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Development of a mixed-species biofilm model and its virulence implications in device related infections

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ABSTRACT: It is becoming increasingly accepted that to understand and model the bacterial colonization and infection of abiotic surfaces requires the use of a biofilm model. There are many bacterial colonizations by at least two primary species, however this is difficult to model *in vitro*. This study reports the development of a simple mixed-species biofilm model using strains of two clinically significant bacteria: *Staphylococcus aureus* and *Pseudomonas aeruginosa* grown on nanoporous polycarbonate membranes on nutrient agar support. Scanning electron microscopy revealed the complex biofilm characteristics of two bacteria blending in extensive extracellular matrices. Using a prototype wound dressing which detects cytolytic virulence factors, the virulence secretion of 30 single and 40 mixed-species biofilms was tested. *P. aeruginosa* was seen to out-compete *S. aureus*, resulting in a biofilm with *P. aeruginosa* dominating. *In situ* growth of mixed-species biofilm under prototype dressings showed a real-time correlation between the viable biofilm population and their associated virulence factors, as seen by dressing fluorescent assay. This paper aims to provide a protocol for scientists working in the field of device related infection to create mixed-species biofilms and demonstrate that such biofilms are persistently more virulent in real infections. J Biomed Mater Res Part B: Appl Biomater 00B: 000–000, 2018.

Key Words: biofilm, wound dressing, lipid vesicles, Pseudomonas aeruginosa, Staphylococcus aureus

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INTRODUCTION

Modern society has benefited from the application of invasive medical devices in healthcare, treatment, and recovery of patients, for example 30 million indwelling urinary catheters were used in United States alone annually.[1] Likewise, a global increase in the use of various invasive devices, such as central venous catheters, mechanical heart valves, pacemakers, prosthetic joints, dialysis catheters, dental implants, and contact lens has been reported.[2] The primary purpose of these devices is to aid treatment and bring better healthcare outcomes to the patients, but infection associated with them have become significant.[2] Wound dressings, for example, are surface dwelling devices and have been used for the protection/healing of wounds. They are polymeric materials in contact with skin and tissue and whilst deigned to prevent infection, can often facilitate surface bacterial growth. Such microbial contamination, probably originated from patient's skin flora, increases the risk of device-induced host infection, negatively impact the quality of treatment, and potentially affect the intended function of the device. Bacterial contamination primarily exists in an extracellular polymeric substance (EPS) network, adhered onto the indwelling device surface as a biofilm.

Bacteria, of single or multispecies biofilms, recovered from infected indwelling devices mostly include *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Escherichia coli*, *Proteus mirabilis,* and *Klebsiella pneumoniae*.[3] Many bacteria achieve the mutual benefits in biofilms, from sharing of resources to avoidance of host immune clearance and a better protection against the environmental threats such as antibiotics/antimicrobials. As bacteria grow into a biofilm, the changes in gene expression and regulation often results in increased pathogenicity and overall virulence activity of biofilm, and increased antimicrobial resistance compared with free floating planktonic cells.[4,5] Device related infections involving multi-species biofilm are more persistent: it becomes critical to understand and identify the factors associated with the formation of mixed-species biofilms and their enhanced pathogenicity over the infected hosts.[6]

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Infection due to an invasive device depends on three main *in situ* factors: bacteria, device, and host, but among them the bacteria factor is of primary importance. As such, the role of bacteria in biofilm formation on a device surface, from initial surface contamination to irreversible establishment of polymeric network, is the subject of research interest. Understanding device related infections requires the development of *in vitro*/*ex vivo* biofilm models that mimic the mixed-species nature and their growth on surfaces. Many modern wound dressings claim to control local bacterial population, for example by elution of silver ions. However, the clinical evidence for the efficacy of such dressings is often mixed, with single trial studies frequently reporting success, but systematic reviews such as those by the Cochrane Library being more cautious in their conclusions.[7] It is notable that most *in vitro* evaluations of wound dressings still use very simple test methods to evaluate efficacy, such as a reduction in viable colony forming units (CFU) after immersing swatches of dressing in planktonic culture of a single species/strain of bacteria and visualization of zones of inhibition around dressings on culture plates.[8] There are more systematic tests that have been developed to quantify the general efficacy of antimicrobial fabrics, such as the Japanese Industrial Standard,[9] but these still have methodological flaws if being used to quantify the likely behaviour of a wound dressing on a patient.

The occurrence of bacteria in the biofilm state in wounds has only gained clinical acceptance fairly recently,[10,11] although the formation of biofilms on abiotic surfaces such as ventilator tubing has long been observed.[12] In particular, *S. aureus* and *P. aeruginosa* are the two bacterial pathogens most commonly isolated in many microbial studies of acute wounds, and it is generally accepted that *S. aureus* is an early stage colonizer, where *P. aeruginosa* is frequently isolated later, especially in burns.[13] Studies of chronic co-infected *S. aureus* and *P. aeruginosa* wound suggest that such coinfections are more virulent than monospecies infections, suggesting a synergistic relationship between the two organisms.[6] Gabrilska and Rumbaugh[14] review various *in vivo* and *in vitro* biofilm models of mixed-species infection ranging from very simple static models such as microtitre plate assays to the Calgary peg model, which utilizes the Robbins device for controlled nutrient feeding, to more complex models such as the Lubbock chronic wound model.[15] A burn specific biofilm model on gauze, which was designed to be transferred to a lab animal, has been developed to try and create the common microbiology of burns, the Zurich burn biofilm model.[16]

A prototype diagnostic dressing (PDD) targeting the bacterial virulence factors has been developed that has been used to test the biofilm virulence.[17] It consists of phospholipid vesicles containing self-quenched carboxyfluorescein, dispersed in a hydrogel matrix.[18] Biofilm virulence factors, such as delta toxin, phenol soluble modulin, and rhamnolipid lyse the lipid vesicle membrane thus releasing carboxyfluorescein which is diluted within the hydrogel matrix, giving a clear visual indication of the presence of critical colonization: bacterial density dependent molecular mechanisms switch on virulence factor secretion at high bacterial density.[12] The mixed-species biofilm model developed in this study was a colony biofilm model[19] and intended to test and validate the PDD, but has potential utilization for alternative wound dressings or other medical devices with antibacterial or bacterial sensing capabilities.

This article presents the methodology for preparing mixed-species model biofilms. We discuss the test results of virulence from such biofilms comprised of two strains of *S. aureus* and *P. aeruginosa* randomly chosen from a library of 20 *S. aureus* and 10 *P. aeruginosa* strains and inoculated onto a nanoporous polycarbonate (PC) membrane placed on nutrient agar. The pores allow diffusion of small nutrient molecules to the growing biofilm but impede cells spreading into the underlying agar. The PDD were placed on the 44- to 48-h aged biofilms and used to quantify the relative virulence factor production of single and mixed-species biofilms. Viable cells were also recovered from the biofilms and used to quantify the number of CFU of each species of bacteria in the biofilm of different start inocula.

MATERIALS AND METHODS

Materials

Phospholipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was purchased from Avanti Polar Lipids. Cholesterol, 5,6-carboxyfluorescein, fetal calf serum (HyClone), 4–(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), hexamethyldisilazane (HMDS), paraformaldehyde, glutaraldehyde, and agarose were purchased from Sigma Aldrich, U.K. 10,12-Tricosadiyonic acid (TCDA) was acquired from Alfa Aesar, U.K. Two types of modified HEPES buffers, containing NaCl and NaOH, with or without 50 mmol dm^{-3} 5,6-carboxyfluorescein were prepared in deionized water (18.2 MΩ cm, Millipore) and sterilized. Luria broth (LB), tryptic soy broth (TSB), brain heart infusion (BHI), Baird Parker (BP), and cetrimide culture agars were purchased from Fluka and Fisher Scientific, U.K. Broths were prepared in deionized water, sterilized, and used in liquid culture of bacteria. Likewise sterile BHI agar plates were prepared in petridishes and used as a nutrient substrate for the growth of biofilm. The dressing mold was made of a square plastic plate with an array of short cylindrical studs to create an embossed hydrogel dressing. Biofilms were formed on 19 mm diameter nanoporous PC membranes with the average pore diameter of 200 nm (Whatman). Artificial wound fluid (AWF) was aseptically prepared using fetal calf serum mixed in equal volume with a sterilized mixture of 0.85% NaCl (w/v) and 0.1% peptone (w/v), and prepared in aliquots of 500 μL small tubes followed by storage at −20°C until further use.

Preparation of vesicles

Aseptic preparation of vesicles was previously reported.[20] In summary, 100 mmol dm^{-3} of lipids, cholesterol, and TCDA in chloroform were mixed in the ratio of 55:20:25 (%vol) respectively. One milliliter of lipid mixture was dried in a vacuum desiccator before thoroughly rehydrated in 5 mL of sterile HEPES buffer containing 50 mmol dm^{-3} 5,6-

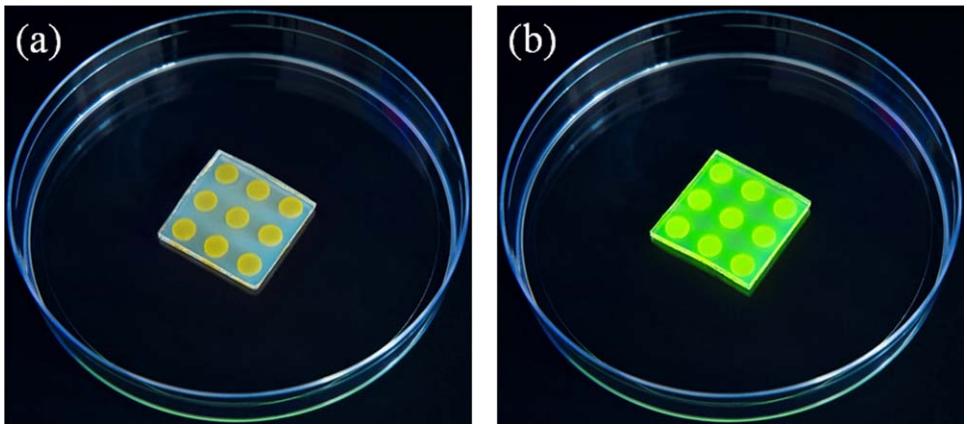

FIGURE 1. Prototype diagnostic dressing (PDD) as seen under a (254 nm) UV light showing (a) no fluorescent activation for noninfected and (b) fluorescent activation for infected conditions.

carboxyfluorescein. Following heating in hot water bath and three freeze-thaw cycles, the solution was then extruded three times at 55°C through a nanoporous polycarbonate membrane (200 nm pore) using an extruder (LF-50, Avestin). The nonencapsulated dyes were removed using gel filtration columns (DNA grade, NAP-25, GE Healthcare, U.K.) and the vesicles were stored at 4°C for 6 days before cross-linking under UV light (254 nm, Hamamatsu, Japan). Cross-linked vesicles were stored at 4°C until further use.

Prototype diagnostic dressing production

Agarose gel (2%) was used as a dressing material to hydrate the vesicles. The production of prototype diagnostic dressing (PDD) was previously described and the PDD, in activated and nonactivated forms was shown in Figure 1.[18] In short, sterilized molten agarose in HEPES buffer was aseptically molded to result a 2 mm thick square gel slab carrying an array of cylindrical wells in it. These wells were individually filled with a mixture of agarose (0.7%) and vesicles (35 μL/well).

Bacterial culture

Twenty *S. aureus* and ten *P. aeruginosa* clinical strains were used to produce the biofilm model with the two additional strains—*E. coli* (DH5α) and *S. aureus* (Agr-) for the negative controls. DH5α was a nonpathogenic bacterium used for laboratory cloning, and Agr- was a mutant strain in which pathogenic Agr activity was eliminated. *S. aureus* (MRSA 252) was also used for the initial assessment of mixed-species biofilm models. All clinical strains used in this study are listed in Table I.[4,5,21] Bacteria were individually cultured from a single colony in 10 mL of broth in a shaker incubator for 18 h at 37°C, resulting in 10^9 CFU mL^{-1} in a final culture. Then 18-h cultures of *S. aureus* and *P. aeruginosa* were diluted to 10^3 and 10^5 folds in HEPES respectively and used as initial inocula for biofilm growth.

In vitro single and mixed-species biofilm models

Thirty-two single-species biofilms (Table I) and forty mixed-species biofilms (Table II) were prepared in triplicate for this study. The protocol for making biofilms was described in our previous study.[18] Nanoporous PC membranes were disinfected in 70% ethanol for 15 min, allowed to dry and positioned on BHI agar with the smooth side facing up. Pretreatment on each membrane was 30 μL of AWF spread and dried. For single-species biofilm, diluted bacteria were premixed with sterile HEPES in equal volume and 50 μL of the mixture was used on each membrane. For mixed-species biofilms, diluted bacteria of each pair of selected strains was pre-mixed in equal volume before pipetting 50 μL of the mixture onto each PC membrane. The agar plates were then incubated at healthy skin temperature (33°C) for 44–48 h.[22]

Biofilm characterization

All biofilms were characterized by scanning electron microscopy (SEM) and plating and colony counting. For SEM study, biofilms were fixed in a mixture of 1.5% glutaraldehyde and 3% paraformaldehyde in water for an hour. They were then sequentially dehydrated in 50, 70, 80, 90 and 100% ethanol for 10 min each before drying in HMDS for 30 s. Then the biofilms were mounted on an aluminum stud and dried in a vacuum desiccator for 18 h. Following the sputtered coating with gold, they were imaged by SEM (JEOL SEM6480LV). For quantitative analysis, each biofilm was placed in a tube with 5 mL of sterile HEPES and vortexed for 1 min before further stripping in a sonicating water bath for 2 × 15 min. After an additional 1 min vortex, the biofilm extract was serially diluted in HEPES and plated on LB and TS agars for *P. aeruginosa* and *S. aureus* single-specie biofilms respectively.

TABLE I. List of Bacterial Strains Used in Development of Single and Mixed-Species *In Vitro* Biofilms (*MRSA strain)

S. aureus				*P. aeruginosa*		Controls
2	25	67	112	45124	45468	*E. coli* DH5α
3	38	69	114	45291	45498	*S. aureus* Agr-
10	49	71	126	45311	45506	MRSA 252
16	52	82*	160	45400	45666	
21	56	101	233	45445	45701	

TABLE II. List of *S. aureus* and *P. aeruginosa* Strains for Mixed-Species Biofilms

No	Strains	No	Strains	No	Strains	No	Strains
1	2 + 45498	11	82 + 45124	21	21 + 45400	31	38 + 45191
2	233 + 45666	12	16 + 45311	22	10 + 45124	32	56 + 45498
3	82 + 45291	13	10 + 45311	23	49 + 45666	33	101 + 45468
4	67 + 45445	14	160 + 45445	24	126 + 45701	34	38 + 45468
5	2 + 45400	15	3 + 45124	25	112 + 45445	35	21 + 45291
6	49 + 45311	16	71 + 45468	26	82 + 45506	36	233 + 45701
7	25 + 45666	17	49 + 45701	27	160 + 45506	37	56 + 45400
8	69 + 45400	18	16 + 45124	28	52 + 45506	38	52 + 45498
9	71 + 45311	19	25 + 45701	29	126 + 45666	39	112 + 45666
10	160 + 45124	20	114 + 45445	30	82 + 45311	40	112 + 45498

Strains were randomly selected and paired and refer to Table I for strain number and bacteria species.

For mixed-species biofilms, the extract was plated on selective BP and cetrimide agars for *S. aureus* and *P. aeruginosa,* respectively. All experiments were tested in triplicate with fluorescent, and CFU data were presented in average value with standard deviation statistically analyzed by student's *t* test.

In situ dressing response to growing biofilm study

S. aureus (MSSA 16) and *P. aeruginosa* (45311) were grown in a mixed-species biofilm at the interface between the PDD and the nanoporous PC membrane on BHI agars in a NUNC-6 well plate. A single ply sterile cotton gauze was used between the membrane and the PDD to provide a space for the biofilm to grow. While incubating at 33°C, the PDD fluorescence in triplicate was measured 4 hourly up to 36 h using a FluoStar Omega microplate reader (BMG Labtech, U.K.), with excitation and emission wavelengths of 485 nm and 520 nm, respectively. For *in situ* quantitative analysis, biofilms were grown in parallel, and each biofilm removed every 4 h for stripping, plating, and colony counting to determine the viable biofilm cells. The populations of *S. aureus* and *P. aeruginosa* in each stage of mixed-species biofilm growth was then correlated to the PDD fluorescence.

Biofilm cells reduction assay

For a therapeutic efficacy testing, the biofilm cell reduction by the hydrogel dressing containing octenidine hydrochloride was studied using two single-species biofilms of selected strains of *S. aureus* (MSSA 2) and *P. aeruginosa* (45124). Octenidine has been used as an effective antiseptic agent/coating against *S. aureus* and *P. aeruginosa* biofilms.[23,24] Two types of hydrogel, with and without 2% octenidine hydrochloride were provided from Scapa Healthcare (First Water) U.K. Aquacel Ag^{TM} (1.2% ionic Ag from ConvaTec) in hydrated form was used as a positive control. Each hydrogel, cut into 3 × 3 cm^2 was rehydrated in 1.5 mL sterile HEPES for 30 min. Rehydration aids hydrogels better release of octenidine. Likewise, each Aquacel Ag^{TM} dressing, cut into the same size was rehydrated in 1 mL of sterile HEPES. Then 24-hold 12 single-species biofilms in triplicate were transferred onto hydrated hydrogels, Aquacel Ag^{TM} (positive control) and agarose gel (negative control) before incubation for 24 h at 37°C. Hydrogels were removed in the end and biofilms were stripped for plating and colony counting.

RESULTS

Evaluation of mixed-species biofilm

Despite using the equal initial inocula, *P. aeruginosa* had reached a biofilm population 5 to 6 orders of magnitude greater than *S. aureus*. Figure 2(a–c) shows 48-h old, mixed-species biofilms grown from a fixed initial inoculum of *S. aureus* (10^5 CFU) with the varying inocula (10^5, 10^3, and 1 CFU) of *P. aeruginosa*. A relatively higher inoculation ensured *S. aureus* to be able to compete with *P. aeruginosa* in biofilm. It appeared that *S. aureus* colonized the surface first, followed by a late growth of *P. aeruginosa* as seen in Figure 2. Equal initial inocula produced the biofilm with excess *P. aeruginosa*, but two orders of magnitude higher initial inocula of *S. aureus* over *P. aeruginosa* provided the biofilms with a balanced population. Hence the initial inocula of *S. aureus* (10^5) and *P. aeruginosa* (10^3) CFU per membrane were used in this study.

Characterization of single and mixed-species biofilms

SEM revealed the biofilm of cocci (*S. aureus*) and rod (*P. aeruginosa*) bacteria in Figure 2(d,e). It shows a mixed-species biofilm with an abundant EPS network. *S. aureus* appeared in small clusters as well as within the larger agglomerates of *P. aeruginosa*. The reason for scattered biofilm appearance of *S. aureus* was not well understood but the motility might be the reason for a broader coverage of *P. aeruginosa*, compared with the nonmotile *S. aureus*.

Single-species biofilms and their virulence activity

Figure 3(a) shows the viable cell count recovered from eight selected single-species biofilms (the remaining results can be found in Supporting Information Figure S1). It was noticed that a matured biofilm always reached an average cell density of 10^{10} CFU which was one order of magnitude higher than the same species cultured in planktonic mode (planktonic data not shown). The lipolytic activity of these biofilms, quantified by fluorescent activation of PDD, was shown in Figure 4(b). The virulence effect of the identical strains cultured in both planktonic and biofilm modes was also measured. About the half of *S. aureus* strains in

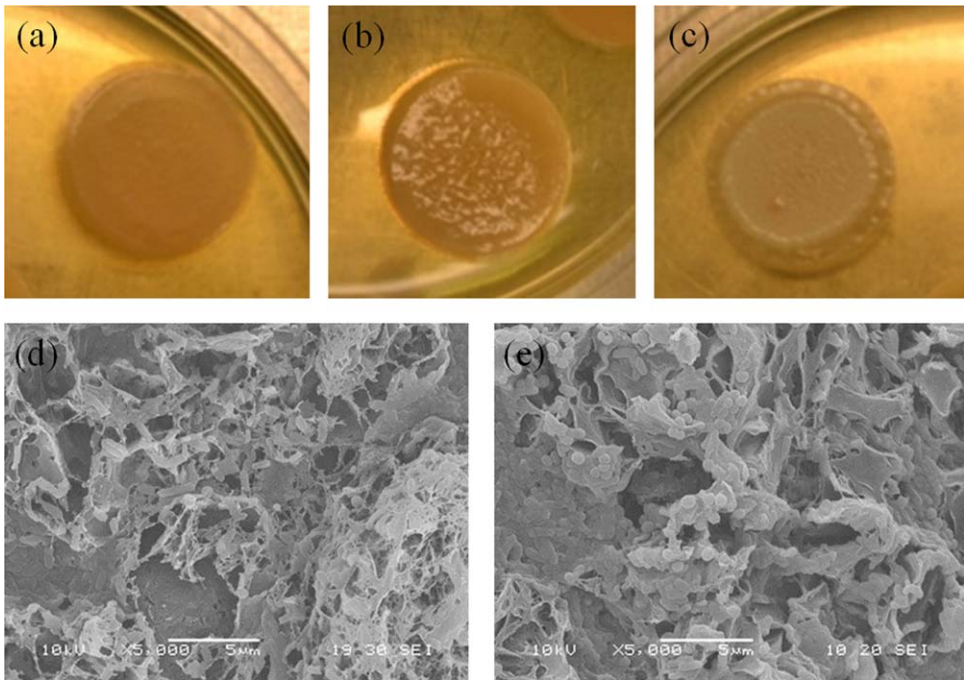

FIGURE 2. Effect of equal and unequal initial inocula of *S. aureus* (MRSA 252) and *P. aeruginosa* (45445) in 48 h mixed-species biofilms: (a) Equal initial inocula of *S. aureus* and *P. aeruginosa* (10^5 CFU each), (b) Unequal inocula of *S. aureus* (10^5 CFU) and *P. aeruginosa* (10^3 CFU), and (c) Unequal inocula of *S. aureus* (10^5 CFU) and *P. aeruginosa* (1 CFU). Note an inner circle of uneven biofilm surface in (b) belongs to *S. aureus* colonies (white) which was later outgrown by *P. aeruginosa* colonies (yellow) to the edge of membrane. SEM images of (d) single-species biofilm of *P. aeruginosa* (45311) and (e) mixed-species biofilm following an initial inoculation of 10^5 CFU of *S. aureus* (MSSA 16) and 10^3 CFU of *P. aeruginosa* (45311). All biofilms were grown on nanoporous PC membrane on BHI agar surface at 37°C for 48 h (Scale bar: 5 μm).

planktonic growth showed virulence, but the same strains when grown into single-species biofilms elicited a weaker virulence response (Figure 4). In contrary *P. aeruginosa* strains, regardless of the growth mode, showed a high virulence, as measured by the PDD.

Mixed-species biofilms and their virulence activity

Forty mixed-species biofilms were grown (Supporting Information Figure S2) and most of them displayed an inner circular zone where *S. aureus* grew before being outgrown by *P. aeruginosa*. The viable cell counts recovered from five selected mixed-species biofilms were presented in Figure 3(b) (the remaining data in Supporting Information Figure S3). Most of them triggered the fluorescent response, above 40% threshold, in 24 h [Figure 5(a) and the remaining data in Supporting Information Figure S4]. PDD fluorescence, as seen under a UV lamp, in response to the controls and the selected mixed-species biofilms were also shown in Figure 5(b,c). It was also noticed that *S. aureus* strains which previously were not much lytic against PDD in single-species biofilms were found to be associated with virulence in mixed-species biofilms. The increase in virulence activity of mixed-species biofilm was seen in *P. aeruginosa* (45291) with *S. aureus* strain of MSSA 82 (Figure 5). These *S. aureus*

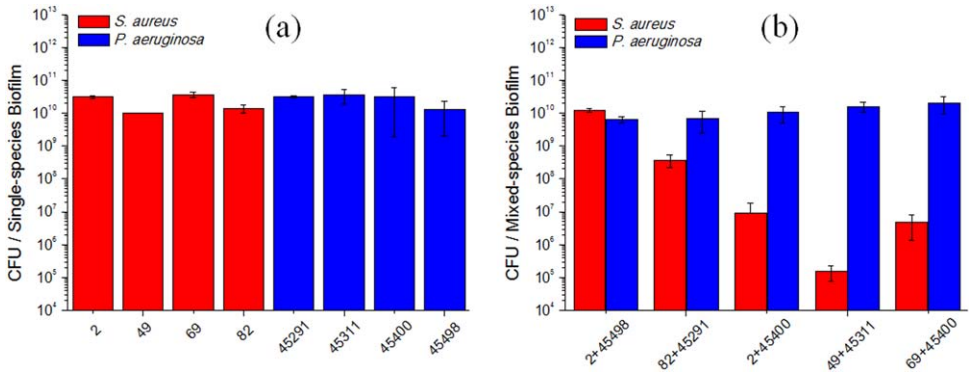

FIGURE 3. (a) Total number of viable cells (in CFU/biofilm) recovered from eight selected single-species biofilms and (b) five mixed-species biofilms. Despite using higher the initial inocula of *S. aureus*, CFU of *S. aureus* in mixed-species biofilms vary, in comparison to CFU of *P. aeruginosa*. The remaining CFU data of all single and mixed-species biofilms were presented in Supporting Information Figure S1 and S3 respectively.

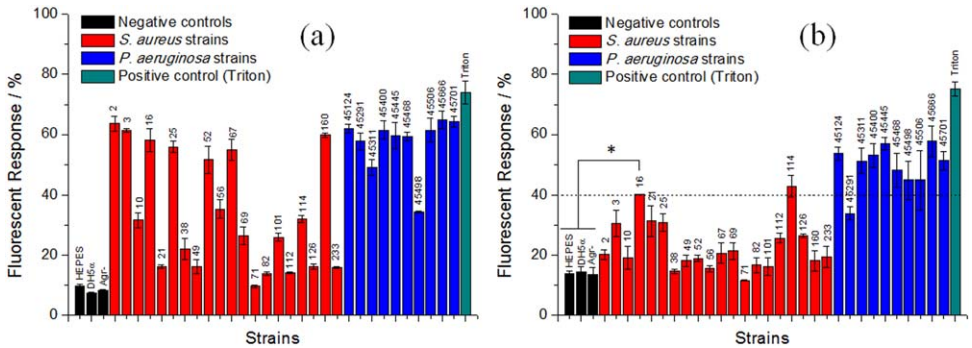

FIGURE 4. Effect of bacterial growth mode on fluorescent response of lipid vesicles. (a) Fluorescent response of lipid vesicles after incubation with planktonic culture of 20 *S. aureus* in TSB and 10 *P. aeruginosa* strains in LB (18 h culture) for 24 h, and (b) fluorescent response of PDD to single-species biofilms after incubation at 33°C for 24 h. Biofilms were formed at 33°C for 44 h before putting them on PDD. Fluorescent response was calculated with respect to the reference dressings containing 250 μ*M* 5,6-carboxyfluorescein. Fluorescence above the dotted line exhibits visible switch on from PDD (*$p < 0.05$). Numbers on top of each column represent the respective strain number of the bacteria tested.

and *P. aeruginosa* strains in their single-species biofilm were relatively less virulence and only activated the PDD fluorescence less than the threshold fluorescent response [Figure 4(b)], but their resultant response was >40% when they were grown in mixed-species biofilms. Similar increase in the PDD fluorescent was also observed in *S. aureus* 49 and 69 strains when they were grown into mixed-species biofilms. It was hypothesized that the virulence secretion is closely related to the population density of bacteria.[25] Employing quorum sensing (QS) mechanism, a biofilm cells awaits to reach a threshold population which then infects and causes an irreversible damage to the host if the infection is left untreated.[26,27]

Real-time PDD response to biofilm cells (*in situ* analysis)

As most of *P. aeruginosa* strains out-competed *S. aureus* in mixed-species biofilms, a time-dependent study of *in situ* PDD response to biofilm growth was carried out using (16 + 45311) as a mixed-species model. *S. aureus* (MSSA 16) was selected as it displayed relatively high response (Figure 4). Figure 6(a) shows the *in situ* biofilm cell counts and the respective fluorescent response in every 4 h. Both bacteria showed a growth retardation between 4 and 8 h before exponential growth. *S. aureus* (MSSA 16) growth reached to a turning point after 16 h, reaching a peak CFU of 10^9 before a declining down to 10^6 in the end. Interestingly, at the 16 h time point, *P. aeruginosa* (45311), with only a population of 10^7 CFU outnumbered and started its elimination against a relatively larger population of *S. aureus*. *P. aeruginosa* however reached and maintained above 10^{10} CFU after 20 h of incubation. Figure 6(a) also depicted the kinetic response to growing biofilms *in situ*. The fluorescence response was observed when CFU of both species reached 10^9 after 16 h of incubation.

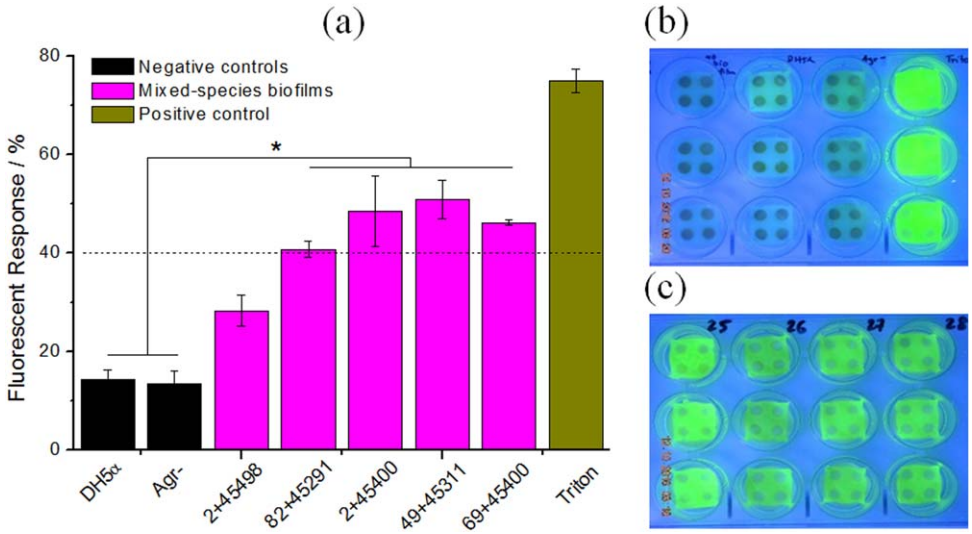

FIGURE 5. Fluorescent response of PDD after incubation with mixed-species biofilms at 33°C for 24 h. (a) Fluorescent response of PDD in triplicate to each mixed-species biofilm is calculated based on positive control dressing with 250 μ*M* 5,6-carboxyfluorescein. Dotted line indicates the notable dressing switch on (*$p < 0.005$). (b) Control plate with no biofilm, *E. coli* (DH5α) biofilm, *S. aureus* (Agr-) biofilm and positive control Triton (first to fourth columns, from left to right) and (c) PDD fluorescent response to selected mixed-species biofilms (right).

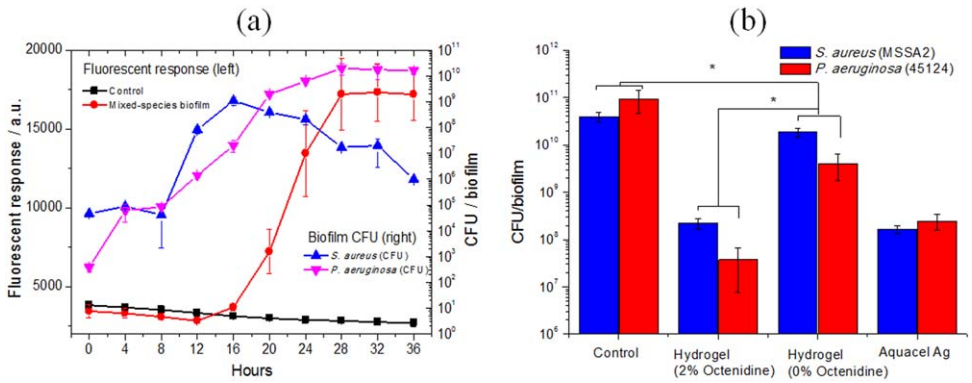

FIGURE 6. (a) Time dependent variation of biofilm cells (CFU) and *in situ* fluorescent response of PDD throughout the biofilm formation of *S. aureus* (MSSA 16) and *P. aeruginosa* (45311) in mixed-species biofilms and (b) Reduction of viable biofilm cells (CFU) in single-species biofilms of *S. aureus* (MSSA 2) and *P. aeruginosa* (45124) using 2% octenidine hydrochloride containing hydrogel. Negative controls are biofilms only and biofilms on neutral hydrogel. Positive control is biofilms on silver containing fabric (Aquacel Ag^{TM}). All tests are in triplicate. 2% Octenidine containing hydrogel reduced the viable biofilm cells (CFU) to two orders of magnitude lower than controls, showing slightly more effectiveness against *P. aeruginosa* single-species biofilm of *P. aeruginosa*. (*$p < 0.005$)

Biofilm cells reduction assay

It is accepted that the biofilm cells are much more resistant to antibiotics/antiseptics than free-floating planktonic cells of same species.[28] To realize the concept of therapeutic dressing, an attempt was made to see the effect of octenidine hydrochloride treatment in two single-species biofilms and the results are shown in Figure 6(b). It was observed that 2% octenidine containing hydrogels reduced viable cells count of both biofilms by approximately two orders of magnitude compared with control hydrogels without octenidine. Octenidine also showed more effectiveness against *P. aeruginosa* than *S. aureus*. The efficacy of octenidine against biofilms was similar to the positive control Aquacel Ag^{TM} dressings.

DISCUSSION

This study pointed out the growth-mode-dependent virulence activity of bacteria. In single-species biofilms, most of *S. aureus* appeared to be relatively less virulent than the biofilms of *P. aeruginosa* despite biofilm population reached above 10^{10} CFU (Figure 3). Such a growth-mode-dependent virulence could be explained by a genetic regulation followed by QS. In *S. aureus*, accessory gene regulator (*agr*) system controls the downregulation of virulence genes as bacteria cells grow into biofilm, resulting in the reduced virulence activity.[12,29]

The fluorescent activation of each PDD in Figure 5(a) was directly related to the virulence factors associated with biofilms.[18] Previous *P. aeruginosa* studies suggested that rhamnolipid was identified as the primary virulence factor which were highly cytolytic against lipid bilayer membranes.[18] *P. aeruginosa* deploys rhamnolipid as the biosurfactants to internalize the host cell membrane[30] and for the uptake of hydrophobic molecules for the biofilm growth.[31] It was also reported that *P. aeruginosa* used two QS systems, namely *las* and *rhl* to control the expression of coding Type 4 pilus-dependent twitching motility which played a vital role in facilitating host infection.[32] Despite *las* activity of *P. aeruginosa* biofilms decreased with time, the *rhl* system responsible for the expression of rhamnolipid, remained active for more than a week.[33] This might explain the active virulence activities of *P. aeruginosa* in all growth conditions as observed in Figure 4.

In mixed-species biofilms, *P. aeruginosa* clearly outcompeted *S. aureus* regardless of the unequal initial inocula, resulting in the imbalance biofilm cell population, and it was the direct outcome of the competition between *S. aureus* and *P. aeruginosa* [Figure 3(b)]. The multifactorial mechanism of *P. aeruginosa*-mediated elimination of *S. aureus* in cystic fibrosis infection was proposed[34] and *P. aeruginosa* secreted a whole range of molecules to overcome *S. aureus,* including anti-staphylococcal inhibitor molecules,[35,36] proteases,[37] and extracellular virulence factors.[38] Such enhanced biofilm virulence was attributed by the co-growth and competition between two species of bacteria.[39,40] *P. aeruginosa*-mediated elimination of *S. aureus* was population dependent, as it occurred when both biofilm cells reached 10^8 CFU in *in situ* growth.

CONCLUSION

A method development of a simple and portable mixed-species biofilm has been reported. The primary limitation of growing an *in vitro* mixed-species biofilm with a balanced population of both *S. aureus* and *P. aeruginosa* was overcome by a method of unequal initial inocula. Mixed-species biofilms comprising 10 *P. aeruginosa* and 20 *S. aureus* clinical strains were grown on nanoporous PC membranes and tested. Biofilms grown for 48 h evidenced the cohabitation of both resident species growing together within the EPS network, with the population of *P. aeruginosa* higher than *S. aureus* in most biofilms. Using 30 single and 40 mixed-species biofilms, the effects of biofilm growth modes in their biofilm virulence activities were tested using an assay of colorimetric PDD containing lipid vesicles.

It was observed that the *P. aeruginosa* was the key pathogen, dominating the overall virulence of mixed-species biofilms. Such a virulence attenuation was strain-dependent and related to the strains of species and the viable bacteria population. *In situ* growth of a selected mixed-species biofilm correlated between the viable biofilm population and their time-dependent activation against the lipid vesicles in PDD. A hydrogel containing 2% octenidine was found to be able to reduce biofilm cells in single-species biofilms of both bacteria within 24 h interface. Such an *in vitro* mixed-species biofilm model could be the choice for researchers in their biofilm related clinical studies for the device related infections.

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