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A novel flow-system to establish experimental biofilms for modelling chronic wound infection and testing the efficacy of wound dressings

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ABSTRACT

Several models exist for the study of chronic wound infection, but few combine all of the necessary elements to allow high throughput, reproducible biofilm culture with the possibility of applying topical antimicrobial treatments. Furthermore, few take into account the appropriate means of providing nutrients combined with biofilm growth at the air-liquid interface. In this manuscript, a new biofilm flow device for study of wound biofilms is reported. The device is 3D printed, straightforward to operate, and can be used to investigate single and mixed species biofilms, as well as the efficacy of antimicrobial dressings. Single species biofilms of *Staphylococcus aureus* or *Pseudomonas aeruginosa* were reproducibly cultured over 72 h giving consistent log counts of 8–10 colony forming units (CFU). There was a 3–4 log reduction in recoverable bacteria when antimicrobial dressings were applied to biofilms cultured for 48 h, and left *in situ* for a further 24 h. Two-species biofilms of *S. aureus* and *P. aeruginosa* inoculated at a 1:1 ratio, were also reproducibly cultured at both 20 °C and 37 °C; of particular note was a definitive Gram-negative shift within the population that occurred only at 37 °C.

1. Introduction

Chronic wounds exhibit a perpetual state of non-healing with inevitable recalcitrant infection. Biopsies of a variety of wounds have found that over 78% of chronic wounds contain biofilm, which is associated with unsuccessful anti-infective treatment (James et al., 2008; Kirker and James, 2017; Malone et al., 2017). Consequently, persons with chronic infected wounds are often afflicted for many months or years, with the most severe cases necessitating physical debridement of tissues and eventual amputation. Numerous antimicrobial wound dressings are commercially available and form a part of chronic wound management strategies. To date there are no universally accepted, robust means of testing new antimicrobial dressings for their efficacy, particularly against biofilms.

A number of *in vitro* biofilm models are available and utilised with varying success to study wound biofilms. These include the Lubbock system (Sun et al., 2008), the Modified Robbin's Device (Kharazmi et al., 1999; Millar et al., 2001), the Calgary Device (Ceri et al., 1999; Harrison et al., 2006), Constant Depth Film Fermenters (CDFF) (Hill et al., 2010), drip-flow reactors (Goeres et al., 2009), flow chamber and bubble traps (Tolker-Nielsen and Sternberg, 2014), and more recently, microfluidic systems (Wright et al., 2015). The Lubbock system and Calgary Device are static biofilm models; the former is most representative of the wound environment as biofilms are grown on filters on top of plugs of agar that are placed onto an agar-filled Petri dish which allows for the application of wound dressings. The Calgary Device allows for the culture of up to 96 biofilms in a static system, with the biofilm submerged in media, which is not truly representative of the wound environment.

Chronic infected wounds commonly produce exudate, which further complicates accurate modelling of wound infection *in vitro* (Junka et al., 2017). The Modified Robbin's Device, CDFF, drip flow reactors, flow chamber/bubble-trap systems and microfluidic devices have tried to address the requirement for flow within biofilm models and are sufficiently versatile to allow for the modelling of diverse biofilms including oral, wound, genitourinary tract and respiratory tract biofilm (Pratten, 2007; Hope et al., 2012; Diez-Aguilar et al., 2017; Melvin et al., 2017). The Modified Robbins Device, CDFF and microfluidic systems are available commercially but the initial cost of purchasing these devices and/or equipment can be prohibitive. Detailed descriptions for in-house construction of flow chamber/bubble-trap biofilm models and drip-flow reactors are available; this makes them cheaper options but requires a degree of technical expertise. Furthermore, the "home-made" nature of such devices can affect reproducibility.

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The design of several of the biofilm models, described above, are such that cultured biofilms remain submerged in media throughout experiments. This is a disadvantage for the study of wound biofilms, which are typically not submerged but grow at the air-liquid interface of the wound bed, being "fed" from beneath by wound exudate. CDFFs and drip flow reactors allow for the growth of a biofilm that is more representative of a wound and it is possible to apply wound dressings to the former. CDFFs also allow for high-throughput, reproducible biofilm growth. However, with the CDFF, all cultured biofilms are duplicates and fed through one inlet, meaning that it is only possible to study biofilms comprised of the same microorganism(s), simultaneously. Drip-flow reactors have tried to address the problem: several biofilms are cultured concurrently, but fed independently; however, cross-contamination is common (Azeredo et al., 2016).

A new biofilm flow system is presented here (Duckworth Biofilm Device; DBD), that has a series of "wells" for the growth of 12 biofilms across four separate channels. This allows triplicate biofilms to be cultured so as to prevent cross contamination between individual channels. Furthermore, the device allows ease of sampling during experiments without disrupting continuing biofilm growth. Biofilms are cultured on a semi-permeable substratum that is fed with media from beneath. Biofilms can be cultured on cellulose (MF-Millipore; cellulose acetate/cellulose nitrate) disks for recovery and enumeration, or on glass coverslips for microscopic analysis; this approach also allows for the application of wound dressings. The DBD can be produced by additive layer manufacturing and is re-usable (sterilisable by autoclave or disinfection, depending on the material; see methods). It is a single part instrument with a lid and does not require technical expertise to utilise i.e. does not need to be constructed by the user.

Herein we describe the design and preliminary testing of the DBD, which is proposed as a new biofilm flow system for the study of wound biofilms and for the testing of antimicrobial dressings.

2. Materials and methods

2.1. Device design and manufacture

Computer aided design (CAD) was undertaken using Autodesk Inventor (Autodesk Inc., California, USA). Electronic CAD files are available as both. ipt (openable using CAD software) and. stl (openable by 3D printing software). To request a copy please contact the corresponding author. Manufacture of the flow cell used in these experiments used a Renishaw RenAM 500 M (Renishaw, Wotton-under-Edge, UK) and was in aluminium alloy ($AlSi_{10}Mg$). This device was sterilisable by autoclave. Some surface tarnishing was visible following repeated sterilisation; however, there was no apparent functional loss over 50 sterilisation cycles.

The DBD has since been printed using Accura ClearVue Resin at 0.1 layers (PDR, Cardiff Metropolitan University; http://pdronline.co.uk/). This can be sterilised without affecting the dimensional accuracy of the device by formaldehyde at 80 °C, low temperature steam at 75 °C, or gamma irradiation. Decontamination of Accura ClearVue Resin devices in this study used Gerrard Ampholytic Surface Active Biocide (GASAB) disinfectant, prepared at a 1:100 concentration, as per the manufacturer's instructions (Fisher Scientific, UK). GASAB was flowed through the device at a rate of 5 mL min^{-1} for 30 min, followed by submersion in GASAB for 16 h. Following disinfection, the device was washed with sterile distilled water, at a flow rate of 5 mL min^{-1} for 30 min.

2.2. Setting up and running the Duckworth Biofilm Device

The DBD has one input portal, connected to a flask of fresh media; from the entry reservoirs, the flow splits into four separate channels (Fig. 1A and B). Spent media exits via a single portal, by peristaltic pump (MasterFlex L/S Digital Pump System with EASY-LOAD II Pump Head, Cole-Palmer) (Fig. 1C). Silicone tubing was from Cole-Palmer (13 mm, MasterFlex; London, UK) and held into the device using sterile plastic 1 mL pipette tips (Fig. 1C). Each of the four channels of the device have three biofilm support wells (Fig. 2A); these are comprised of a 1 mm "ledge" that is open to the media flowing beneath. It is necessary to fill the device with media by either pipetting into each well or by flowing the media through at a rate of 1 mL min^{-1}.

A disk of noble agar measuring 10 mm in diameter (cut from a 15 mL agar plate in a standard sized Petri dish using a sterilised steel, leather press punch) inserted into the well, rests on the support ledge, and acts as a porous matrix support for biofilm growth (Fig. 1A and 2 A). Critically, the dimensions of each well constrain the size of the agar disk meaning that the spatial position of each biofilm relative to the nutrient flow is identical. A cellulose membrane (diameter = 13 mm, pore size = 0.22 μm) on top of the disk of noble agar provides a surface for biofilm growth (Millipore, UK) (Fig. 2A). Bacterial suspension (20 μL) equilibrated to an appropriate optical density was used to inoculate the surface of the cellulose membrane. The device ran at a flow rate of 0.322 mL min^{-1} (equivalent to 0.083 mL min^{-1} per channel).

Under these conditions 500 mL of media is sufficient to complete one 24 h run. The device has a lid, which was kept in place whilst the flow cell was running. A 0.22 μm syringe filter was inserted into the aperture at the centre of the lid (Fig. 2B). Setting up and running the device as described above (Fig. 2C) allowed for the culture of 12 biofilms simultaneously without contamination of the nutrient flow. The design of the device enabled the removal and recovery of bacteria from biofilms, either simultaneously or at specific time points, without disturbing the continuing experiment.

2.3. Optimising biofilm growth

Preparation of the DBD took place in a class 2 laminar flow cabinet. Twelve agar disks were cut from a Petri dish filled with 15 mL noble agar at a concentration of 1.5% (w/v), using a 10 mm leather press punch, sterilised prior to use, by autoclave, and transferred to the device using a sterile scalpel. One cellulose disk was placed on top of the agar disks using sterile forceps; each disk was inoculated with 20 μL bacterial suspension (either *Pseudomonas aeruginosa* or *Staphylococcus aureus* individually, or a 1:1 ratio of both bacteria) equilibrated to 1×10^5 CFU. Once the lid was in place, a sterile 0.22 μm syringe filter was inserted into the aperture. The device was re-located to the bench top (20 °C) or incubator (37 °C) where the peristaltic flow rate was set to 0.332 mL min^{-1} (equivalent of 0.083 mL min^{-1} per channel). At appropriate times, the cellulose disks were removed from the top of the agar disks, using sterile forceps, and transferred into 10 mL sterile PBS. These were vortexed (2200 rpm, 20 s) to dislodge and homogenise the biofilm. Serial dilutions (10^{-1} to 10^{-12}) were prepared using PBS, and were enumerated using the total viable count method of Miles and Misra (Miles et al., 1938). At the end of each experiment, the $AlSi_{10}Mg$ device underwent decontamination by autoclaving (135 °C, 1 atm, 5 min), it was subsequently washed with GASAB and sterilised for use by autoclaving (121 °C, 1 atm, 20 min). The Accura ClearVue Resin device was decontaminated using GASAB as previously described.

2.4. Manufacture of alginate film dressings containing chlorhexidine hexametaphophate

Alginate (PROTANAL LF10/60FT (FMC Health and Nutrition, Philadelphia, USA)) (2 wt% aq.) was prepared containing chlorhexidine hexametaphosphate nanoparticles (CHX-HMP) (manufactured as previously described (Barbour et al., 2013)) equivalent to 0, 3 or 6 wt% cf. alginate. These were poured (17.5 g) into standard size Petri dishes and the water evaporated at r.t. over 3 days. These were crosslinked with the addition of $CaCl_2$ (30 mL, 0.18 M, 2 wt% aq., 25 min). The crosslinked alginate films were removed, washed with deionised water and