuming Equal Variances) for *S. aureus* K1 and *A. baumanii* 8s

	Average Height (µm)			
	Positive Control		Test	
	S. aureus K1	*A. baumanii 8s*	*S. aureus* K1	*A. baumanii 8s*
Mean	20.39	24.13	5.46	9.30
S.D.	4.52	4.91	2.34	3.05
Observations	9.00	9	9.00	9
P(T<=t) two-tail	*S. aureus* K1 1.08266E-11		*A. baumanii* 8s 6.63446E-11	

Reproducible results obtained in above experiments with two different cultures a Gram positive and a Gram negative, indicate that this system can be used for quantitative assessment of biofilms grown under flow conditions. PDMS being optically clear allowed visualization of biofilm using microscopy. Microscopy based Z stacking or focus stacking technique used allowed non-destructive quantification of biofilm formed within the device. As compared to currently available methods, fabrication of this flow cell device is less expensive and assembly of system is easy. This system can also be used for screening of anti-biofilm agents targeting catheter associated biofilms.

Reproducible results obtained in above experiments with two different cultures a Gram positive and a Gram negative, which were proved to be statistically significant, indicate that this system can be used for quantitative assessment of biofilms grown under flow conditions. PDMS being optically clear allowed visualization of biofilm using microscopic methods. Microscopy based Z stacking technique allowed non-destructive quantification of biofilm formed within the device. As compared to currently available methods, fabrication of this flow cell device is less expensive and assembly of system is easy. Also, effect of plant extracts as anti-Quorum Sensing (QS) molecules and their effect on biofilm formation was studied as a proof of concept with this model. This makes system useful for screening of anti-biofilm agents targeting catheter associated biofilms.

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