Received: 1 July 2023 | Accepted: 11 October 2023

DOI: 10.1111/1751-7915.14360

MICROBIAL BIOTECHNOLOGY Applied Microbiology International

REVIEW

Three dimensional printed biofilms: Fabrication, design and future biomedical and environmental applications

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Funding information
Arnold and Mabel Beckman Foundation; National Science Foundation's Convergence Accelerator program

Abstract

Three dimensional printing has emerged as a widely acceptable strategy for the fabrication of mammalian cell laden constructs with complex microenvironments for tissue engineering and regenerative medicine. More recently 3D printed living materials containing microorganisms have been developed and matured into living biofilms. The potential for engineered 3D biofilms as in vitro models for biomedical applications, such as antimicrobial susceptibility testing, and environmental applications, such as bioleaching, bioremediation, and wastewater purification, is extensive but the need for an in-depth understanding of the structure–function relationship between the complex construct and the microorganism response still exists. This review discusses 3D printing fabrication methods for engineered biofilms with specific structural features. Next, it highlights the importance of bioink compositions and 3D bioarchitecture design. Finally, a brief overview of current and potential applications of 3D printed biofilms in environmental and biomedical fields is discussed.

INTRODUCTION

Microorganisms, which may exist in a single cell state or colonies, are the most copious organisms on earth. When microorganisms colonize, rather than staying in a planktonic cell state, biofilms are formed. The schematic in Figure 1 explains the biofilm lifecycle as presented by Rumbaugh and Sauer (2020). Biofilms are three dimensional (3D) aggregates of cells encompassed in self-produced extracellular polymeric substances (EPSs), which makes up over 50%–90% of the biofilm structure (Donlan, 2002; Li et al., 2022). Microorganisms benefit from the 3D structure of biofilms due to intercellular communication, close contact with nutrients, stability and growth and protection from harsh conditions such as antimicrobial agents and rapid changes in their surrounding environments. EPSs are typically composed of a variety of biopolymers including polysaccharides, proteins and nucleic acids (Li et al., 2022). The heterogeneous structural properties of the EPS bestow biofilms with distinct functions including surface adhesion sites, the ability to aggregate into clusters, preservation of enzymatic activity and regrowth after harsh treatments. Although biofilms are a favourable structure for some biotechnologies used in wastewater treatment (Xu & Jiang, 2018), bioleaching (Zhang et al., 2019) and bioremediation (Catania et al., 2020), biofilms can also be detrimental to human health. Biofilms are responsible for the development of chronic infections, which are 100–1000 times more resistant to antimicrobial agents (Olsen, 2015), and in the United States alone, there is an estimate of 17 million

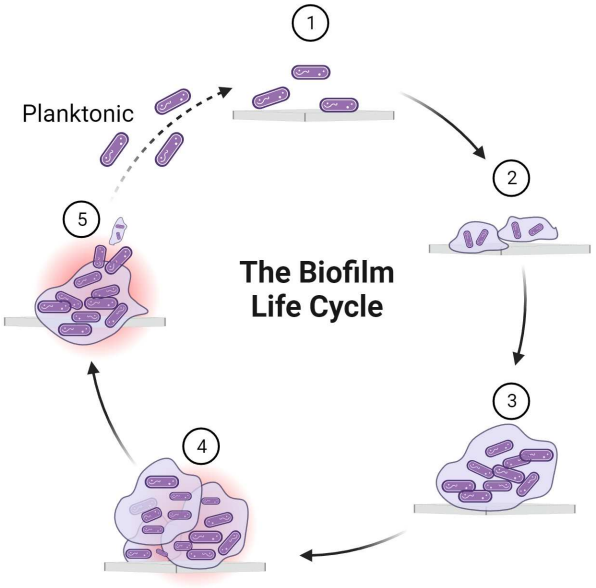

FIGURE 1 Schematic of the biofilm life cycle on a substrate, a cyclic process initiated by planktonic cells. The steps presented in biofilm life cycle are 1. reversible cell attachment, 2. irreversible cell attachment, 3–4. biofilm maturation, 5. biofilm dispersion.

new biofilm-associated infections leading to $94 billion in healthcare costs per year (Wolcott et al., 2013). Therefore, there is an increasing need for antimicrobial drug testing, specifically antimicrobial susceptibility testing (AST), which calculates the minimum inhibitory concentration of an antimicrobial agent. To accurately test *in vivo* conditions, AST requires biorelevant 3D models that mimic natural biofilm formation (Ning et al., 2019).

Traditionally, two dimensional (2D) planktonic cultures are used to study the efficacy of antimicrobials but 2D cultures are more sensitive to treatments and do not reflect the increased resistance of 3D biofilms to antimicrobial agents (Ning et al., 2019). Misleading results of 2D cultures have clinical implications which may lead to chronic infections. This has been seen in patients diagnosed with cystic fibrosis where the treatment of *Pseudomonas aeruginosa* is effective until microbial aggregation and biofilm formation occurs (Malhotra et al., 2019). In addition to increased sensitivity of antimicrobials, limitations of 2D planktonic cultures include lack of complexity of the 3D *in vivo* environment, limited host defence mechanisms and lack of structural thickness. The fabrication of 3D biofilms would allow for a biorelevant architecture for antimicrobial drug testing. 3D bioprinting has emerged as a versatile method for the fabrication of 3D bioarchitectures composed of cells or microorganisms, biological factors and substrates. 3D printed biofilms are in the early stages of development for antimicrobial testing and future research should focus on overcoming limitations with the fabrication process such as shear stress induced on the cells during printing and stability post-printing. Additionally, 3D printed bacteria are surrounded in a deposited polymer network instead of only producing their own EPS, which may affect bacteria proliferation. The 3D scaffold produced through bioprinting creates a complex microenvironment that better mimics the structural, mechanical and biological performance of native conditions as opposed to conventional laboratory grown biofilms, which are grown in liquid media or agar (Ning et al., 2019). 3D printed biofilms are fabricated in biofilm form ready, whereas laboratory grown biofilms take days to reach a film state. This is due to the precise control over structural features, such as pore dimensions, pore geometries, porosities and cellular densities, which are allowed by 3D printing strategies. Not only is 3D bioprinting in the nascent stage for the fabrication of mammalian cell living materials but also for the development of 3D scaffolds composed of algae (Malik et al., 2020), bacteria (Schmieden et al., 2018) and plant cells (Seidel et al., 2017). While several reviews have been published recently on the topic of 3D bioprinting of microbes (Crivello et al., 2023; Hayta et al., 2021; Li et al., 2022), this review will focus on the importance of the material composition and printing parameters of 3D printed biofilms to highlight the importance of structure–function relation achieved through 3D printing, as well as highlight biomedical and environmental applications of biofilms. With further development of the spatial patterning of extracellular matrix (ECM) components 3D printing would allow for highly robust and biorelevant engineered biofilms. Additionally, the spatial control of cells and biological additives allowed by 3D printing strategies offers the possibility of achieving programmable biological functionalities within 3D printed biofilms, such as synthetic gene circuits (Ze et al., 2022).

In addition to structural features, the mechanical and biological characteristics of engineered scaffolds are crucial factors to consider in achieving mature, stable, biorelevant structures. Under applied stresses, a biofilm exhibits elastic and fluid-like behaviours, known as viscoelasticity (Charlton et al., 2019). The matrix viscoelasticity, a key mechanical property of natural biofilms, prevents the disruption or dispersal of microorganism aggregates. Biofilms are unique in that they are comprised of living organisms and a dynamic EPS composed of proteins, DNA and polysaccharides, which dictate the film's matrix viscoelasticity. This important property can be investigated through rheological studies in which the storage and loss modulus of a material is determined. Depending on the bacterial species, biofilm stiffness ranges from a few 100 to several kPa (Hayta et al., 2021). When engineering 3D biofilms, the composition of bioink determines the viscoelastic properties of the printed 3D films. When selecting the appropriate polymer for a bioink, it is crucial to choose an ink with favourable rheological properties that mimic that of natural

biofilms. In addition to matrix viscosity, characteristics of natural biofilms such as bacterial density, distribution of nutrients and signalling molecules, and the location of water channels oxygen molecules are dynamic variables that effect biological and mechanical phenotypes in biofilms. With the precise design control and rapid prototyping allowed by 3D printing strategies, these dynamic variables can be tested to produce a robust biofilm system (Balasubramanian et al., 2019).

This review summarizes 3D printing techniques for the fabrication of engineered biofilms with specific structural features. Next, it highlights bioink compositions, including material, bacterial species, and biological additives currently used for successful 3D fabrication of biorelevant biofilms. This review will also discuss current applications of engineered biofilms, highlighting the advantages of 3D bioarchitectures, as compared to their 2D counterpart. Finally, gaps in the literature encompassing 3D printed engineered biofilms will be discussed.

FABRICATION METHODS OF 3D PRINTED BIOFILMS

The fabrication of robust 3D biofilms requires specific structural features and spatial distribution of biologics within the engineered living scaffolds. Thus, a fabrication technique resulting in control of structural and spatial features is essential for successful mature film manufacturing (Balasubramanian et al., 2021). In addition to spatial and temporal resolution, the fabrication of living scaffolds requires suitable physicochemical environments making traditional manufacturing methods, such as machining, an unsuitable technique (Wangpraseurt et al., 2022). To allow for a biocompatible environment, two methods of biomanufacturing are traditionally used: additive manufacturing and moulding. Moulding is a low-cost technique in which biomaterials are cast in a manufactured mould (Occhetta et al., 2013). Although a simple method allowing for rapid prototyping, moulding cannot capture sophisticated and complex geometries needed to achieve mature living scaffolds. Therefore, additive manufacturing has evolved as a fabrication technique allowing for the spatial and temporal control of complex geometries as well as a biocompatible manufacturing environment.

Additive manufacturing, also known as 3D bioprinting, has extensively been used in the fields of tissue engineering and regenerative medicine for the fabrication of biomimetic human tissue, such as skin (Admane et al., 2019), cartilage (Müller et al., 2017), bone (Dang et al., 2020) and cardiac tissue (Noor et al., 2019). Additionally, the fabrication of biomimetic scaffolds through bioprinting techniques, allows for a more precise model, as compared to 2D culture and animal models, for studying drug delivery and disease modelling (Vanderburgh et al., 2017). This is due to the control over the placement and geometry, in spatially predefined locations, of cells, biomolecules and biomaterials within the 3D scaffold. Bioprinting also allows for the fabrication of patient-specific 3D geometries constructed from magnetic resonance imaging or computerized tomography scans due to architectural control (Ramadan & Zourob, 2021). Depending on the additive manufacturing technique, pore sizes and pore geometries of bioprinted scaffolds can be fabricated on a micro or nano scale (Chan et al., 2021; Cui et al., 2022). The precision of feature size, as well as cellular densities, are crucial in achieving mature, biomimetic living scaffolds. Naturally occurring biofilms have a heterogeneous makeup in pore size, density and porosity. It has been reported that the density in the bottom layers of naturally occurring biofilms is 5–10 times higher than the top layers (Zhang & Bishop, 1994). Additionally, porosity has been shown to increase from 58%–67% in the bottom layers of naturally occurring biofilms to 85%–93% in the top layers (Zhang & Bishop, 1994). The pore size also increases from 0.3–0.4 μm in the bottom layers to 1.7–2.7 μm in the top layers (Zhang & Bishop, 1994). 3D printing strategies allow for the fabrication of functionally graded scaffolds, which would mimic the heterogeneous nature of biofilms. Some extrusion-based bioprinting techniques allow for the use of multiple print heads, resulting in multiple materials and cellular densities within a single printed scaffold. More recently, bioprinting techniques have been utilized for the fabrication of microorganism living materials. This section of the review will focus on multiple bioprinting techniques which have been deployed for biofilm fabrication. Table 1 summarizes the 3D printing strategies discussed in this review and their controllable parameters.

Bioprinted scaffolds are fabricated layer by layer using either a top-down or bottom-up approach. Main bioprinting strategies can be generally categorized into two types: material deposition bioprinting and light assisted bioprinting. Material deposition strategies include extrusion-based bioprinting and inkjet-based bioprinting while light-assisted strategies include laser assisted printing (LAB) and stereolithography (SLA)-based bioprinting. These bioprinting techniques are described in the schematic in Figure 2.

Material deposition based bioprinting

Extrusion-based bioprinting is the most commonly used 3D printing strategy for the fabrication of cell or microorganism-laden biomaterial scaffolds (Balasubramanian et al., 2021; Huang, Liu,

TABLE 1 Summary of 3D printing strategies including controllable parameters, advantages and disadvantages.

3D bioprinting strategy	Controllable parameters	Bioink viscosities	Achievable pore sizes	Strengths	Limitations
Extrusion-based	Pressure	$6–30 \times 10^7$ mPa·s (biomaterial)	100 μm–1 mm	Wide range of materials	Shear stress on cells
	Speed			Variable cell densities	
	Temperature			Rapid prototyping	
	Nozzle diameter			Multiple printheads	
Inkjet	Pressure	3.5–12 mPa·s (biomaterial)	50–75 μm droplets	Fast fabrication	Low-viscosity ink
	Speed			High cell viability	Nozzle clogging
	Voltage			Good resolution	Low mechanical strength
	Dwell time				Low cell density
	Nozzle diameter				
Bioplotting	Pressure	1.3–382 Pa (biomaterial)	100 μm–1 mm	Multicell fabrication	Shear stress on cells
	Speed				
	Temperature				
	Nozzle diameter				
Stereolithography (SLA)	Layer height	>5 mPa s (resin)	As low as 25 μm	High precision and accuracy	Lack of compatible materials
	Wavelength			Low print time	Lengthy post-processing
	Post-cure time and temperature				High-intensity UV light
Laser-assisted bioprinting (LAB)	Wavelength	40–200 mPa s (biomaterial)	20–325 μm	High precision and resolution	Expensive
	Pulse duration			Biomaterial deposition in solid or liquid phase	Long fabrication times
	Pulse energy				Thermal cell damage
	Focal geometry				Sedimentation of cells
	Focal spot size				
Digital light processing (DLP)	Layer height	>5 mPa s (resin)	35–200 μm	High precision and accuracy	Cytotoxic effect of photoinitiators.
	Wavelength			Low print time	Limited photosensitive polymers

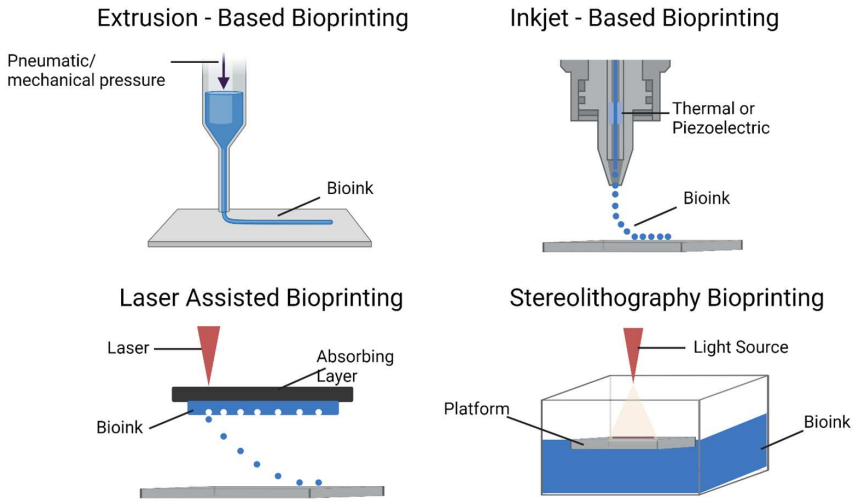

FIGURE 2 Schematics of material deposition, including extrusion-based and inkjet bioprinting, and light assisted bioprinting techniques, including laser assisted and stereolithography.

et al., 2019; Ning et al., 2019). The principle behind extrusion-based bioprinting includes a pneumatic or mechanical (piston or screw) force and an extrusion head, which can move in the *x*, *y* and *z* directions, to dispense a continuous strand of bioink onto a substrate. Post-printing, the biomaterial is typically cross-linked by UV light, chemicals or enzymes to achieve a mechanically sound structure. Advantages of extrusion-based strategies include the printability of hydrogels and thermoplastics, a wide range of extruding nozzle diameters to support specific feature sizes (100 μm–1 mm) and the ability for rapid and low-cost prototyping (Jeong et al., 2020). Additionally, extrusion-based systems allow for the use of multiple printhead resulting in the ability to print multiple materials and/or microorganisms into the same scaffold. This allows for the fabrication of robust mechanical and biological engineered living materials to mimic their natural counterpart.

Extrusion-based bioprinting is highly versatile in that a wide range of materials can be printed with precise patterning and, therefore, is strongly suitable for the fabrication of 3D biofilms. Extrusion-based strategies have been used for the fabrication of *Escherichia coli* encapsulated alginate scaffolds resulting in the development of engineered biofilms. Ning et al. successfully produced mature *in vitro* biofilm models to investigate the relationship between biofilm thickness and response to antimicrobial treatment (Ning et al., 2019). 1- and 2-mm scaffolds were printed and exposed to tetracycline, mimicking a course of antimicrobial treatment. Results showed that *E. coli* retained viability in 2-mm printed biofilms while tetracycline eradicated *E. coli* in 1-mm scaffolds. An additional study investigated the relationship between ECM composition of *E. coli*-laden alginate 3D printed biofilms and their resistance against Ethanol or Virkon S. The results revealed the importance of ECM composition and its relationship against disinfectants. Biofilms expressing curli or curli and cellulose demonstrated greater resistance against disinfectants as compared to biofilms expressing only cellulose (Balasubramanian et al., 2021).

Another material deposition-based bioprinting strategy includes inkjet-based bioprinting. Inkjet bioprinting is a non-contact printing method in which picoliter volume droplets containing 10–100 cells/droplet are printed either in a continuous strand or drop on demand (DOD) onto the printing substrate (Matai et al., 2020; Ng et al., 2022). DOD can be created through thermal or piezoelectric pulses allowing for the ejection of droplets from the print nozzle. An advantage of inkjet-based approaches is the high resolution achieved in the final printed part. Typically, DOD allows for bioink droplets to have a diameter of 10–150 μm. However, drawbacks of inkjet-based technologies include the need for low-viscosity bioinks (3.5–12 mPa·s) to avoid clogging the nozzle, heterogeneous drying of droplets and the risk of low cell viability caused by the thermal and shear stresses induced on the cells during printing (Jeong et al., 2020; Matai et al., 2020). Despite these drawbacks, inkjet-based bioprinting has been explored for the fabrication of 3D living scaffolds due to the high printing resolution. DOD printing was employed to produce *Ecklonia cava* encapsulated alginate microparticles and results showed the ability to precisely control the number of microorganisms in each droplet (Lee et al., 2019). Although microorganism density did alter droplet viscosity and elasticity of the alginate microparticle, continuous growth of *E. cava* was observed for 45 days after printing. One of the first studies to print multiple strains of *E. coli* in a single biofilm deployed DOD printing for biofilm fabrication. Kumar et al. 3D printed multiple strains of the gut bacterium *E. coli* in various configurations (Krishna Kumar et al., 2021). For samples printed with a homogeneous mixture of strains, toxin-producing strains largely eliminated

susceptible non-producers, yet for strains printed in an adjacent pattern, susceptible strains persisted. These results further reveal the importance of spatial patterning in engineered biofilms.

Light assisted bioprinting

In light-assisted bioprinting, 3D structures are printed, cross-linked and solidified through photopolymerization. Light-assisted strategies include stereolithography, LAB, 2-photon polymerization and digital light processing-based 3D printing. In general, the bioink, composed of a prepolymer, photo initiators and living microorganisms, is exposed to light and solidifies at the exposed locations allowing the unexposed bioink to be washed away (Wangpraseurt et al., 2022). This process is highlighted for laser-assisted and stereolithography bioprinting in Figure 2. Due to this fabrication method, light-assisted bioprinting can achieve submicron-sized features and high resolutions (You et al., 2018). Additionally, because the bioink is not physically extruded from a nozzle, shear stresses are not applied to the cells resulting in higher cell viability. However, viability may be negatively affected due to longer printing times with some light-based techniques such as direct laser writing, exposure to laser light and long exposure to photoinitiations (Barreiro Carpio et al., 2021). Cell spatial control in the fabricated sample is more difficult to achieve in light-assisted bioprinting because the cells are suspended in a liquid bath, allowing movement of cells during the printing process (Barreiro Carpio et al., 2021). Through 2-photon polymerization, gelatine scaffolds containing low- or high-density inner cavities of *Staphylococcus aureus* surrounded by a square gelatine scaffold containing *P. aeruginosa* were successfully fabricated to study the underlying mechanisms of cellular communications between the two bacterial species (Connell et al., 2013). More recently, Dubbin et al. used stereolithography approaches to produce *E. coli*-laden polyethylene glycol diacrylate (PEGDa) scaffolds with thickness as low as 10 μm while achieving high post-print viability. The ability to print two different strains of *E. coli* in a predetermined pattern through stereolithography techniques was demonstrated (Dubbin et al., 2021).

DESIGN OF 3D PRINTED BIOFILMS

Prior to biofilm fabrication, three crucial steps must take place to achieve a desired 3D printed biofilm: (1) development of the bioink and (2) geometric design of the scaffold (3) optimization of printing parameters. These steps are portrayed in the schematic in Figure 3.

Extensive literature exists on 3D bioprinting, particularly of mammalian cell laden hydrogels, but the processes of bioink development, scaffold geometric design and printing parameter optimization for translating a 2D design to a 3D model are seldomly discussed. This is apparent in the 3D bioprinted biofilm literature. Understanding of the process parameters is vital for accurate fabrication of user-defined 3D structures that mimic natural biofilms (Matai et al., 2020). Several factors influence print fidelity, that is the geometric retention of a single extruded strand of bioink, as well as the printed part as a whole, as compared to the computer aided design model (CAD) (Schwab et al., 2020). Several of these factors include bioink viscosity, printing pressure, printing speed, printing temperature and printing distance (*z*-distance). The variability of these factors affects the diameter of the extruded bioink strand and, therefore, influences layer height, overall porosity, mechanical strength and overall scaffold geometry. In the case of material deposition-based 3D bioprinting strategies, the viscosity of the bioink is an important parameter to control to achieve biorelevant 3D architectures (Gopinathan & Noh, 2018). An ideal bioink will behave as a viscoelastic; encompassing liquid-like

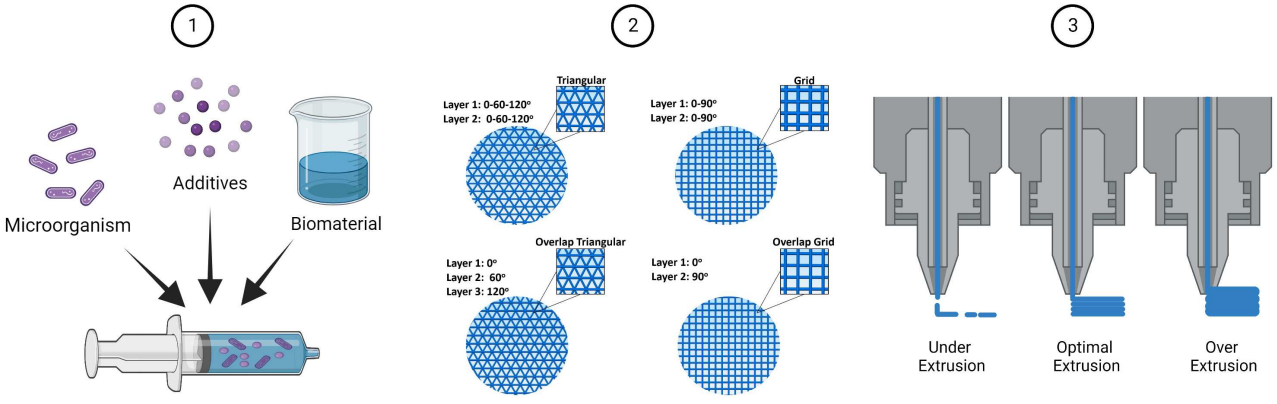

FIGURE 3 Schematic of pre 3D printing process to achieve desirable engineered biofilms. 1. Development of bioink including mixing of biomaterial, additives and microorganisms. 2. Geometric scaffold design using computer aided design and slicing software. 3. Optimization of printing parameters.

behaviours to ensure smooth extrusion out of the print nozzle without clogging while also encompassing solid-like behaviours to ensure a stable structure post printing. Not only is the viscosity of the bioink crucial to yield high fidelity 3D biofilms; the viscosity of the fabricated part is also crucial to ensure full maturation and natural behaviour of the biofilm long term (Charlton et al., 2019). In the subsequent section, we present a brief overview of bioink composition and scaffold design of current 3D printed biofilms found in the literature. Table 2 summarizes these results.

Bioink

A bioink's composition is a driving factor to determine the functionality of a final 3D printed scaffold, due to the bioink's rheological, mechanical and biological properties (Carrow et al., 2015). The polymers that make up a bioink can be natural, synthetic or a natural-synthetic hybrid and depend on the application of the fabricated scaffold. Common natural polymers used in 3D bioprinting include alginate, collagen, gelatine, fibrin, chitosan, hydroxyapatite and hyaluronic acid (Gerdes et al., 2020; Huang, Liu, et al., 2019; Intini et al., 2018; Schaffner et al., 2017; Schwarz et al., 2020). Common synthetic polymers used in scaffold fabrication include polylactic acid, polycaprolactone, polyethylene glycol and polyvinylpyrrolidone (Bruyas et al., 2018; Senatov et al., 2016). The role of these polymers may be to provide (1) structural support, (2) sacrificial material during printing or (3) provide mechanical, chemical or electrical signals post printing (Williams et al., 2018). Almost all current 3D printed biofilms are fabricated with natural hydrogels, with the most common being alginate and gelatine, which can be seen from Table 2. Both alginate and gelatine are widely adopted hydrogels for bioprinting due to their biocompatibility, viscoelasticity and low cost. To achieve a natural polymer bioink with desirable viscoelastic properties for printing, the percent polymer and percent crosslinking agent can be altered until a smooth strand of ink can be extruded out of the nozzle while still producing a structurally sound scaffold. To ensure biofilm sterility, certain measures should be taken throughout the 3D printing process. 3D-bioprinted biofilms are only sterile insofar as the ingredients of the bioink and cell culture media begin as sterile, and the 3D-printer tubing, printhead and nozzle are sterilized with sterilizing chemicals or irradiation. When printing with engineered strains, antibiotics are often included which will aid in sterility. After printing the biofilms can be stored in a closed, sterile container to limit contamination. Studies have shown that 3D-printed bacteria at high density will not be colonized by nearby microbial strains, maintaining biofilm sterility (Johnston et al., 2020).

In addition to ink viscosity, printing parameter optimization can be performed to yield high-fidelity 3D printed biomaterials. Biomaterial extrusion rate is based on the defined printing pressure, temperature and speed. Printed material strands with a larger diameter than the print nozzle experience a greater extrusion rate as compared to the linear print speed and result in excess material deposition (Figure 3). The opposite is true for printed material strands with a diameter less than the print nozzle. Underextrusion of the bioink will result in gaps in the printed strand and lower-than-desired scaffold height (Figure 3). Additionally, increased print speeds could pull the extruded material strand as it is deposited onto the surface, thinning the diameter. When printer parameters are not optimized for a predefined geometry, feature sizes, geometries, overall scaffold dimensions and desired mechanical properties will not be achieved. Bioink compositions ranging from 2%–5% (w/v) alginate and 5% (w/v) gelatine (Cui et al., 2022; Huang, Liu, et al., 2019; Ning et al., 2019; Schmieden et al., 2018) have been developed and resulted in printable bioinks and structurally sound scaffolds post crosslinking. Ning et al. produced a 2% (w/v) alginate bioink with 0.2% calcium chloride as a pre-crosslinking agent followed by post-crosslinking of the microorganism-laden scaffold with 10–40 mM barium chloride and found longer term viability and stability of material with the two-step crosslinking process (Figure 4; Ning et al., 2019). Currently, one study investigates the fabrication of 3D printed biofilms through SLA strategies using a synthetic polymer: PEGDa, seen in Figure 4. Post-print cellular viability was achieved, and the ability to print *E. coli* expressing different fluorescence in distinct pattens was proven (Dubbin et al., 2021).

Biological or mechanical additives may be added to the bioink to achieve a specific structure–function relationship within the 3D printed scaffold geometry. Biological additives include growth factors, proteins and antimicrobials to enhance cellular attachment, viability, proliferation or to induce an antimicrobial response of the 3D printed scaffolds. Common mechanical additives include nanofibers, nanoparticles, hydroxyapatite or a pre-crosslinker to improve the viscosity of the bioink and mechanical strength of the fabricated scaffold. In current literature encompassing 3D printed engineered biofilms, the approach of integrating additives into the printed scaffold is different. Researchers are printing with specific microorganisms which express certain proteins to naturally create biological and mechanical additives. This approach allows for the formation of engineered biofilms expressing a biomimetic ECM with natural biological and mechanical properties. Balasubramanian et al. printed with specific *E. coli* species that expressed either cellulous, curli or curli and cellulous and investigated the effect of curli production in the ECM on disinfectant resistance (Figure 4; Balasubramanian et al., 2021). In another study, researchers exploited the export machinery of *Bacillus subtilis* by fusing the extracellular amyloid-like protein TasA, a subunit of the ECM of *B.*

TABLE 2 Design summary of current additive manufactured 3D biofilms.

Bioink	Microorganism	Fabrication method	Scaffold design (pore size; pore geometry; scaffold height)	Scaffold geometry schematic	Reference
Alginate, Cellulose, Curli	*Escherichia coli*	Extrusion-based 3D printing	NR; 0°/90°; 400 μm		(Balasubramanian et al., 2021)
Alginate	*E. coli*	Extrusion-based 3D printing	2 mm lines		(Schmieden et al., 2018)
Alginate	*E. coli*, *Pseudomonas aeruginosa*, MSSA, MRSA	Extrusion-based 3D printing	100 and 25% infill; 0°/90°; 0.25, 0.5, 1, 2 and 4 mm		(Ning et al., 2019)
Alginate	*Ecklonia cava*	Inkjet 3D printing	80 μm droplets with 400 μm drop spacing		(Lee et al., 2019)
Gelatine, Gelatine methacryloyl	*Streptococcus zooepidemicus*	3D Bioplotting	2.5, 2, 1.5 and 1 mm IFD; 45°/90°/135°; 3 mm		(Cui et al., 2022)
Gelatine, Alginate, TasA-His Tag nanofibers	*Bacillus subtilis*	Extrusion-based 3D printing	NR; 0°/90°; NR		(Huang, Liu, et al., 2019)
Gelatine	*P. aeruginosa*, *Staphylococcus aureus*	Two photon polymerization	100% infill; square; NR		(Connell et al., 2013)
CsgA-α, CsgA-γ, CsgA- αγ hydrogels, nanofibers	*E. coli*	Extrusion-based 3D printing	300 μm; 0°/90°; single layer		(Duraj-Thatte et al., 2021)
			0% infill; square and circle, 10 layers		
			100% infill; conical; 21 layers		
Agarose	*B. subtilis*	FDM with novel printhead	100% infill; hexagon and square; NR		(González et al., 2020)
PEGDa	*E. coli*	Projection stereolithography	800 μm; 0°/90°; 2.5 mm		(Dubbin et al., 2021)
Hyaluronic acid, k-carrageenan, fumed silica	*Pseudomonas putida*, *B. subtilis*, *Acetobacter xylinum*	Direct Ink Writing	NR; 0°/90°; NR		(Schaffner et al., 2017)

Abbreviations: IF, interfilament distance (distance between the adjacent filament centres); NR, not reported.

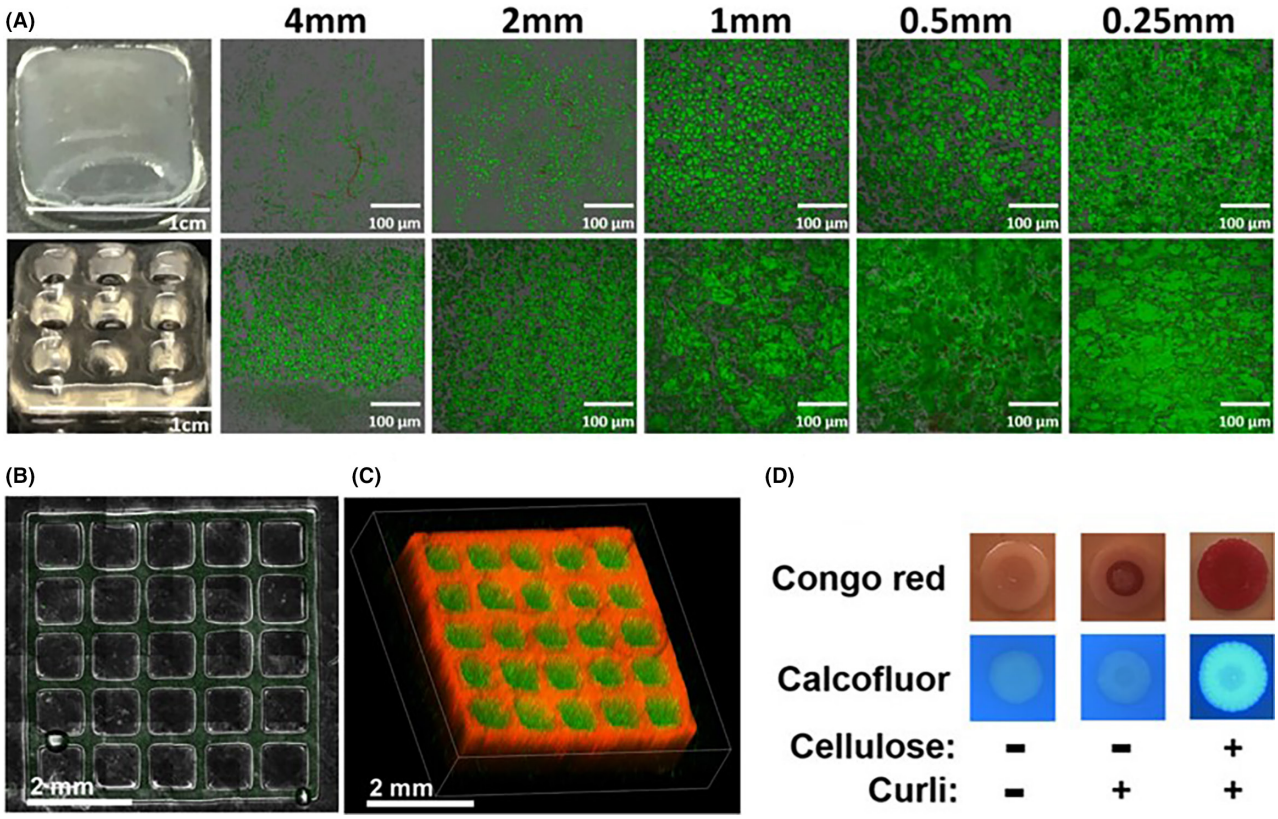

FIGURE 4 Current 3D printed biofilms. (A) Nonporous and porous *Escherichia coli* 3D printed biofilms printed at various thickness (0.25–4 mm). Biofilm growth in response to structure thickness was analysed through fluorescent microscopy (Ning et al., 2019). (B) SLA-printed biofilm laden with *E coli* expressing GFP or mCherry to demonstrate the spatial control of SLA (Dubbin et al., 2021). (C) Fluorescent microscopy of SLA-printed biofilms laden with *E coli* expressing GFP (green) or mCherry (red) (Dubbin et al., 2021). (D) Congo Red and Calcofluor analysis of 3D printed biofilms expressing Cellulous/Curli (Balasubramanian et al., 2021). '3D bioprinting of mature bacterial biofilms for antimicrobial resistance drug testing' by Ning et al. is licensed under CC BY 4.0/Cropped from original/DOI https://doi.org/10.1088/1758-5090/ab37a0, 'Projection Microstereolithographic Microbial Bioprinting for Engineered Biofilms' by Dubbin et al. is licensed under CC BY NC ND 4.0/Cropped from original/https://doi.org/10.1021/acs.nanolett.0c04100, 'Emergent Biological Endurance Depends on Extracellular Matrix Composition of Three Dimensionally Printed *Escherichia coli* Biofilms' by Balasubramanian et al. is licensed under CC BY NC ND 4.0/Cropped from original/https://doi.org/10.1021/acssynbio.1c00290.

subtilis, with other proteins to produce functional nanofibers. The production of these fibres is regulated by the *tapA-sipW-tasA* gene operon. TapA proteins act as a molecular nucleator for the extracellular assembly of these proteins in surface nanofibers (Driks, 2011). The presence of these nanofibers allowed for stable, environmentally responsive engineered biofilms capable of self-regeneration (Huang, Liu, et al., 2019).

The final stage of bioink development is the integration of cells or microorganisms. There are two approaches for the integration of cells or microorganisms with 3D bioprinting strategies: (1) cell-seeding of the scaffold post-printing or (2) integration into the bioink pre-printing. Cell-seeding post printing eliminates imposed shear stresses on the cells as they are extruded through the nozzle, but this approach hinders homogeneous distribution of cells throughout the whole scaffold. Typically, higher cell densities will occur on the surfaces and edges of the scaffold, which can lead to unsuitable oxygen gradients resulting in low cellular viability and proliferation (Fedorovich et al., 2011). When cells are integrated into the bioink pre-printing, a homogenous cell-laden scaffold will be achieved (Kačarević et al., 2018). The moduli, or bioink stiffness, in the latter approach largely affects the cellular encapsulation and long-term viability with low moduli hydrogels (<1 kPa) expressing better cellular encapsulation (Goldshmid & Seliktar, 2017). For all material deposition based bioprinting strategies summarized in Table 2, microorganisms were integrated with the bioink pre-printing and the fabricated scaffolds resulted in long-term viability.

Scaffold design

3D printing strategies allow for the fabrication of a wide range of geometric shapes, patterns and sized scaffolds through the control of pore geometry, pore size, porosity, scaffold height and overall scaffold geometry. These specific design features can be modelled using CAD software and transformed into a readable 3D printer file type through slicing software. Depending

on the application of the cell-laden scaffold, these geometric features can be adjusted to optimize cell viability, proliferation and differentiation. Despite this strong structure–function relationship between scaffold design and cell function, robust investigation does not exist for 3D printed biofilms. Design considerations including pore geometry, pore size, porosity, scaffold height and microorganism density may change depending on the microorganism species being studied. For example, scaffold porosity should differ for an anaerobic species biofilm or aerobic species biofilm. Ning et al. studied the effect of 3D printed scaffold porosity and height on *E. coli* and *P. aeruginosa* proliferation. Results showed a significant decrease in *E. coli* proliferation in solid, thick scaffolds as compared to porous, thin scaffolds. The porous scaffolds allowed for oxygen and nutrient transport throughout the entirety of the 3D printed scaffold which is needed for aerobic bacterial survival. However, facilitated anaerobic *P. aeruginosa* showed significant proliferation and biofilm formation in solid, thick scaffolds (Ning et al., 2019).

In addition to scaffold porosity and height, scaffold pore size and geometry are important design features that may influence microorganism proliferation, but a lack of research exists to justify the effect of pore size and geometry. Although, mammalian cell-laden scaffolds have shown significant differences in cell viability, proliferation and differentiation depending on pore size and geometry for a variety of cell types (di Luca et al., 2016; Krok-Borkowicz et al., 2019; van Bael et al., 2012; Zhang et al., 2020). In current 3D printed biofilm research, almost all porous scaffolds are printed with a 0°/90° strand laydown pattern. This pattern can be seen in the schematics in Table 2. In addition to a 0°/90° strand laydown pattern, 3D printers are capable of printing other geometries, such as 45°/90°/135° and 0°/60°/90°. Moreover, a universal pore size range which produces mature biofilms does not exist. Currently, biofilms laden with the same bacterial species are being printed with pore sizes ranging from 0 to 800 μm, which is summarized in Table 2. In-depth studies regarding the effect of pore geometry and pore size on microorganism proliferation and biofilm formation should be investigated.

CURRENT AND FUTURE APPLICATIONS OF 3D PRINTED BIOFILMS

3D printed biofilms for therapeutic development

Biofilms are responsible for 65%–80% of all chronic infections, including chronic wound infections, chronic lung infections such as cystic fibrosis and chronic infection from biofilm formation on medical devices such as prostheses, catheters and cardiac valves (Macià et al., 2014). Traditional antimicrobial susceptibility testing (AST) is performed with planktonically growing bacteria, which is 100–1000 times less resistant to antibiotics than biofilms (Olsen, 2015), and therefore, the results of these established tests cannot be used to predict therapeutic strategies for biofilm infections. Thus, there is a growing need for *in vitro* biofilm models to study AST specifically for biofilm-growing bacteria. Several *in vitro* methods have been developed but disadvantages of these methods exist (Bahamondez-Canas et al., 2019). Additionally, a lack of standardization and interpretation of results exists and, therefore, limits translation into a clinical setting. Current standard methods include microtiter plate and flow cell systems. The microtiter plate method uses a 96-well plate to grow the biofilm in a ring around the well and crystal violet staining to quantify biomass. Limitations of the strategy exist due to heterogeneous bacterial growth, limited oxygen flow and an absence of a relation between biomass, which is quantified by crystal violet and biofilm viability (Peeters et al., 2008). The flow cell system allows for the formation of thick biofilm, as compared to the microtiter plate method and allows for the delivery of nutrients through a multichannel peristaltic pump. Despite this, the flow cell strategy is time-consuming, requires special handling of fragile equipment and does not allow for rapid manufacturing of biofilm models (Macià et al., 2014). To overcome limitations of current biofilm AST strategies, 3D printed in vitro biofilm models can be employed. 3D printing allows for rapid fabrication of *in vitro* models and allows for the spatial control of scaffold patterning, bacterial density and ensures a homogeneous distribution of bacteria throughout the entire model.

Bacteria species that are resistant to antibiotic treatments when growing in a biofilm are often susceptible to the same antibiotics when living in a planktonic lifestyle. This emergent resistance of biofilm communities is not caused by the mechanisms responsible for planktonic antibiotic resistance (Anderl et al., 2000; Brooun et al., 2000; Williams et al., 1997), including drug efflux pumps, enzymes that modify or neutralize antibiotics or mutations in drug target sites. Instead, antibiotic resistance of biofilms is due to biofilm-specific features such as limited diffusion through the EPS (Gordon et al., 1988; Shigeta et al., 1997), altered bacterial metabolism or growth rates (Das et al., 1998; Heim et al., 2020; Prigent-Combaret et al., 1999; Tuomanen et al., 1986), or changes in the chemical microenvironment found within biofilms (de Beer et al., 1994; Stewart et al., 2019). While an estimated 80% of human bacterial infections involve biofilms (Costerton et al., 1999), typical antibiotic drug development has used planktonic cells to assess drug effectiveness. As a result, treatment guidelines for antibiotic usage can be ineffective due to enhanced drug resistance of biofilm-resident bacteria (Penesyan et al., 2015). In order to develop a new generation of antibiotics that display targeted

effectiveness against biofilm bacteria, new biofilm-specific model systems must be developed for use as a platform for biofilm-specific drug development.

Model biofilm systems that can be used effectively for drug development will need to be created in medium-to-high throughput to support screening of large drug libraries, which could be achieved by automated 3D-printer deposition of biofilms onto clinically relevant test substrates. While current biofilm model systems fail to emulate the diversity and spatial organization observed in clinical patient-derived biofilms, 3D bioprinting techniques offer a unique opportunity to develop multispecies biofilm-on-a-chip models that reproducibly recapitulate the spatial patterning of constituent bacteria species seen in native biofilms (Kim et al., 2020). 3D-bioprinting approaches would additionally allow the creation of an entirely new modality of anti-biofilm treatments. While the common approach to treating biofilms is to eradicate all resident bacteria, this approach can have undesirable secondary effects due to the importance of healthy microflora communities to food digestion, gastrointestinal and oral tissue function and regulation of the immune system and host epithelium (Young, 2017). Therefore, it is a high priority to develop treatments that suppress disease-causing biofilm bacteria in favour of commensals. The presence of commensal biofilms is protective against colonization by potentially pathogenic microbes (Kreth et al., 2005; Vollaard & Clasener, 1994). 3D bioprinting will allow the development of model commensal biofilms with high fidelity and flexibility, which can be used prophylactically in patients to compete with and suppress harmful bacteria species. This approach will be particularly useful in cases where the host microbiome has been eradicated through prior medical antibiotic or chemotherapy treatments, or in the case of medical implants where no commensal bacteria have been able to become established yet.

In addition to the use of bacterial species for the fabrication of drug delivery systems, cellulose-producing bacteria may be utilized for the biosynthesis of bacterial cellulose, a natural, renewable and 3D nanomaterial (Martirani-VonAbercron & Pacheco-Sánchez, 2023). Bacterial cellulose is being explored in the field of biomedicine, specifically as a wound dressing due to its biocompatibility, biodegradability, high water-holding capacity and absorption of exudates from injured skin tissue. Through precise manipulation of the pore volume within the bacterial cellulose, water-holding capacity and water release rate of wound dressing can be altered.

Environmental applications

Bio-based economies and, more recently, a circular bioeconomy have been implicated as a sustainable societal vision for combating climate change across the world (Lange et al., 2021). As part of that circular bioeconomy, interest in the deliberate use of microbial biofilms in biotechnology is widespread, especially because of their inherent robustness to external environmental stressors and ability to self-heal. Biofilms are useful in a broad range of applications including wastewater treatment, environmental remediation (e.g. permeable reactive barriers), antiseptic testing and development, microbial fuel cells, mining, biomaterials and biomimetic engineering (Huang, Peng, et al., 2019; Krasowski et al., 2021; Mahto et al., 2022; Mishra et al., 2022; Pandey et al., 2021). Some examples include bioremediation of hydrocarbons, pesticides, heavy metals and organo-pollutants in water treatment (Mishra et al., 2022). Recent breakthroughs in the production of bioelectricity have utilized anaerobic biofilms in the anode of microbial fuel cells (Armstrong, 2023). In addition to producing bioelectricity, the anaerobic biofilms in the microbial fuel cells recover nutrients producing biofertilizer, treating wastewater and killing pathogens. However, most current biofilm engineering technologies rely on biofilms naturally forming over time and, as such, offer only rudimentary control of biofilm characteristics and functions—mainly due to the heterogeneity and complexity of biofilms and their processes (Mukherjee & Cao, 2021)—and as such, robust applications of biofilm technologies, though desired, are limited. Therefore, developing reliable methods for fabricating and controlling biofilms, while maximizing their functionalities and self-healing capabilities, is critical for biofilm-based biotechnologies to be widely deployed. Though there are currently limited studies conducted, 3D printing of biofilms with desired microbial ecology, functions and/or responses to specific environmental stimuli has the potential to drastically change the current landscape of environmental biofilm-based biotechnologies. Some key considerations for environmental applications of 3D printed biofilms include the need to consider more realistic multi-species biofilms instead of single-species biofilms, compatibility of the scaffold/bioink chemistry with the surrounding environmental conditions (e.g. alginate requires a certain amount of divalent cations compared to monovalent cations), effects of fluctuating environmental conditions, interactions with naturally occurring microbial consortia and long-term reliability of desired functions in the printed biofilm. Additional fabrication methods may need to be considered to scaleup the current micron to millimetre-sized biofilm models for application in environmental biotechnology. Although this approach has not been applied to bacterial species, Malik et al. successfully 3D printed 1000 × 500 mm algae-laden alginate structures using a robotic arm fitted with a 3D printing nozzle (Malik et al., 2020). Similar technologies may be employed for the fabrication of large-scale biofilm models. With further study, 3D printed biofilms will transform many environmental biotechnologies, such as wastewater treatment, bioremediation and bioleaching, and

enable greater levels of design, control and predictability even under the changing climate. 3D printing may allow for the fabrication of biofilm models that overcome current limitations of natural uncontrolled dynamic biofilm development (Mukherjee & Cao, 2021). With the development of a controlled synthetic process, the use of environmental biotechnology will greatly expand.

CONCLUSION AND FUTURE DIRECTION

Biofilms offer unique advantages, as compared to their planktonic counterparts, in terms of their adaptability, structural viscoelastic properties and ability to fight off disinfectants. These properties have proved advantageous in the fields of wastewater purification, bioleaching, bioremediation and corrosion protection. Adversely, the unique properties of biofilms prove detrimental to the biomedical industry, leading to chronic wound infections, chronic lung infections and chronic infections from biofilm formation on medical devices. The potential of engineering 3D biofilms to aid in the understanding of their structure–functional relationship exists and could provide further insight for their environmental and biomedical applications. 3D bioprinting technologies allow for the fabrication of living materials with user defined structural features. Bioprinting strategies, namely material deposition based and light assisted strategies, have been employed for the fabrication of 3D engineered biofilms. The structural features and spatial control of cells throughout the engineered films are crucial user defined parameters to ensure the 3D printed constructs mimic their native counterparts. The need for an in-depth understanding of the structure–function relationship between the complex construct and the microorganism response still exists. Once fully understood, 3D bioprinted biofilms with precise structural features can be harnessed for the development of in vitro models to study antibiotic testing to reduce the risk of chronic infections in healthcare settings. With future development of 3D printed in vitro biofilm models would capture the essence of natural forming biofilms, allowing for a more precise model for study drug delivery and therapeutic development as well as harnessing the favourable properties of biofilms for environmental applications. The fabrication of biomimetic biofilms through 3D bioprinting strategies would allow for rapid development of these models. In addition, 3D printing strategies would allow for the fabrication of biofilms with multiple organism species to more closely mimic the interaction of biofilms and their surrounding environments. Naturally occurring biofilms contain multiple species, yet multispecies models do not exist, and therefore, there is a lack of knowledge on how mixed models may affect antimicrobial susceptibility, in healthcare settings. In addition, engineered biofilms are beneficial in wastewater treatment, bioremediation and bioleaching. With the fabrication of multispecies engineered biofilms, bacterial interactions within a single engineered biofilm could be studied for the first time to further harness the positive characteristics of biofilms for wastewater treatment, bioremediation and bioleaching. In addition to studying multispecies biofilms for antimicrobials and environmental applications, the interactions of multispecies biofilms may result in the admixture fusion of different functional proteins and multifunctional biofilms, which may alter chemical or mechanical properties based on environmental triggers. There still exists many research opportunities for the advancement of single and multispecies 3D engineered biofilms and potential applications but overall 3D printing strategies would provide biomimetic models of biofilms, both biologically and mechanically, for therapeutic development and environmental applications.

AUTHOR CONTRIBUTIONS

Emily Lazarus: Conceptualization (equal); writing – original draft (lead); writing – review and editing (equal). **Anne S. Meyer:** Conceptualization (equal); writing – original draft (equal); writing – review and editing (equal). **Kaoru Ikuma:** Conceptualization (equal); writing – original draft (equal); writing – review and editing (equal). **Iris V. Rivero:** Conceptualization (equal); project administration (lead); writing – original draft (equal); writing – review and editing (equal).

ACKNOWLEDGEMENTS

This work was supported by the Arnold & Mabel Beckman Foundation and the National Science Foundation's Convergence Accelerator program.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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How to cite this article: Lazarus, E., Meyer, A.S., Ikuma, K. & Rivero, I.V. (2024) Three dimensional printed biofilms: Fabrication, design and future biomedical and environmental applications. *Microbial Biotechnology*, 17, e14360. Available from: https://doi.org/10.1111/1751-7915.14360