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Attachment and optimization of *Staphylococcus aureus*, *Staphylococcus epidermidis,* and *Pseudomonas aeruginosa* biofilms to a 3D printed lattice

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ABSTRACT

A lattice was designed and fabricated using three-dimensional (3D) printing that allows for the facile transfer of biofilms formed from either *Staphylococcus aureus*, *Staphylococcus epidermidis*, or *Pseudomonas aeruginosa* into a fresh cell culture flask. To enhance biofilm production onto the filaments, three protein-based treatments were compared: fetal bovine serum (FBS), bovine serum albumin (BSA), and fibrinogen (Fb). Protein treatments included either supplementing the growth broths or pre-coating the lattice prior to immersion into the broth. *S. aureus* and *P. aeruginosa* biofilms were observed on all tested filaments that contained the supplement Fb. *S. epidermidis* required BSA to form biofilm. Ultimately, polycarbonate (PC) was chosen as the optimal material for lattice creation since it can be autoclaved without warping key design features. In addition, this 3D printed design may facilitate biofilm transfer from the bacterial culture to different cell culture platforms.

1. Introduction

Bacterial biofilms created *via* different methods have been widely studied for decades. A significant amount of literature is available describing the different methods for developing biofilms (Lebeaux et al., 2013). Various *in vitro* and *in vivo* models have been used to elucidate how biofilms cause pathogenesis in mammals (Buhmann et al., 2016; Colomer-Winter et al., 2019; Lebeaux et al., 2013; MacKintosh et al., 2006). Each type of biofilm model has its advantages and disadvantages; for example, static models allow detailed studies of biofilm formation, yet there is a limit to nutrients in these cultures and measurable time points. On the other hand, the open or dynamic models allow continuous biofilm formation and possibilities for assessment of different aspects of the biofilm system, but they demand specialized equipment and technical skills. Models for mimicking the complexity of the *in vivo* setting are rather complicated (Gutierrez-Murgas et al., 2016; Lebeaux et al., 2013; Leipert et al., 2017; Pericolini et al., 2018).

Several static biofilm models used in conjunction with studies of immune cell responses have been described in the literature. For most of these models, a bacterial biofilm is typically grown onto the surface of a material such as an agar plate (Schommer et al., 2011), filter disk (Hernandez-Jimenez et al., 2013; Mittal et al., 2004; Mittal et al., 2006), within a cell culture well plate, or on various materials (Mittal et al., 2004; Mittal et al., 2006). What can be challenging to find in some of these reported studies is the level of washing applied prior to biofilm transfer. Effective washing of the biofilm is necessary to remove planktonic bacteria. Vigorous washing steps may lead to loss of the biofilm prior to transfer to secondary cell culture and further study. The extracellular matrix produced by a biofilm is frequently fragile leading to challenges during handling. A secondary challenge with biofilm models is the challenges associated of the issue of lack of reproducibility (Azeredo et al., 2017).

In this work, we sought to achieve two primary objectives. The first was to 3D print a lattice that allowed attached biofilm to undergo the necessary vigorous washing steps for removing planktonic cells prior to placement in cell culture without losing or destroying the biofilm. The secondary objective was to assess the reproducibility of the biofilm formation and attachment process onto the 3D printed material after completion of the washing step. To help create a transferrable and reproducible biofilm formation platform, 3D printed materials were considered, compared, and then tested as they can be rapidly made to fit specific design needs for a biofilm transfer scenario. A significant advantage of using 3D printing is the availability of low-cost materials with different chemistry. Inexpensive 3D printers are common in many

Abbreviations: PP, polypropylene; TPU, thermoplastic polyurethane; PETG, polyethylene terephthalate glycol; PLA, polylactic acid; PC, polycarbonate; LF, layfoam; T_m, melting point; T_g, glass transition temperature..

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different research settings allowing for rapid and uniquely designed product creation without the need for significant specialized equipment or skills. More importantly, various design features (brush-like filaments in this work) can be added into the print enabling vigorous washing of the biofilm and enabling the facile transfer of the biofilm to different cell culture types. Individual filaments were analyzed for cost-effectiveness, biofilm attachment affinity, and temperature resistance needed for autoclave sterilization. For the third objective, different protein supplements were evaluated for their ability to promote significant biofilm attachment onto the 3D printed devices. Each added protein supplement, fetal bovine serum (FBS), bovine serum albumin (BSA), and fibrinogen (Fb), was used to obtain an attached biofilm that had reproducible biomass with three different bacterial species commonly observed as biofilms in healthcare settings - *Staphylococcus aureus*, *Staphylococcus epidermidis,* and *Pseudomonas aeruginosa*. There are several protein supplements that have been described in the literature for promoting biofilm attachment such as FBS and BSA. BSA can be used to increase biofilm attachment in *S. epidermidis* (Linnes et al., 2012) and 30 *v*/v% of FBS improves biofilm growth for different bacterial species (Leonhard et al., 2018; Linnes et al., 2012).

2. Materials and methods

2.1. Materials

Staphylococcus epidermidis (35984), *Staphylococcus aureus* (25923), and *Pseudomonas aeruginosa* (700829) were purchased from ATCC (Manassas, VA, USA). The following items were procured from Becton Dickinson (BD, Bacto, Franklin Lakes, NJ, USA): tryptic soy broth (TS), brain heart infusion broth (BHI), and agar. Fibrinogen (Fb) fraction 1 was purchased from Chem-Impex International Inc. (Wood Dale, IL, USA). Fetal Bovine Serum (FBS) from Sigma-Aldrich (St. Louis, MO, USA), and Bovine Serum Albumin (BSA) were obtained from Rockland-Inc (Pottstown, PA, USA). Hank's solution was obtained from Lonza (Basel, Switzerland). Filaments (all 1.75 mm o.d.) for 3D printing were sourced as follows: Polypropylene (PP) from Verbatim (Boulevard Charlotte, NC, USA), blue polyurethane (TPU) from Hatchbox (Pomona, CA, USA), clear polyethylene terephthalate glycol (PETG) from eSun (Shenzhen, China), lay-foam (LF) from Kai Parthy CC-Products (Koeln, Germany), white polylactic acid (PLA) from eSun, and clear polycarbonate (PC) from Gizmo Dorks LLC (Temple City, CA, USA). Table 1 shows the filaments and their chemical properties that were used in this study.

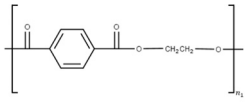
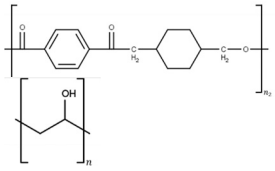
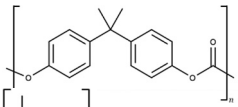
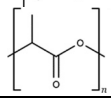
2.2. Optimization of the experimental bacterial plate

All bacterial species obtained from ATCC were received as lyophilized powders. A stock solution was prepared by suspending the powder in 5 mL of either TS (*S. epidermidis*) or BHI (*S. aureus* and *P. aeruginosa*) followed by 24 h of incubation at 37 °C. After the incubation period, the stock was centrifuged at 1200 RPM (337 xg RCF), the supernatant was decanted, and 1.5 mL of fresh media with 30% glycerol was added and the stock was stored at −80 °C. Bacterial cultures were prepared by removing a sample from the stock with the edge of a wood stick, then inoculation into 5 mL disposable culture tubes and incubating with either BHI or TS for 24 h at 37 °C. The bacterial count was approximated using measurements of the resulting cultures at 600 nm optical density (O.D.) with a target value of 1.0. A streak plate was prepared using the appropriate bacterial culture with either BHI or TS agar plates (See Supplemental Information Figs. S1-S3). The plates were incubated for 24 h at 37 °C and stored in the refrigerator at 4 °C for one week. This resulted in a bacterial plate allowing a single colony of either *S. epidermidis, S. aureus,* or *P. aeruginosa* to be obtained.

2.3. Optimization of culture medium and filaments

Filaments tested to determine biofilm adhesion included PP, TPU, PETG, PLA, and PC (Table 1). A pre-coating method was compared to a control that did not include protein precoating. All the filaments were tested with – (1) a pre-coating with FBS and (2) a non-coated method.

Table 1
Polymer filaments assessed for use in 3D Printing.

Name	Name/Structure	T_g(°C)	T_m(°C)	Properties
Polypropylene (PP)		−10	170	Hydrophobic, flexible
Thermoplastic polyurethane (TPU)	Proprietary (highly dependent on chemical formulation)	~60*	< 210*	Flexible, varied hydrophobicity
Polyethylene terephthalate glycol (PETG)		75	220	Hydroscopic
Lay foam (LF) (Fiber + PVA)		80	200	Unknown/proprietary fiber encased in polyvinyl alcohol (PVA). PVA is water-soluble.
Polycarbonate (PC)		147	155	Hydroscopic, rigid, and autoclaved printed structures do not deform.
Polylactic acid (PLA)		60	180	Hydroscopic

* Dependent on the chemical formulation. The exact chemical formulation of Hatchbox TPU is not known.

The pre-coated method included incubating the polymer filament in a solution containing 10*v*/v% FBS and 90 v/v% TS (*S. epidermidis*) or 10v/v% FBS and 90 v/v% BHI media (*S. aureus* and *P. aeruginosa*) for 24 h at 200 RPM (Benchtop shaker) at room temperature. Following the pre-coating incubation period, the coated filament was immersed into fresh culture medium containing either 5 or 10 mg/mL of fibrinogen (Fb) with the appropriate broth (TS or BHI). Then, with a single swipe of the intended bacterial colony, which was obtained from the previously prepared bacterial plate, a solution was prepared. The filament was incubated within this solution at 37 °C at 200 RPM (Benchtop shaker) for an additional 24 h.

Non-coated filaments were prepared *via* filament incubation in the culture medium with a supplement of either 5 mg/mL or 10 mg/mL of Fb and a single swipe of the respective bacterial colony for 24 h. To promote biofilm formation, Fb was optimized to an initial 5 mg/mL of Fb using published information (Bonifait et al., 2008). Comparisons of biofilm growth were performed for protein pre-coating *vs.* non-coating methods using five primary filaments (not 3D printed) with varied supplemental concentrations of Fb, FBS, and BSA within the bacterial culture. The FBS and BSA concentrations were optimized using information from other published works (Leonhard et al., 2018; Linnes et al., 2012).

The optimized concentration of Fb was found to be 7 mg/mL with 1% *w/v* NaCl. Sodium chloride was used to increase the solubility of Fb by increasing the ionic strength of the solution. The concentrations of FBS in the culture medium tested were 10%, 20%, and 30% *v*/v%. FBS-containing solutions of 10%, 20%, and 30% of FBS were only performed with *P. aeruginosa*. Due to the high cost of FBS, all experiments were modified and BSA was substituted into the bacteria culture media to promote biofilm formation. Variable concentrations of BSA such as 1%, 2%, and 3% w/v were also explored. Ultimately, the optimized culture medium contained 7 mg/mL of Fb with 3% w/v BSA and 1% w/v of NaCl.

2.4. Biofilm growth lattices

Biofilm growth lattices were printed simultaneously in batches of 30 on a Prusa i3 MK2.5 3D-printer from Prusa Research (Prague, Czech Republic) modified to have a 3030-aluminum frame. A 0.25 mm brass nozzle from E3D Online (Chalgrove, Oxfordshire, United Kingdom) was used for all prints. Simplify3D (Cincinnati, OH, USA) was used as the slicer. Growth lattices were printed using PC filament. Table 2 gives the biofilm growth lattice print settings. Fig. 1 shows a diagram of the biofilm growth lattice geometry. The lattice uses a 24.25 mm long double-sided array of 0.5 mm wide hooked prongs, which are only two extrusion widths thick (0.5 mm) to minimize the size of the lattice and increase surface area for biofilm attachment. The entire structure is 100 mm long and 1.75 mm wide. The print bed surface is composed of polyetherimide (PEI) and then coated with a layer of Elmer's glue stick, which is primarily composed of polyvinyl alcohol (PVA). Heating the print bed to 110 °C along with the PVA coating was required to promote the adhesion of the PC to the print surface. This assists in reducing print failure due to print detachment from the print bed. The design is thin and printed flat on the print bed to avoid a large temperature gradient across the print. Any temperature gradient across the print can cause warping of the printed PC, and subsequently, cause print detachment and failure. It is advised to use a heated print chamber to decrease this temperature gradient. However, this is not required for these print settings and geometry.

Table 2
Important simplify 3D print profile settings for polycarbonate (PC).

Settings Tab Location	Setting	Value
Extruder	Extrusion Width (manual)	0.25 mm
Layer1	Primary Layer Height	0.18 mm
	Top Solid Layers	5
	Bottom Solid Layers	5
	Outline/Perimeter Shells	11
	First Layer Height	75%
	First Layer Width	105%
	First Layer Speed	25%
Temperature	Heated Bed	110 °C
	Primary Extruder	270 °C
Cooling	Fan, Layer 1	0%
	Fan, Layer 2+	30%
Speeds	Default Printing Speed	50 mm/s
	Outline Underspeed	50%
	Solid Infill Underspeed	40%
	X/Y Axis Movement Speed	60mm/s
Advanced	External Thin Wall Type	Allow Single Extrusion Walls
	Internal Thin Wall Type	All Single Extrusion Fill
General Settings		
No Supports		
No Raft		

2.5. Autoclave 3D design

Non-printed filaments (lattice filament) and 3D printed lattices were individually packed in aluminum foil prior to autoclaving. The autoclave was set up at 121 °C for 30 min. Materials incompatible with autoclave conditions were submerged in 70% ethanol made with autoclaved water for 24 h prior to the experiment and then irradiated using a UV lamp for 20 min.

2.6. Biofilm disruption

After incubating the 3D printed lattice in the appropriate bacterial broth for 24 h to obtain a biofilm, the lattice with accompanying biofilm was removed. Following removal, the attached biofilm was rinsed using a syringe containing 3 mL of Hank's solution to remove planktonic bacteria. The attached biofilm was then placed into a 5 mL solution of Hank's and incubated at 37 °C for 3 min. To disrupt the biofilm to allow biomass measurement, the attached biofilm was incubated in 37 °C autoclaved water. This was followed by 2 min in a sonic bath followed by vortexing. Aliquots (200 μL) of the remaining fluid were measured at 600 nm.

2.7. Data analysis

All assays were repeated in triplicate and the data is represented as the mean ± SD. Statistical analysis was performed using ANOVA with the SigmaPlot software package (Version 14, site license to the University of Arkansas). Statistically significant values were defined as $p < 0.05$.

3. Results and discussion

Different polymeric filaments (Table 1) were initially tested to determine if biofilm would attach to them prior to the 3D printing. When 3D printing for biological applications, there are a few critical chemical and physical properties to consider: glass transition temperature (T_g), melting point (T_m), hydroscopy, flexibility, and the ability to withstand autoclave conditions. Materials that could not be autoclaved were irradiated using a UV lamp and submerged in a 70% ethanol/water (*v*/v) solution. Some filament materials exhibit hydroscopy resulting in occasional chaotic bubble formation from the water throughout the plastic material, leading to significant print defects or failures either during or after 3D print. A flexible material requires lower print speeds as it can easily bend and wrap itself around the extrusion gearing causing a print failure. For printing, the T_g is important for improving print bed adhesion as the cooling of the filament during the print can cause detachment and print failure. For autoclaving, the T_g needs to be considered as any T_g below approximately 120 °*C* will deform or completely lose the printed geometry during the sterilization process. The exception to this rule is a

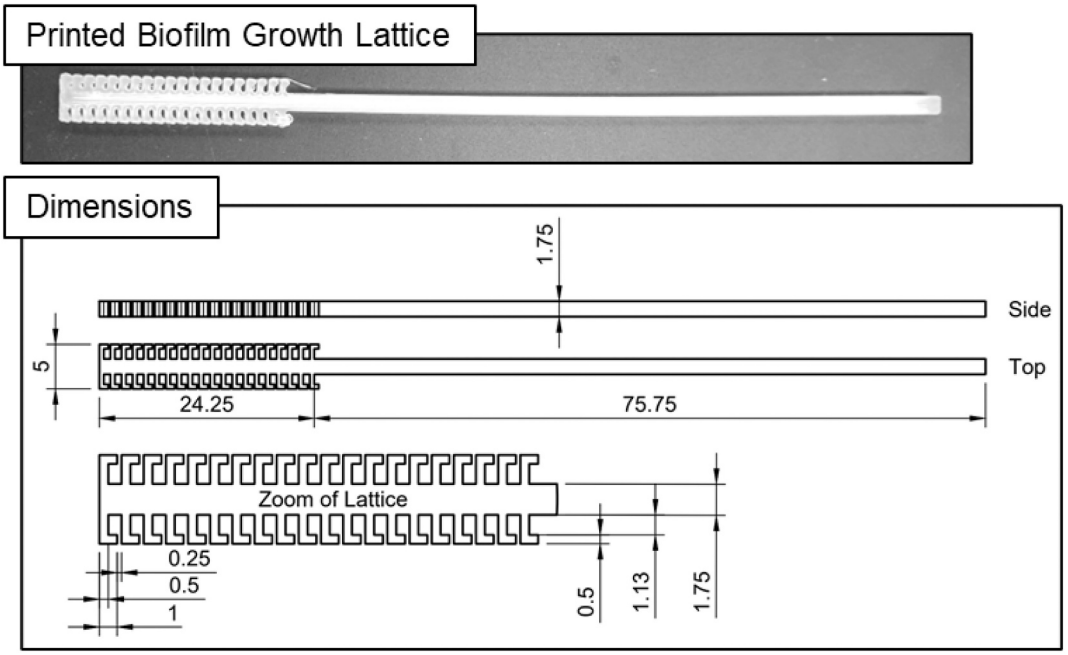


Fig. 1. Biofilm growth lattice geometry. All dimensions are in mm.

flexible filament, such as PP, where the T_g is −10 °C. However, PP deforms when autoclaved. The only plastic used in this research capable of maintaining the printed geometry throughout the autoclaving process is PC due to its high T_g of 147 °C. The T_m is only considered based on the limitations of the extruder and printer being used. In the case of this research, the maximum possible print temperature is 300 °C. Out of all the filaments, PLA is the most printed material as it does not warp due to polymer expansion and contraction during heating and cooling but will warp considerably under autoclave conditions with a T_g of 60 °C. After studying the different filaments, a wide variety of supplements were considered to enhance biofilm formation.

In this study, fibrinogen (Fb), fetal bovine serum (FBS), or bovine serum albumin (BSA) were used with the 3D printed filaments to determine which protein coating would be optimal for biofilm attachment.(Leonhard et al., 2018; Linnes et al., 2012) The bacterial species *S. aureus*, *S. epidermidis,* and *P. aeruginosa* were used due to their human health relevance with respect to device infection and since the longer-term goal is to transfer these into human macrophage cell lines for further study (Bryers, 2008; Srivastava and Bhargava, 2016; Watters et al., 2016).

3.1. Optimization of culture medium broth

To obtain optimum planktonic growth data, both TS and BHI were tested as media for culturing *S. aureus, S. epidermidis,* and *P. aeruginosa*. Optimum broths were those that exhibited the highest turbidity following a 24-h incubation. Table 3 shows *S. aureus* and *P. aeruginosa* achieved maximum growth of ~1.0×10^9 CFU/mL (O.D. 600 nm, 1.2) in BHI at 37 °C at 200 RPM (Benchtop shaker), while *S. epidermidis* showed maximum growth at ~6.0×10^8 CFU/mL (O.D. 600 nm 0.8) in TS at 37 °C at 200 RPM (Benchtop shaker). Thus, *S. aureus* was cultured using BHI, *S. epidermidis* was cultured using TS and *P. aeruginosa* was cultured using BHI as a culture medium.

Table 3
Bacteria growth after 24 h of incubation at 37 °C, $N = 3$.

Bacteria	Broth	O.D. 600 nm ± SD.
S. aureus	Tryptic Soy	1.20 ± 0.09
	Brain Heart infusion	1.4 ± 0.09
S. epidermidis	Tryptic Soy	0.80 ± 0.05
	Brain Heart infusion	No growth
P. aeruginosa	Tryptic Soy	0.80 ± 0.05
	Brain Heart infusion	1.1 ± 0.4

3.2. Optimization of culture medium to increase biofilm formation

After determining the optimum growth media for each species of bacteria, biofilm growth trials were performed. When filaments were incubated in a culture medium without any supplements (control), no observable biofilm growth onto the filament was observed (Table 4). For this reason, the addition of additional protein (BSA, Fb, and/or FBS) to the media was used to promote biofilm formation. Fb was used as in the medium because others have demonstrated the use of Fb to promote biofilm attachment (Colomer-Winter et al., 2019; Xu et al., 2017). After Fb was added to the culture medium, additional supplements (FBS and BSA) were evaluated. Commonly used filament materials for 3D printing (Table 1) were tested with the various media and bacterial species to test whether biofilm would attach to the lattice filament.

Two experimental trials were performed to assess the level of observable biofilm formation onto the filaments: non-coated and pre-coated filaments. The non-coated lattice filaments were prepared by incubating them in broth containing both Fb (7 mg/mL) and bacteria (one colony), Table 4. Pre-coated lattice filament was prepared by incubating the filament in broth containing Fb (7 mg/mL), then relocating it into a fresh broth medium with the bacteria (one colony) after 24 h of incubation. A visual inspection scoring system denoted in Fig. 2 was used with the biofilms to assist in differentiating each culture method.

For the protein pre-coated filaments which were incubated in media with Fb for 24 h and then transferred to a fresh inoculated bacteria culture, there was no observable biofilm attachment onto these materials. However, biofilm formed on filaments placed into bacterial broths supplemented (non-coated method) with protein as shown in Table 4. There was no observable biofilm on samples without the presence of the supplement (Fb). However, when Fb has been added to the culture with PP and PC lattice filament, biofilm formation is observable with both bacteria (*S. aureus* and *P. aeruginosa*). PP and PC could be used to employ both in 3D printing designs that can withstand autoclaving for use in

Table 4
Optimization using a wide variety of lattice filaments (non-printed filaments) incubated with 10 and 5 mg/mL of fibrinogen to study biofilm attachment.[†]

	Media	[Fibrinogen]	Non-Coated Filaments				
			Polypropylene	TPU	PETG	Poly-Lactic Acid	Polycarbonate
S. aureus	BHI	10 mg/mL	++	++	+	+	++
		5 mg/mL	N/O	N/O	N/O	N/O	N/O
		Control	N/O	N/O	N/O	N/O	N/O
S. epidermidis	TS	10 mg/mL	N/O	N/O	N/O	N/O	N/O
		5 mg/mL	N/O	N/O	N/O	N/O	N/O
		Control	N/O	N/O	N/O	N/O	N/O
P. aeruginosa	BHI	7 mg/mL	+	+	++	+	++
		Control	N/O	N/O	N/O	N/O	N/O

The attachment was defined as +++. ++. + and N/O (not observable), from greatest to no attachment, respectively, as shown in Fig. 2.
[†] Control experiments include only the media and no additional protein supplement.

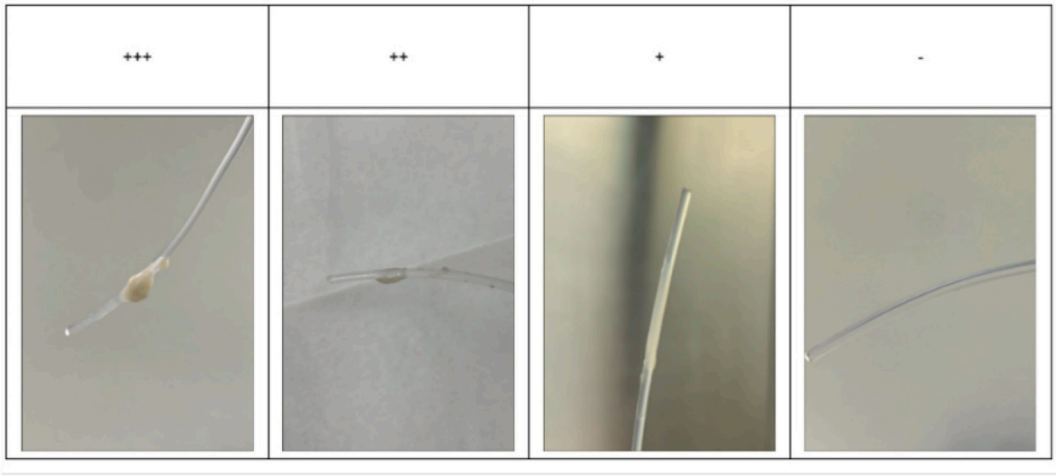


Fig. 2. Qualitative description that was used to characterize biofilm attachment onto the non-printed polymer filaments for Tables 3–5. The attachment was defined as +++ (highest biofilm formation), ++ (middle observable biofilm), + (lowest biofilm formation) and N/O (no observable biofilm formation).

further *in vitro* analyses; however, PC maintained a higher structural integrity of its fine features post-autoclaving. For this reason, the PC filament material was chosen to continue with different experiments to understand how protein supplements affect biofilm attachment to it.

3.3. Designing of biofilm growth lattices

One of the primary problems with using non-printed filament (lattice filament) is the biofilm tends to slide off from its attached surface when removed from the culture. The surface of the 3D printing filament is typically smooth and typically has a low visible roughness as can be seen in Fig. 1. A hooked lattice design was chosen to provide some structural integrity to the biofilm while facilitating its removal from the 25 mL culture tube without biofilm detachment during the removal process. This design was successful in the first iteration. The array of hooked structures making up the lattice extends from the bottom of the 25-mL test tube filled with 4 mL of media to above the fluid level (Fig. 3). Past observations in our lab have demonstrated that biofilm appears to prefer to develop at the air-liquid interface making this design optimal. During the lattice design, we ensured part of the 3D printed filament would straddle the air-liquid interface.

After elucidating the relationship associated with filaments and biofilm adhesion using Fb as a supplement, the growth medium was optimized for consistent biofilm growth on a 3D printed filament using two others supplement such as BSA and FBS. FBS and BSA have been suggested as protein supplements to enhance biofilm growth when present in culture media (Campoccia et al., 2006; Leonhard et al., 2018; Linnes et al., 2012; McCourt et al., 2014; Proctor et al., 1982). More recent publications have depicted that albumin inhibits biofilm formation; however, our study provides evidence that the combination of Fb, glucose, and BSA produces a biofilm from ~0.2 AU (Linnes et al., 2012; Re et al., 1994).

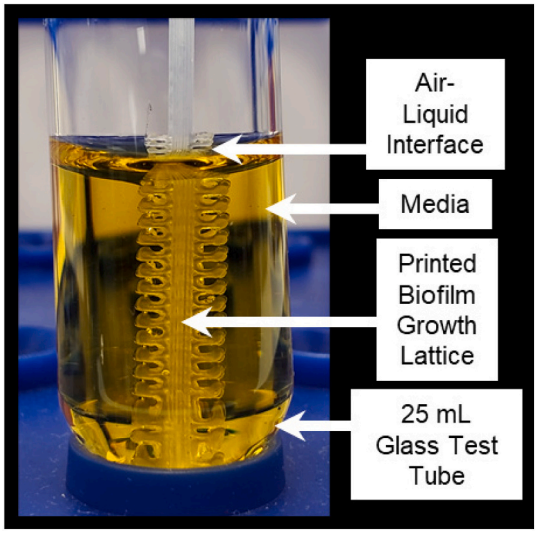


Fig. 3. Polycarbonate biofilm growth lattice immersed in a 25-mL test tube. The lattice structure *vs.* the fluid level is depicted.

3.4. Optimization of supplement increase biofilm formation

For this experiment, the media contained two types of supplements: 30% FBS or 3% BSA with 1% glucose and 7 mg/mL Fb with 1% NaCl. BSA and FBS were candidates because previous studies have shown to affect the biofilm architecture in clinically isolated strains of *P. aeruginosa* (Dutta Sinha et al., 2017). Table 5 shows inclusion of BSA and FBS resulted in increased biofilm biomass. In comparison with FBS, BSA use led to reproducible biofilms that could be transferred between cultures without loss of biofilm from the 3D-printed lattice. The use of BSA also significantly reduces experimental costs. For the next phase of experimentation, BSA was evaluated as a biofilm promoter.

BSA concentrations and incubation times were important parameters to adjust to obtain optimal biofilm growth. For this experiment, the biofilm was transferred from the culture growth medium, rinsed using a syringe with Hank's solution, and finally placed in a conical tube, and sonicated before OD measurement. In addition, we altered the concentration of BSA from 1% w/v, 2% w/v, and 3% w/v, and checked the biofilm every hour; however, after 24 h of incubation, the biofilm maintained its structural integrity during transfer and then its biomass was measured using the biofilm disrupt method as shown in Table 6.

After completing the media optimization for biofilm-filament adhesion, the ability to obtain replicate formation of sufficient biofilm mass was necessary to allow transfers of the biofilm. Our goal was to have a lattice for biofilm formation that allowed the transfer of the biofilm/lattice from the bacterial culture to a cell culture flask without noticeable detachment of biofilm from the lattice. After significant trial and error, the most reproducible results were obtained when biofilms disrupted from their surfaces resulted in an OD 600 measurements of 0.20–0.25. Biofilm/lattice adhesion that gave <0.20 OD 600 measurements was too fragile or frequently detached from the 3D printed lattice. Biofilms that resulted in ~0.2 OD 600 after removal *via* sonication were manageable and could be transferred without loss of the biofilm. Fig. 4 shows the biomass reproducibility for the PC lattice is shown for all three bacterial species using seven lattice experiments.

As a result, 3% *w/v* of BSA, 1% w/v sodium chloride (NaCl), 1% w/v glucose, and 7 mg/mL of Fb was an optimal concentration as shown in Fig. 4 and Table 5. Moreover, Fig. 4 showed no significant differences between the biofilm count (CFU/mL) among the three separate species. Having no significant difference in the biofilm biomass (CFU/mL) was essential for transfer and consistency in further studies. After the incubation for approximately 24 h, it was possible to obtain an approximate count of 1.5 to 2 × 10^8 CFU/mL (Fig. 4). The minimum incubation times necessary to reach the needed biofilm density to prevent fragility were *S. aureus* (21h), *S. epidermidis* (14 h), and *P. aeruginosa* (19 h) $N = 5$. Pictures of the biofilms formed for the various species are shown in Fig. 5.

Culture media enriched with 7 mg/mL of Fb and 3% BSA showed consistent biofilm attachment. After optimizing the concentration of Fb (7 mg/mL), BSA (3% *w/v*), and glucose (1% w/v), the time point necessary to obtain O.D. 600 of ~0.2 was determined, which functions to provide biofilms suitable for co-culture (*in vitro* model) for future studies (Fig. 5). The reason for O.D. 600 of ~0.2 is that at this level of biomass, the biofilm did not incur loss or damage during transfer processes. Below O.D. 600 values of 0.2 ABS, the biofilm was often lost (fell off) during the transfer from one culture flask to another.

Table 5
Optimization of culture media to obtain consistency in the biofilm grown using 3D printed lattice with 7 mg/mL of Fb and 30% *v/v* FBS or 3% *w/v* BSA to study biofilm attachment.[†]

	Media	Growth Factor	Fb. Conc.	Non-Coated Filaments	
				Polypropylene	Polycarbonate
S. aureus	B.H.I.	FBS 30%	7 mg/mL	++	++
		BSA 3%	7 mg/mL	+++	+++
			Control	N/O	N/O
S. epidermidis	T.S.	FBS 30%	7 mg/mL	N/O	N/O
		BSA 3%	7 mg/mL	++	++
			Control	N/O	N/O
P. aeruginosa	B.H.I.	FBS 30%	7 mg/mL	++	++
		BSA 3%	7 mg/mL	+	++
			Control	N/O	N/O

The attachment was defined as +++. ++. + and N/O (not observable), from greatest to no attachment, respectively, Fig. 2.

[†] Control experiments include only the media and no additional protein supplement.

Table 6
Bacteria growth after 24 h of incubation at 37 °C under different protein supplement conditions.

Bacteria	Media with 7 mg/mL of Fb, 1% glucose and 1% NaCl	O.D. 600 nm ± S. D.
S. aureus in BHI	3% BSA	0.20 ± 0.01
	2% BSA	0.30 ± 0.06
	1% BSA	0.50 ± 0.01
S. epidermidis in TS	3% BSA	0.20 ± 0.01
	2% BSA	0.20 ± 0.02
	1% BSA	0.50 ± 0.01
P. aeruginosa in BHI	3% BSA	0.20 ± 0.01
	2% BSA	0.30 ± 0.01
	1% BSA	0.20 ± 0.02

[†] After incubation and formation onto the 3D printed lattice, the biofilm was sonicated and measured at 600 nm. For each species, $n = 3$.

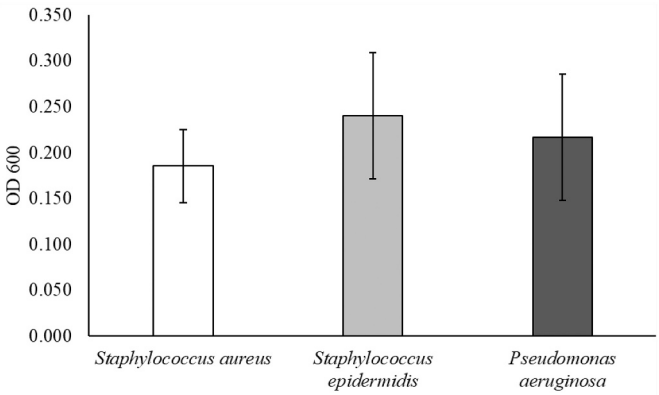


Fig. 4. Reproducibility of biomass (OD 600 nm) for (A) *S. aureus*-biofilm, (B) *S. epidermidis*-biofilm, and (C) *P. aeruginosa*-biofilm. Biofilms were grown onto a PC lattice using 3% BSA, 1% glucose, 1% sodium chloride, 7 mg/mL of Fb in BHI (*S. aureus/P. aeruginosa*) or TS (*S. epidermidis*) at 37 °C. Data were analyzed using SigmaPlot and ANOVA was used with no significance. The data represent mean ± SD, $N = 7$.

4. Conclusion

In this study, fibrinogen (Fb), fetal bovine serum (FBS), or bovine serum albumin (BSA) were used with the 3D printed filaments to determine which protein coating would be optimal for biofilm attachment. The bacterial species *S. aureus*, *S. epidermidis,* and *P. aeruginosa* were used due to their human health relevance with respect to device infection and since the longer-term goal is to transfer these into human macrophage cell lines for further study (Bryers, 2008; Srivastava and Bhargava, 2016; Watters et al., 2016).

PC filament has shown to be a likely candidate for use in future *in*

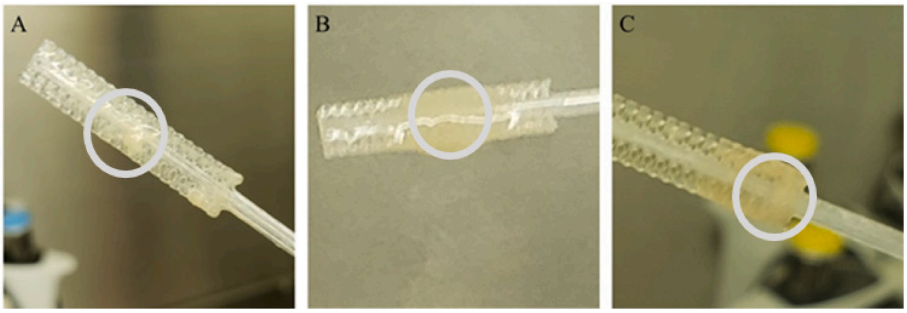


Fig. 5. (A) *S. aureus*-biofilm, (B) *S. epidermidis*-biofilm, and (C) *P. aeruginosa*-biofilm were grown using PC lattice, 3% BSA, 1% glucose, 1% sodium chloride, 7 mg/mL of Fb in BHI or TS at 37 °C. PC lattice was chosen because it can be autoclaved without warping of key design features due to its glass transition temperature of 140 °C.

vitro models: First, the PC lattice has shown an increased biofilm attachment compared with the other filaments. Second, the 3D printed PC lattice can be easily autoclaved without loss of fine features, which makes a more fitting sterilization technique. Finally, supplements, such as 7 mg/mL of Fb, 1% glucose, and 3% BSA into the culture medium, increased biofilm attachment onto the 3D printed lattice. This optimized method showed an easy handling biofilm attachment that can be transported from one place to another. For future studies, an *in vitro* model will be used to study the bacteria-biofilm using *S. aureus*, *S. epidermidis,* and *P. aeruginosa* with the immune system.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2022.106644.

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