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3D Bioprinting: A Powerful Tool to Leverage Tissue Engineering and Microbial Systems

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Abstract

Bioprinting covers the precise deposition of cells, biological scaffolds and growth factors to produce desired tissue models. The main focus of bioprinting is the creation of functional three- dimensional (3D) biomimetic composites for various application areas. Successful creations of model tissues depend on certain parameters such as determination of optimum microenvironment conditions, selection of appropriate scaffold, and cell source. As the cell culture-based assays have vital roles in the biomedical field, bioprinted tissue analogs would provide unprecedented chances to study, screen, and treat diseases. Today's 3D bioprinting technology is able to print cells and scaffolds simultaneously, which provides the opportunity for disease modeling. This paper presents a general overview of the current state of the art in bioprinting technologies and potential 3D cell culture systems now being developed to model microbial infections, host-pathogen interactions, niches for microbiota, biofilm formation, and assess microbial resistance to antibiotics.

Keywords: Infection, 3D cell culture, bioprinting, bioink, host-pathogen interactions, microbiota

1. Introduction

Modern printing techniques that allow precise control over product design and development have revolutionized many areas, including art, education, material manufacturing, engineering, and medicine [1,2]. Bioprinting simultaneously combines living cells and biomaterials through a computer-aided (CAD) additive manufacturing process to generate two dimensional (2D) and even 3D bioengineered living constructs that mimic natural tissue characteristics. Due to its high repeatability and accuracy in microscale fabrication resolution, bioprinting technique is commonly used for tissue engineering (TE), regenerative medicine (RM), microbiology or other biological studies [3,4]. The applications of 3D bioprinting are specifically challenged with complexities such as the selection of biomaterials, cell types, growth and differentiation factors, and technical difficulties related to handling of living cells (due to their sensitivity to *in vitro* environment). Addressing these complexities requires the integration of engineering, biomaterial science, cell biology, physics, and medicine [2–6]. In this review article, we give a comprehensive summary of the current applications of 3D bioprinting technologies in modeling host-pathogen interactions and infectious disease mechanisms, niches for microbiota and researches of microbial resistance to antibiotics. We also highlighted the potential implementation of other 3D cell culture techniques varying from scaffold-free and scaffold-based into bioprinting techniques to screen viral and bacterial infections on the mimicked 3D tissue models.

2. Bioprinting

2.1 Concept

In 3D bioprinting, biological materials, biochemicals, and living cells are precisely positioned to build 3D structures. Several approaches for 3D bioprinting, including biomimicry, autonomous self-assembly, and mini-tissue building blocks, are developed to fabricate 3D functional living human constructs with biological and mechanical properties suitable for modeling diseases and clinical restoration of tissue and organ function [2]. The bioprinter systems require distinct specifications of high resolution, high-throughput, ability to control dispensing of multiple bioinks (i.e., homo-/hetero-cellular, bioprintable, and biocompatible biomaterials) with different viscosities concurrently, ease of use, nontoxicity, cell viability, affordability. Consequently, for the precise dispensation of a

bioink, a bioprinter should include three essential elements: (1) a robotic motion (hardware) system, (2) bioink dispensers, and (3) computer-based software-enabled operational control to print bioink with satisfactory resolution [4–8]. Computer-aided design (CAD) software is required to generate a blueprint of tissue/organ design for the mechanical motion of a robotic system as the preprocessing step and dispensing systems. The motion system provides the movement to the bioprinter in x-, y-, and z-axes as the processing step, and finally, the dispensing system (pneumatic-, mechanical- or fluidic-driven) controls the accurate deposition of the medium. Then the bioink is deposited, solidified, and stacked layer-by-layer in the 3D bioprinter as the postprocessing step [1,3,8] (Fig 1).

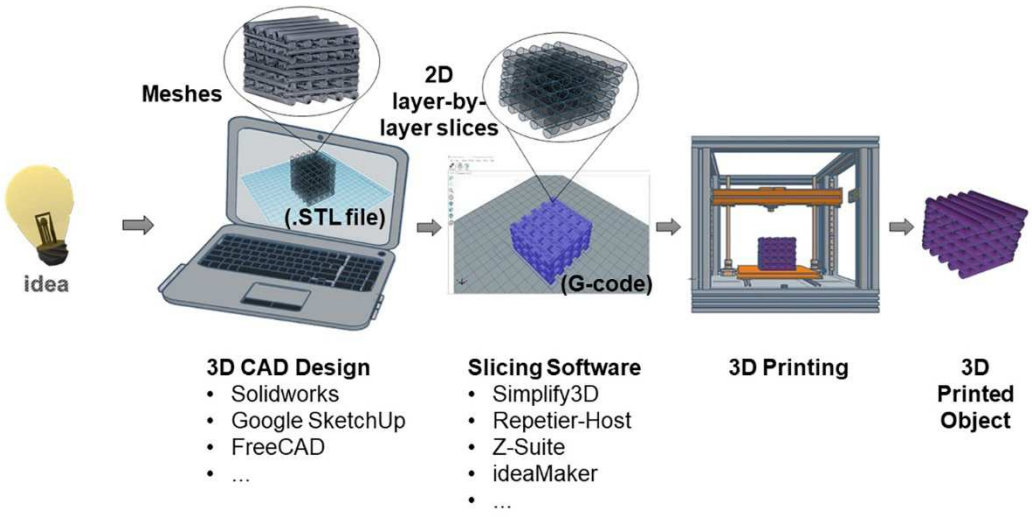


Figure 1. Illustration of 3D (bio)printing processes from the software designs of target tissue/organs to printed models.

2.2 History

The discovery of woodblock printing and the subsequent development of the industrial-scale printing in the 15th century facilitated the rapid reproduction of text and images and the dissemination of information. Printing had a revolutionary effect on society, affecting education, politics, religion, and language across the globe [2]. In 1986, additive manufacturing (AM), rapid prototyping (RP), free form fabrication (FFF), and 3D printing were initially conceived by Charles W. Hull. After two years, bioprinting was first demonstrated by Klebe as cytoscribing technology, a method of micro-positioning biologics including collagen and fibronectin. In that study, cytoscribing was carried out using a Hewlett Packard (HP) inkjet printer and a graphics plotter for specific positioning of cells [9].

With the first attempt of generating cartilage tissue in the shape of an ear on the dorsal of a mouse in 1997 [10], Vacanti and Langer opened up a great venue, where tissue engineering started to emerge in generating tissues in 3D [11]. In 1999, cells were printed with a laser-based bioprinting process by Odde and Renn [12], demonstrating that cells could be patterned in 3D to develop tissue analogs with complex anatomy biomimetically [1,8]. In the earliest 2000s, Rolf Muelhaupt's group at Freiburg Materials Research Center introduced an AM fabrication technique using 3D plotting of thermo-reversible gels in a liquid medium. This group was the first to report the deposition of living cells using an extrusion approach [13]. Afterward and in 2002, an evolution in bioprinting took place when bioengineer Makoto Nakamura realized that the ink droplets in an inkjet printer were the same size as human cells [4,14]. In 2003, Boland and his coworkers started inkjet-based bioprinting by modifying an HP inkjet printer, and cells were successfully printed and patterned [8,15,16]. Until 2005, despite all 3D printers were expensive, proprietary and in industrial scale, costly, and closed nature of the 3D printing industry limited the accessibility of the technology to the exploration that could be done by end-users. The Fab@Home project which was initiated as the first multi-material 3D printer available to the public satisfied the need. Since its open-source release in 2006, it has created a versatile and low-cost printer to accelerate technology innovation and its migration into the consumer space [17] **(Fig 2)**. Several researchers then attempted 3D printing of tissue scaffolds with and without cells, and several spin-off companies have emerged to commercialize breakthrough technologies worldwide [4,5,8,18–20].

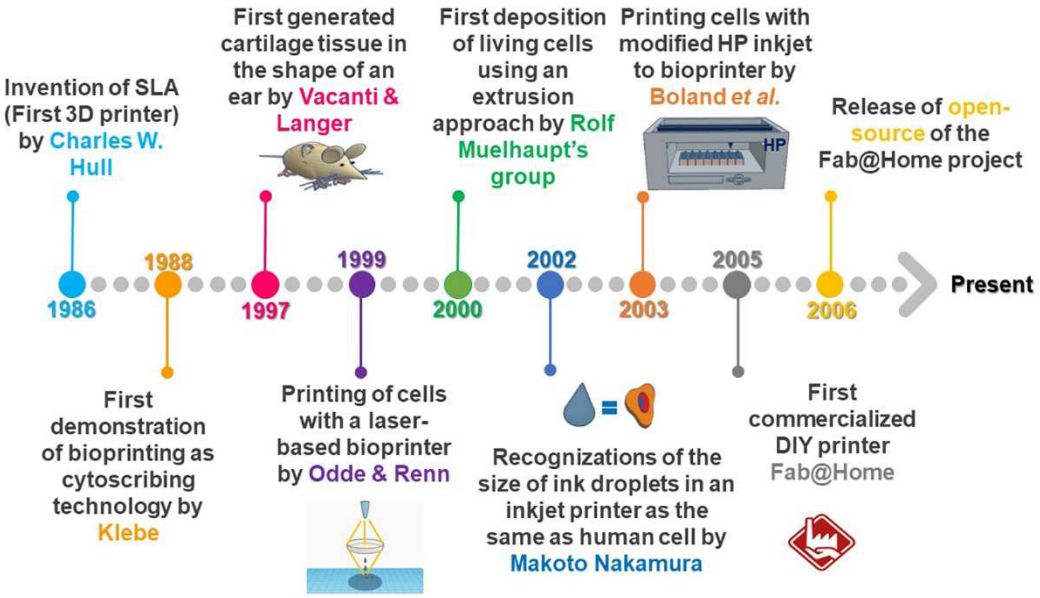


Figure 1. Major milestones in the development of bioprinting technology.

2.3 Techniques

The most promising technologies applied in bioprinting process require specific self-assembly and self-organizing capabilities of cells, and there are three major groups of techniques commonly used in manipulating cells in bioprinting: layer-by-layer (stereolithographic), line-by-line (extrusion-based), and droplet-based bioprinting [8].

The stereolithography (SLA) is a solid freeform, nozzle-free bioprinting method with the high printing quality, and speed, utilizes photopolymerization, a process in which a UV light or laser is directed in a pattern over a path of photopolymerizable liquid polymer, cross-linking the light-sensitive polymers into a hardened layer [21–24]. SLA operates *via* a layer-by-layer process, where each 2D layer is cured in its entirety before moving to the next layer of the construct. As each layer is polymerized, the printing platform can be lowered further into the polymer solution allowing for multiple cycles to form a 3D structure. However, SLA has numerous restrictions such as the lack of biocompatible and biodegradable polymers, harmful effects from toxic photocuring reagents, the inability of complete removal of the supporting structure and the inability to form horizontal gradients in the constructs have been reported resulting from using this method [21,22].

Extrusion-based (solid free-form/fusion deposition) bioprinting is the most common and affordable biological and non-biological 3D printers for the fabrication of complex, multi-layered scaffolds and tissue constructs in biomedical applications. They use the potential energy of mechanical-, pneumatic- or solenoid micro-extrusion-driven system to extrude the bioink through a nozzle, to defeat surface tension-driven droplet formation, and print the cylindrical filament-formed bioink [22,25]. They can print vertically [22] and high viscosity bioinks such as complex polymers, cell spheroids, and clay-based substrates and very high cell densities for tissue formation [25]. However, they are only applicable for printing viscous liquids [22] and poses the potential for the distortion of cellular structure and loss of cellular viability [25].

In contrast, droplet-based bioprinting utilize thermal-, piezo- or acoustic-driven mechanisms to deposit droplets of cell suspension in a high-throughput manner and assembled drop-by-drop [1,2,8,23]. The approaches used in **droplet-based bioprinting** can be classified into (1) inkjet bioprinting, (2) acoustic droplet election, (3) micro-valve bioprinting, and (4) laser-assisting bioprinting (LAP).

The first inkjet printers used for bioprinting applications were modified versions of commercially available 2D ink-based printers [2,23,26], and they have been more popular due to their essential properties such as wide availability

with low cost, ability for highly precise and fast printing [21,22,27], printing of low viscose biomaterials [22] with concentration gradients in 3D constructs [21]. Conversely, they generate unstable droplets at high printing frequencies due to inability to provide a continuous flow and slow printing process [22,27], and they cause thermal, mechanical, and shear stress to the cells [21,27] and cell desiccation/ sedimentation [28]. Moreover, they have poor vertical structure printability, and they can print limited printable materials because of the necessity of low viscosity materials and low cell densities [21,22]. Inkjet bioprinting can be continuous (CIJ), electrohydrodynamic (EHD) jet or drop-on-demand (DOD) inkjet bioprinting, which, are commonly used non-biological and biological applications. In CIJ bioprinting, the pressure is applied to force the bioink through a nozzle, which subsequently breaks up into a stream of droplets to minimize its potential energy and surface tension [26,29].

On the other hand, DOD inkjet bioprinting uses a non-contact technique that may use thermal, piezoelectric, electrostatic, or electromagnetic forces to expel successive droplets of bioink onto a substrate, replicating a CAD design with a printed tissue [1,29]. Moreover, DOD inkjet bioprinters are preferable than CIJ bioprinters for tissue bioprinting purposes because of their properties such as economical, handy to control, and accessible to pattern biologics. However, DOD needs high pressures to eject droplets through a nozzle with a small orifice diameter, which is harmful to cells. Oppositely, electrohydrodynamic (EHD) jet bioprinters utilize an electric field resulting from the electrical potential difference between the printhead and the substrate, to pull the bioink droplets through the printhead orifice as limiting the need for substantially high pressure, shear stress and induced cell damage [26,29].

Acoustic droplet ejection bioprinting relies on a gentle acoustic field generated by an acoustic actuator to eject droplets of cell-laden bioink solution through a nozzle. It is a quick, easy and viable method without mechanical stress on cells as depositing picoliter quantities of the medium or hydrogel encapsulating a single cell in a droplet because bioink is an open pool rather than in a nozzle, thus eliminating the exposure of cells to detrimental stressors such as heat, high pressure, and high voltage [26,29–31]. However, viscous bioinks are not dispensable, and there are no complete commercial systems available [26].

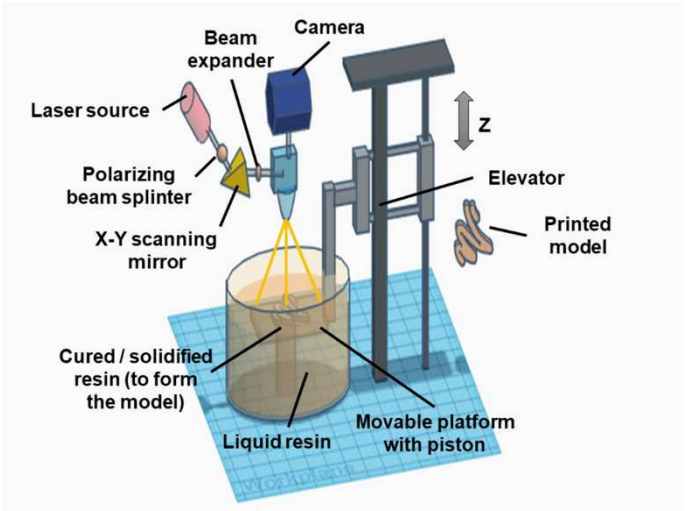
Micro-valve bioprinting, which is a reliable, cheap, and secure method, operates with interchangeable electromechanical/solenoid valves to generate droplets of cell-laden bioink when a voltage pulse is applied to the valve [26,27,29,31]. Depending on the pressure and gating time, bioink with a wide range of printable viscosity [27] is dispersed continuously or drop on demand. Thus, cell damage because of high shear stress on cells during droplet ejection is limited, but cells can be sedimented, and larger droplets (50–300 μm) diameters than nozzle orifice diameter leading to a lower resolution are created [26,27,29].

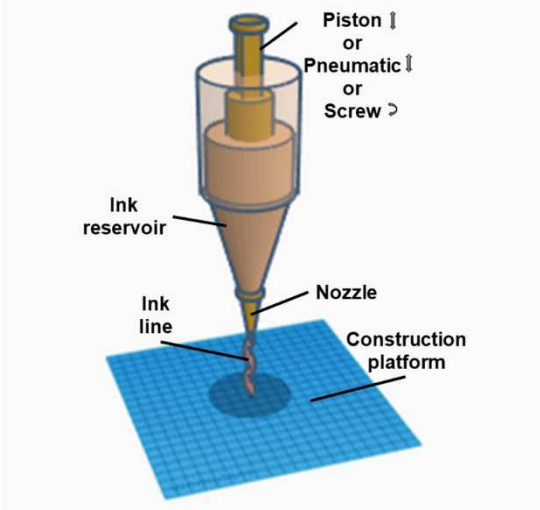
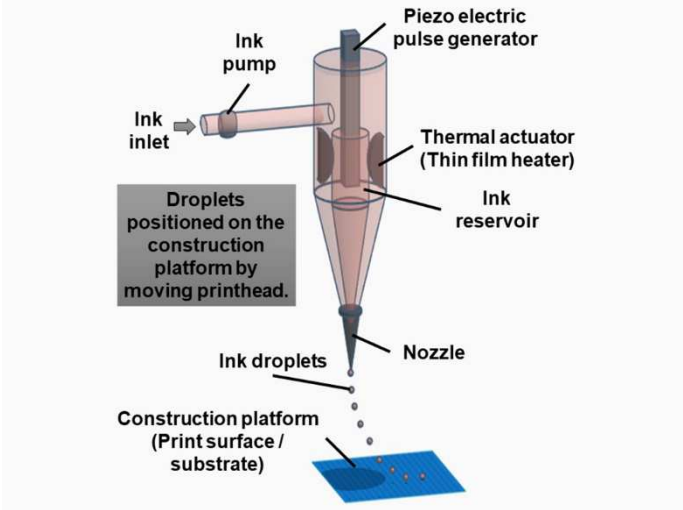
LAP utilizes laser energy to selectively print and precisely pattern cells onto a substrate to deposit cells from a donor slide to a receiver slide without the need for a nozzle [8,21,22,27]. Initially developed to pattern metals (i.e., computer chip fabrication) with high resolution [25], laser-induced forward transfer (LIFT) technology has been successfully applied to biological material, such as peptides, DNA and cells with high cell viability [2,27].

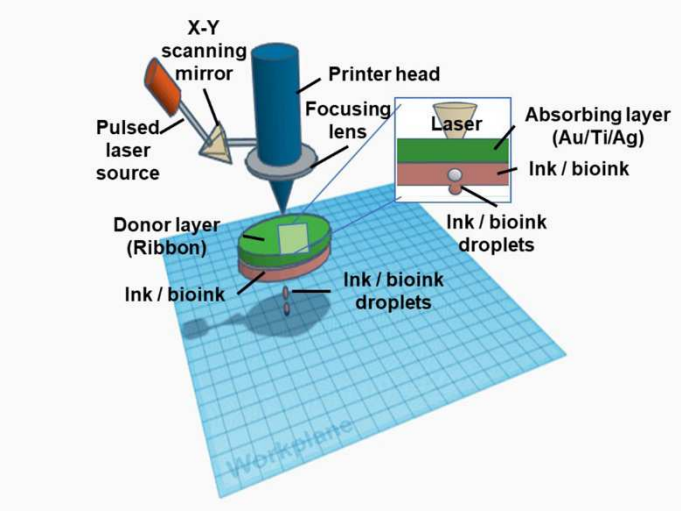
LAP uses laser pulses to heat and vaporizes a solution containing bioactive contents (e.g., growth factors, cells), depositing the contents onto the scaffold with biomaterials in a wide range of viscosity [22,27] without damage from the laser, which could have a destructive effect on these factors. However, this process is costly and slow [21,22]. On the other hand, it can cause thermal damage due to nanosecond/ femtosecond laser irritation [22] and toxic effect on the cells because of the needed metal film [21,27]. Moreover, because of the non-uniform thickness of the transparent layer (ribbon), cell homogeneity is reduced at the low cell density, and it is challenging to incorporate multiple types of biologics [27].

Depending on the type of ink selected and the complexity of the final tissue construct, each bioprinting technique has specific properties and advantages/disadvantages depending on printability, resolution, deposition rate, scalability, bioinks, and biocompatibility, ease of use, printing speed and price, and commercial availability [8,23,25]. Comparison of the conventional bioprinting methods can be found in the table below **(see Table 1)**.

Table 1. Comparison of the conventional bioprinting methods

Printing Technology	Advantages and Disadvantages
Stereolithography 	+ Printing time independent of complex geometries + Good vertical printability + Low cost [22] + Light-sensitive hydrogels can be printed layer-by-layer [21] + Solid freeform and nozzle-free technique [21,22] + High accuracy [21,22] - Applicable to photopolymers only - Lack of biocompatible and biodegradable polymers [21] - Lack of printing multi-cells [22] - UV light source is harmful to DNA and human skin and toxic to cells during photocuring [21,22]

Microextrusion 	+ Good vertical printability [22] + Capable of printing various biomaterials and the ability to print high cell densities [22,25] - Only applicable for viscous liquids [22] - Potential distortion of cellular structure and loss of cellular viability [25]
Inkjet 	+ Wide availability + Ability to introduce concentration gradients in 3D constructs [21] + Ability to print low viscosity biomaterials [22] + Fast fabrication speed + Low cost [21,22] + High printable resolution/precision [21,22,27] - Inherent inability to provide a continuous flow and slow build process [22] - Droplet instability at high printing frequencies - Potential cell desiccation/ sedimentation [27] - Poor vertical structure printability - Limited printable materials/requires low viscosity materials and low cell densities - High cost and time consuming [21,22] - Thermal, mechanical, and shear stress to the cells [21,27]
Laser-assisted printer (LAP)	+ Nozzle-free, non-contact, easy process [8,21,27] + High printing resolution and highly precise delivery control of ink droplets [21,22] + Deposition of biomaterials in a wide range of printable



viscosity [22,27]

+ High cell viability [27]

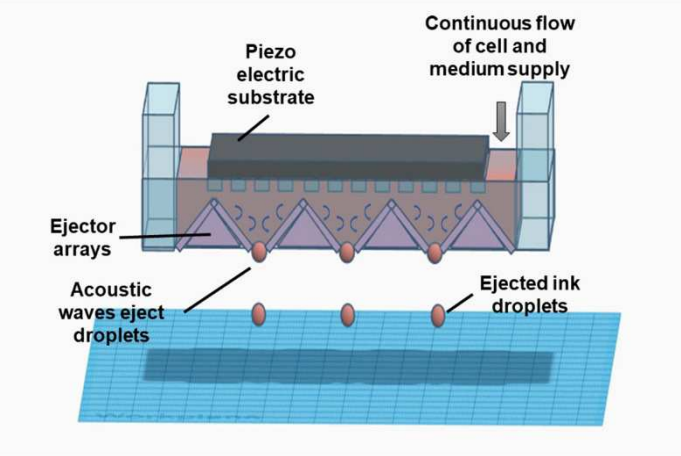
- Thermal damage due to nanosecond/ femtosecond laser irritation [22]

- Difficult to incorporate multiple types of biologics

- Non-uniform transparent layer (ribbon) thickness and poor cell homogeneity of the coated 'ribbon' at a low cellular density [27]

- Requires a metal film which is subject to cytotoxic metallic particle contamination [21,27]

Acoustic droplet ejection



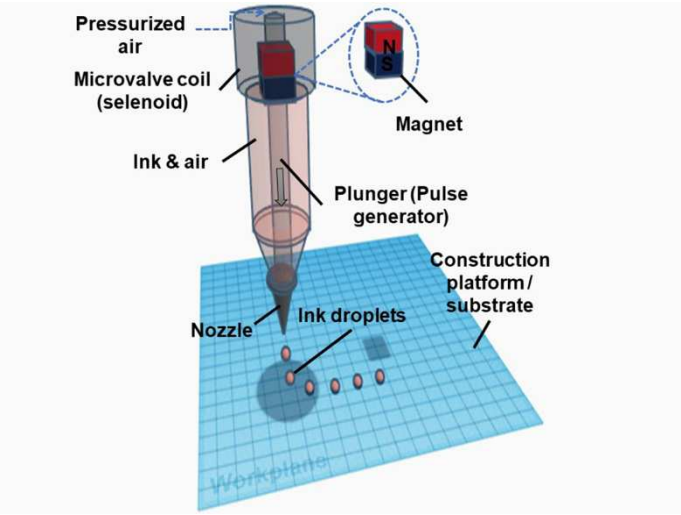
+ No mechanical stress on cells during droplet ejection

+ Easy and fast to fabricate [26,30]

- Viscous bioinks are not dispensable

- Unavailability of complete commercial systems [26]

Microvalve (electromechanical/solenoid)



+ Low cost

+ Interchangeable nozzles which are cleanable from viscose bioinks [26]

+ A wide range of printable viscosity

+ A reliable system for high-throughput printing

+ Easy operation [27]

- Significantly larger droplet diameters than nozzle orifice diameter [26]

- Cell sedimentation [27]

- High shear stress on cells during droplet ejection [26,27]

2.4 Materials – bioinks

There is a global research trend in medicine to develop biomaterials for creating biomedical devices, drug delivery, cell encapsulation, and implantation. Biomaterials are used to create an artificial extracellular matrix (ECM) to provide structural and functional support for the cells and tissue constructs **(Fig 3)**. Different potential biocompatible materials are ranging such as naturally-derived, chemically-synthesized polymers including their modifications and composite materials. In the case of bioprinting, biomaterials need to be incorporated with bioactive molecules and viable cells to create functional structures. Bioinks are a distinct class of biomaterials made up of cellular material, additives (such as growth factors, signaling molecules), and supportive scaffolds which biomimic ECM structure [25,32]. Bioinks need to possess certain characteristics such as specific fabrication temperature, gelation (cross-linking) kinetics, swelling, and bioactive components in addition to biocompatibility, bioprintability, affordability, scalability, practicality as well as resolution, mechanical/structural integrity, bioprinting/post-bioprinting maturation times and biodegradability. Therefore, hydrogels are generally used to mimic the natural ECM in the physiological body due to their high-water content and high permeability to oxygen, nutrients, and other water-soluble compounds, ability to protect cells/drugs and to be modified with specific ligands to create an environment for cell adhesion/proliferation [25,32,33].

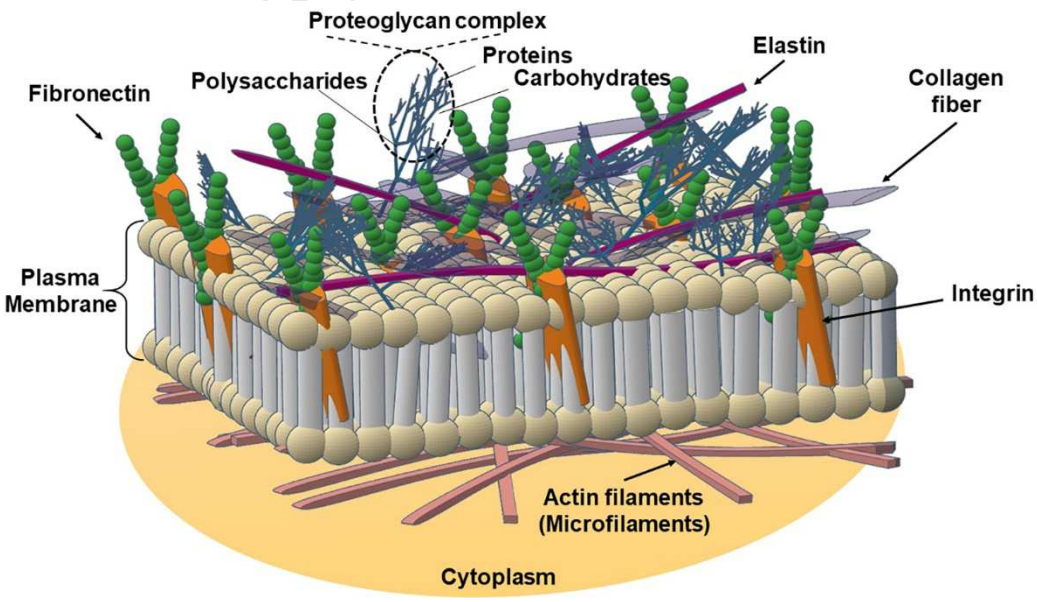


Figure 3. Illustration of ECM structure having a dynamic 3D network of extracellular macromolecules, particularly proteoglycan complexes, collagen fibers, elastin, and other matrix glycoproteins.

Hydrogel-based bioink materials should have specific properties such as good shape fidelity, high zero-shear viscosity (paste-like consistency) [32] and controlled cross-linking to facilitate bioprinter deposition, suitable swelling characteristics, and short-term stability. These properties are required to ensure that tissue structures such as pores, channels, and networks do not collapse [2]. During bioprinting, a hydrogel with suspended cells is processed into a precisely defined shape, which is successively fixed by gelation, a physical cross-linking reaction depends on meshes of high molecular polymer chains, ionic interactions, and hydrogen bridges because of compatibility with biological systems such as growth factors and living cells [4].

Natural-derived hydrogels such as Matrigel, collagen, gelatin, gelatin methacryloyl (GelMA), fibrin, alginate, chitosan/chitin, hyaluronic acid (HA) have been heavily utilized for regenerative medicine because they usually already contain specific bioactive regions that give them good cellular compatibility with the cells of interest. However, they have issues concerning immunogenicity, characterizing their intrinsic properties, variations in terms of properties between species, tissue, and the batch of production and relatively instability compared to their synthetic counterparts. Therefore, fully synthetic functionalized hydrogels such as Poly (2-hydroxyethyl methacrylate) (PHEMA), Poly (vinyl alcohol) (PVA), Poly (ethylene glycol) (PEG) that are also used as bioinks due to their benefits (e.g., highly tunable and consistent properties, and large-scale production capacity) [34]. Although hydrogels have good bioactivity, they are mechanically weak. Therefore, some thermoplastic materials such as polycaprolactone (PCL) and poly (lactic-co-glycolic acid) (PLGA) and other acellular materials like nanocellulose, hydroxyapatite (HA), and β-tricalcium phosphate (β-TCP) are also used to utilize soft materials, hydrogels with enhancing their mechanical strength and shape fidelity to generate functional, bioprinted tissue constructs made with hybrid bioinks [25,32,33,35].

3. Applications of Bioprinting In Medicine

3D cell culture systems have developed as pioneering methodologies and have reached rising prevalence from a wide range of tissue engineering [36–39], regenerative medicine [25,31], infection biology [36,40,41] areas for the outlook to establish highly quantitative researches on the biological entities (e.g., cells, bacteria, and viruses) with spatially defined artificial ECM microenvironments. 3D cell culture systems provide artificial and functional tissue constructs serving as modular platforms which the most encouraging experimental models; hence, it displays many complex characteristics of *in vivo* systems. Traditionally, a top-down approach has been employed, which cells are seeded on top of the pre-made biodegradable scaffolds that provide sufficient mechanical support for a uniform

monoculture tissue layer. Alternatively, a bottom-up approach has been inducted, relying on the assembly from soluble components together with the cells as building hundred-micrometer-scale cellular constructs under conditions compatible with cell viability [42] **(Fig 4)**.

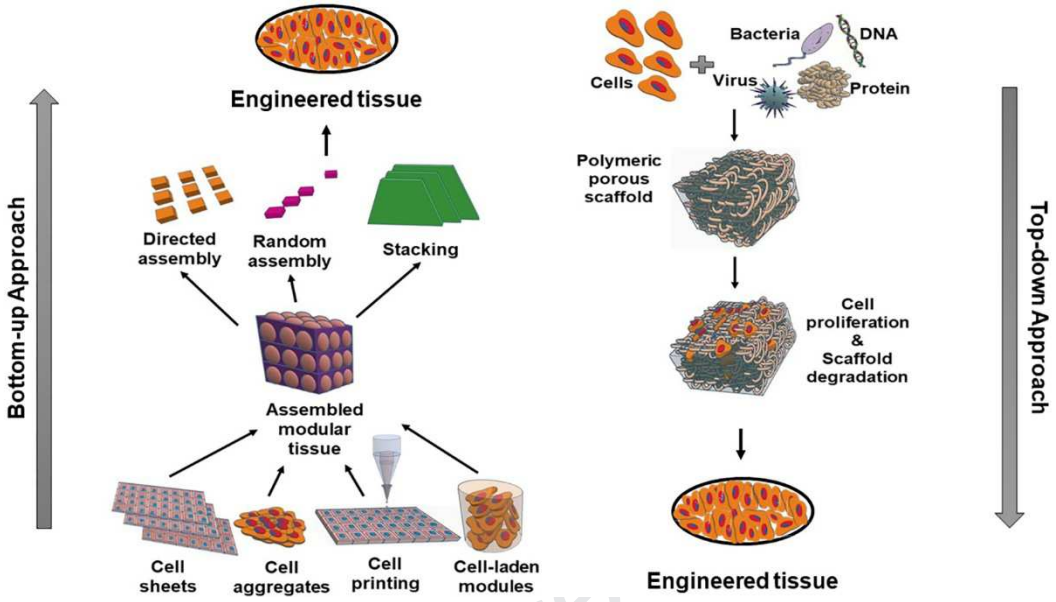


Figure 4. According to the "bottom-up" approach, single cells or organoids/spheroids are used as blocks for complex tissue structures. In contrast, the traditional "top-down" approach proposes to form a tissue structure by planting cells onto scaffolds at a particular shape and size.

Various 3D cell scaffold-free (without biomaterials) culture strategies exist for the development and application of 3D models of human tissues *in vitro* microenvironment, including ultra-low attachment microplates, bioreactors to generate micro-tissues (spheroids). Besides the scaffold-free 3D cell culture methods, many strategies have dependencies on the biocompatible scaffolds, which often require synthesis and fabrication processes **(Fig 5)**. Therefore, reliable techniques have been needed for real-time monitoring of cellular responses, and variation of 3D culturing methodologies (e.g., 3D hydrogel scaffolds, 3D spheroids, 3D organoids) and biomaterials have been described to create connected tissues to improve function and, to overcome barriers resulting from traditional 2D cell culture systems such as well-plate, Transwell® (Corning, USA). Advances in 3D printing/bioprinting technologies have allowed creating complex constructs used in a wide range of medical applications such as dentistry [43–46], drug/pharmaceutical fabrication [47–49], *in vitro* drug screening [50–52], surgical instruments [53–55], medical training and education [56–58], TE and RM [59–62].

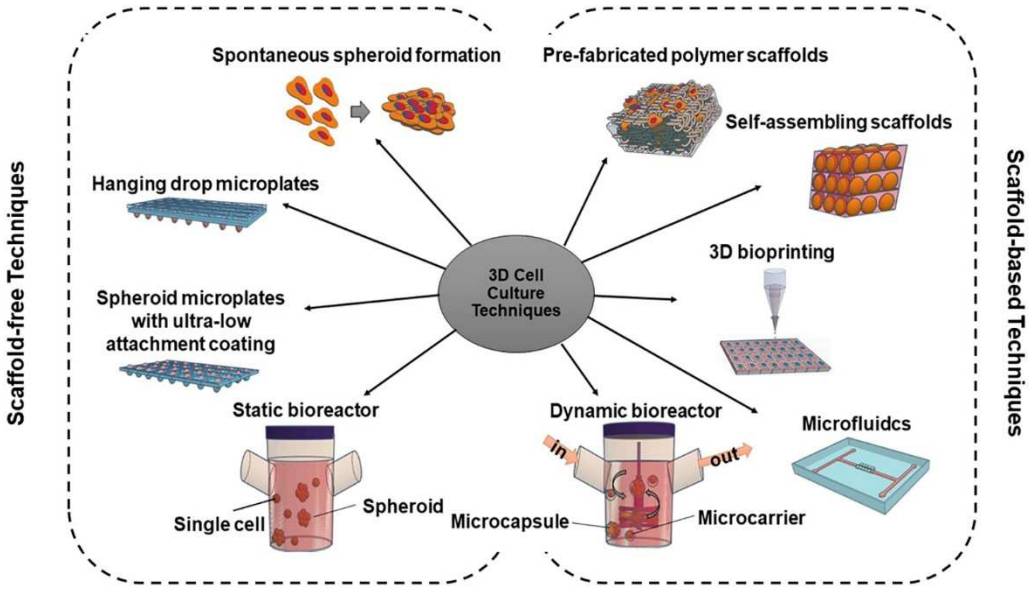


Figure 5. 3D cell culture techniques are leading to create more accurate *in vitro* tissue models.

RM is a multidisciplinary scientific field that has rapidly incorporated TE principles with life sciences to simulate native tissues for replacement of damaged tissues or reparation of malfunctioning organs. Traditional TE strategy follows the top-down approach to keep the 3D shape and mechanical properties of the mimicked tissue, to support in cell attachment, and to provide a substrate for cell proliferation into 3D functioning tissues. In general, the application of scaffolds in RM is straightforward but still subject to some difficulties like the lack of accuracy in cell placement, limited cell density, needs of organic solvents, challenges in integrating the vascular network, insufficient interconnectivity, inability to control distribution and dimensions of the pore, and difficulties in manufacturing patient-specific implants [63]. 3D printing/bioprinting technologies are encouraging to overcome these difficulties of applications of the scaffolds in RM. In the case of infection biology studies in RM, 3D bioprinting strategies are a new paradigm. Although *in vivo* models are still crucial for infection studies, the selection of the model might change all the results. Therefore, 3D *in vitro* models are valuable research tools to generate data in agreement with *in vivo* reports, and they have helped researchers to reconsider part of the knowledge derived from 2D *in vitro* cell cultures experiments.

Currently, 3D cellular *in vitro* models are the most promising models able to acquire information about the host response to infections, especially for difficult-to-culture pathogens. Most *in vitro* infection studies have been performed using cell lines; however, researchers try to develop complex 3D model systems with different cell types including primary/ stem cells, immune cells, e.g., T cells, macrophages under different physical conditions such as different surface/ oxygen tensions, physical forces, geometries of ECM and explore the integration of cellular signals in regulating infection to better recapitulate the native tissue [64–67]. Because of that, the localization of ECM

deposition can impact the process of the *in vitro* infection, reconstituting a protecting barrier and preserving host cell integrity against invasion. Moreover, a significant challenge for the study of host-pathogen mechanisms in 3D is the use of biomaterials that does not affect very similar cell exposure to pathogens and exclude a non-physiologically manner interaction [68]. Therefore, these difficulties have motivated many groups toward the development of bioprinting and new bioink composition approaches.

4. Bioprinted Models For Virus Infections

Viruses are highly contagious and present a conuning public health threat to human [36]. Nowadays, viral infections continue to emerge quickly, causing significant morbidity and mortality worldwide as well as an economic burden [69,70]. Animal models widely used in preclinical studies for viral infections are used to evaluate vaccines and potential antiviral drugs [71–74]. However, having limited fully representative animal host models [75,76], numerous human pathogens need elaborated models to be subjected [70,76–78]. Indeed, one of the significant factors to be considered in selecting an animal model for virus studies is the susceptibility of the animal model to the pathogen. For instance, mice have been commonly used as an animal model for virus studies (i.e., influenza) and evaluation of theranostic efficiency of drugs and vaccines. However, it has become a necessity to use humanized systems to develop models that are closest to reality and progress in diagnosis and treatment as it is difficult to translate data from mice into human physiology. Thus, researchers have focused on engineering approaches to study virus-associated cell culture models to increase knowledge regarding the underlying mechanisms of viral infections and constituted a basis for future studies [36]. Recent studies have shown that advanced 3D cell culture models have the potential to recapitulate the native microenvironments of virus-associated diseases to investigate the structural and functional changes of ECM through the physical, chemical, and biological aspects.

For several pathogenic viruses (i.e., papilloma), epithelia are the site of replication and infection. Therefore, studies related to host-virus interactions are mostly performed *via* 3D organotypic epithelial raft cultures as they present a relevant model for investigating *in vitro* virus replication and pathogenesis as well as studying the effects of antiviral agents [41,79–83]. The source of epithelial tissues may be different parts of the body (larynx, cervix), and the primary/immortalized cell lines can be used in the raft culture models [41,80]. To this respect, human papillomavirus (HPV) has been demonstrated with 3D organotypic epithelial raft cultures as well as human immunodeficiency virus (HIV), human herpesvirus (HSV), varicella-zoster virus (VZV), and adenovirus (AdV). Apart from the physiologically relevant raft culture models, 3D organoids [84], and multicellular spheroid systems [85] have been used to study AdV.

Beyond the static 3D cell culture models, microfluidic platforms offer multi-compartmental 3D structures to mimic native tissues and provide an opportunity to observe physical and biological changes under the dynamic conditions individually. Although the applications of microfluidics in virology are still in progress, their capability to be used for disease modeling has been proven in many studies. For instance, Villenave *et al.* (2017) [86] used coxsackievirus B1 (CVB1) to model enteric virus infection using dynamic gut-on-a-chip microfluidic platform where human villus intestinal epithelium was cultured. It is reported that the platform running under conditions of physiological peristalsis-like motions comprising relaxation of circular smooth muscles while maintaining luminal flow is suitable to model *in vitro* enteric virus infection and investigate mechanisms of pathogenesis. Similarly, the demand for miniaturized cell culture systems serving as a platform for studying hepatitis B virus (HBV) infections on hepatocyte physiology led researchers to focus on microfluidics and HCB associated liver disease was modeled using human HepG2 hepatocellular carcinoma cells and rat hepatocytes [87]. Similar to microfluidics-based studies, dynamic radial flow [88,89] and rotating wall vessel [90,91] cell culture bioreactor systems have been used to study hepatitis C virus (HCV) and hepatitis E virus (HEV) infections (**see Table 2**).

Table 2. An overview of 3D cell culture models for viral applications

3D Model	Study Target	Cell Lines	Virus	Ref.
Spheroids / Organoids	Virus-mediated gene transfer	Epithelial HEK-293 cells	AdV	[84]
	Glioblastoma	Glioma cells		[85]
Organotypic raft cultures	Infection and life cycle investigation	Primary, gingival and immortalized human Keratinocytes	HPV	[80,82]
	Evaluating anti-viral drug efficiency		VZV	[41]
			HIV	[79,81]
	Virus replication		HSV	[80]
Radial flow and rotating-wall vessel bioreactors	Evaluating anti-viral drug efficiency and virus replication	Human hepatocellular carcinoma-derived cell line	HCV	[88,89]
	Viral infection	Hepatoma-derived cell line		[91]
	Virus replication	Hepatocarcinoma cells	HEV	[90]
Microfluidics platforms	Viral infection	Rat hepatocytes and human HepG2 cells	HCB	[87]
		Human Caco2 intestinal cells	CVB1	[86]

Recently, 3D bioprinting, among many popular tissue engineering approaches, brings new insights in the study of virus and development of viral infection models having the ability to mimic *in vivo* viral life cycle along with cell cultures [92,93] and the latest virus studies supported by 3D bioprinting technology have mostly focused on the liver [94], lung [93] and brain [95,96] disease modeling. Bioprinting enables to fabricate cell-laden scaffolds (**see Table 3**) and a 3D printed scaffold may contain many different cell types with various biomaterials within the structure. As the combination of material and cells have to be well defined in the patterned network, it is possible to generate human cell-based scaffolds to reflect human physiology better than animal models for virus studies [69,93,94,97,98].

Table 3. An overview of virus infection models with cell-laden and cell-free bioinks

Bioink Type	Purpose	Cell Line	Virus	Bioink	Bioprinter	Ref.
Cell-laden	Liver infection model	Human bipotent hepatic progenitor cells (HepaRG)	Human adenovirus	Gelatin, sodium alginate, human ECM mixture	Microextrusion	[92]
	Nervous system infection model	Superior cervical ganglia (SCG) and hippocampal neurons	Pseudorabies virus	-	Microextrusion	[95]
	Respiratory system infection model	Human alveolar A549 cells	Influenza A	Gelatin, alginate and matrigel mixture	Microextrusion	[93]
Virus-infected cell-laden	Tracing cells *in vivo*	Mouse induced hepatocyte□like cells (miHeps)	mCherry lentivirus	Alginate	Microextrusion	[94]

Recent research profiles have shown the bioprinted network including arginyl-glycyl-aspartic acid (RGD), the most common tripeptide sequence on ECM, would induce cell migration, adhesion and proliferation [99–101] and that it is essential to prepare well-mixed bioink including suspended cells in growth media and hydrogel solution in bioprinting

process **(Fig 6A)**. Hiller *et al.* (2018) [92] presented a study describing the optimization of a bioink mixture composing of alginate, gelatin, and human ECM to print human HepaRG liver cells with a pneumatic extrusion printer for transduction and infection studies through a liver model. Similarly, Berg *et al*. (2018) [93] manipulated the same bioink mixture by using matrigel instead of human ECM to provide a scaffold for human alveolar A549 cells.

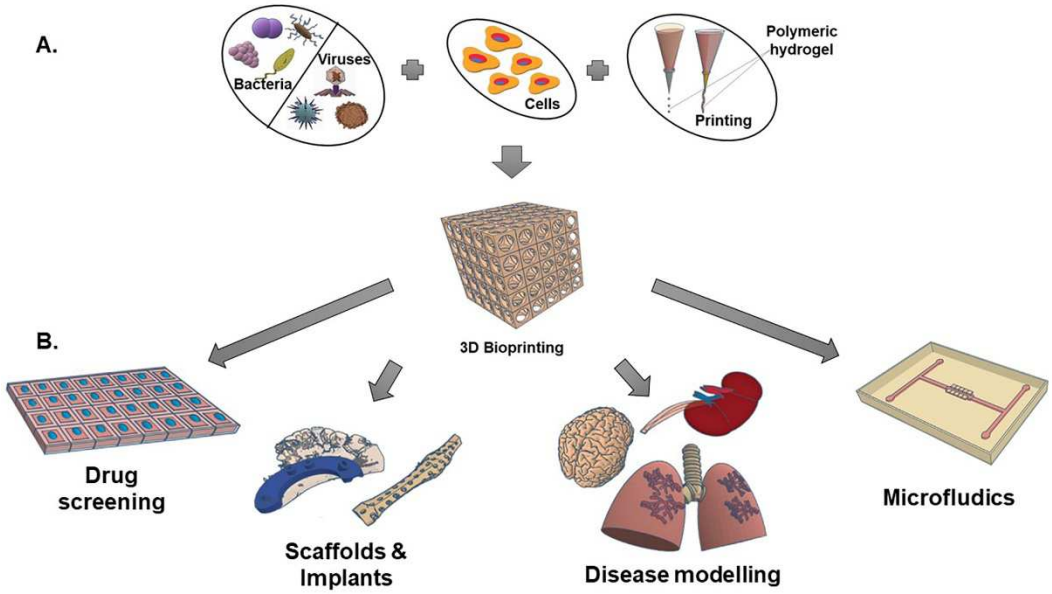


Figure 6. Schematics of the creation of multifunctional living materials. **(A)** Pathogens and/or animal cells are embedded in the bioink formulations to utilize ECM for bioprinting 3D tissue constructs. **(B)** 3D bioprinting technologies are capable of creating complex constructs in a wide range of TE and RM applications such as *in vitro* drug screening, antimicrobial activity of 3D tissue constructs, surgical instruments, host-microbiome interactions, disease modeling, and microfluidics.

Although the incorporation of cells in bioink mixture is still a progressing field of 3D bioprinting-based studies, 3D printing has been used as a manufacturing technique in TE applications, especially for scaffold formation and TE-based cell/gene therapy and implantation, for decades. For instance, Wang *et al.* (2014) [102] used 3D printing technology to produce a virus-activated matrix as a porous bone scaffold to promote endothelial cell activation, migration, and adhesion. They used ceramic, b-tricalcium phosphate (b-TCP) and HA to get ink mixture. A human-safe virus is genetically engineered to generate filamentous phages containing RGD on the side walls. Researchers have recorded that it is possible to induce differentiation of mesenchymal stem cells (MSCs) into osteoblasts by using RGD phages without any additional osteogenic supplements [96,102]. As the gene therapy provide excellent solutions

for many diseases, Yang *et al.* (2017) [103] focused on glioblastoma gene therapy by using vesicular stomatitis virus (VSV) as plasmid DNA encoding VSVMP which can eliminate cancer cells and induce an anticancer immunity response.

5. Bioprinted Models For Bacteria And Biofilm Formation

Cell cultures formed of a single cell type have given significant insight into understanding host-pathogen interactions and infectious disease mechanisms preclinically. However, these limited *in vitro* TE models lack many primary characteristics present in the native, 3D dynamic host microenvironments that are associated with host-pathogen interactions; regulating infection, multicellular complexity, bacterial microbiota, gas exchange, and nutrient gradients, and physiologically relevant biomechanical forces [64] (e.g., fluid shear, stretch, compression). 3D cell culture techniques such as spheroid/organoid cultures [104–108], explant/organotypic cultures [109–113], polymeric scaffolds [40,114,115], natural [116–132] and synthetic hydrogel [133–141] scaffolds, and microfluidics [141–153], programmable and customizable platforms to engineer cell-laden constructs have been under development to mimic host tissues. The development of such 3D tissue systems would allow numerous potential applications including (1) modeling host-bacterial microbiome interactions *in vitro* 3D microenvironment **(see Table 4)** [40,109–114,154–158], (2) testing the antibacterial activity of 3D tissue constructs [37,38,141,143,145,153,159–165] and, (3) biofilm formation [166,167] **(Fig 6B)**.

Table 4. Potential experimental 3D cell culture models that simulate host-microbiome interactions in the human tissues

Model Format	Target Tissues	Potential Bacteria	Ref.
3D spheroids / organoids	• Blood Vessel	• NA	[104,106]
	• Intestinal	• NA	[105]
	• Lung	• *S. typhimurium*	[107,108]
3D organotypic / explant cultures	• Gingiva / Oral	• *F. nucleatum*	[110]
	• Intestinal / Gut	• Recombinant *E. coli (EcN)*	[111]
	• Lung	• *S. aureus*	[156]
	• Skin	• *S. aureus, D. nodosus*	[109,113]

3D Polymeric Scaffold	• Bone	• *E. coli, S. aureus, P. nigrescens, P. gingivalis, S. sanguinis, E. faecalis, S. mutans*	[37,115,159,161,165,166]
	• Skin	• *S. aureus*	[40]
	• NA – various issues	• *S. aureus*	[164]
3D Hydrogel Scaffold	• Blood Vessel	• NA	[120,129,152]
	• Bone	• *P. aeruginosa, E. coli, S. aureus, S. epidermidis*	[125,137,154,167]
	• Intestinal / Gut	• *E. coli*	[114,136]
	• Kidney	• NA	[138]
	• Liver	• NA	[121,124,133,134,139]
	• Lung	• *H. influenzae*	[112,135]
	• Skin	• *S. aureus*	[116,118,119,123,126–128,130–132,140,141,160,162,163]
Microfluidics (chip) systems	• Blood Vessel	• *P. aeruginosa*	[149,155]
	• Bone	• *S. epidermidis*	[144,153]
	• Kidney	• NA	[148,151]
	• Liver	• NA	[142,146,147,150]
	• Skin	• NA	[126]
	• Intestine / Gut	• *Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus paracasei, Bifidobacterium breve, Bifidobacterium longum, and Bifidobacterium infantis*	[168]

Plenty of studies within microbial ecology supported that niche-based, which plays an essential role in mediating, where they perform their neutral processes such as reproduction, mobility, and involvement in cooperative and predatory relationships is jointly responsible for microbial community assembly [169–172]. Bacteria perform these activities because of their adaptive metabolic activity and created biofilms as complex extracellular polymeric

substances (EPS) that warrant survival even in hostile environments where they can communicate *via* short-range physical and chemical signals, interactions, and other adaptive phenotypes, adapt their mechanical properties under stress to match conditions imposed by the surrounding environment [171–174]. Therefore, mimicking natural biofilms is convenient and efficient for biotechnological applications. Bacteria communicate *via* signal molecules, which allow bacteria to monitor and alter functional behaviors in the microenvironment. During interaction with one other, bacteria produce, release, sense, and respond to chemical inducers. This phenomenon is named as quorum sensing, and it regulates bacterial population density by secretion and detecting of extracellular signals [175,176]. In the scope of microbial communications, new approaches have emerged to establish relevant structures for cell-cell, cell-ECM, and host–pathogen interactions. Besides, artificial microenvironments are useful tools as they are supporting microbial cell viability [130,177,178]. Recently, bacteria-associated 3D bioprinting applications have been focused on observation of microscale communications through the spatial configuration of populations, observation of quorum sensing mechanisms and fabrication of suitable biomaterial for microbial microenvironments [173,176,179,180] (**see Table 5**).

Table 5. An overview of artificial ECM for bacterial communication and regulation of microenvironments

Purpose	**Bacteria**	**Bioink**	**Bioprinter**	**Ref.**
Printing bacterial spores on to the flexible material	*Geobacillus stearothermophilus* and *Bacillus atrophaeus*	Guar gum and borax	Inkjet	[178]
Investigation of photopatterned microstructures to single bacteria	*Bacillus subtilis*	Bovine serum albumin (BSA) and riboflavin 5′-monophosphate sodium salt hydrate	Two-photon direct laser writing	[179]
3D printing of bacterial cultures for artificial microenvironment construction	*E. coli*	Sodium Alginate	Microextrusion	[177]
Real-time observation of the quorum-sensing mechanism Observation of cell-cell interactions in bacteria community	*P. aeruginosa* *S. aureus* and *P. aeruginosa*	Bacteria included gelatin and bovine serum albumin	Microscopic three-dimensional printing /	[176] [173]

Demonstration of tunable photoresponsive material manipulation	*P. aeruginosa*	Protein-based hydrogels	multiphoton photolithography	[181]
Developing 3D printed scaffolds for the growth of bacteria	*E. coli* and *S. cerevisiae*	Alginate-gelatin-agar	Microextrusion	[130]
Generating predictive models for microbial growth	*E. coli* and *S. enterica*	Agarose	Microextrusion	[180]

Biofilms are formed in a non-immobilized state at a variety of surfaces and interfaces by depositing a layer of bacteria in nature, and they are initially suspended in a fluid culture medium on the desired substrate in the biotechnological applications. There are several immobilization approaches such as adsorption on surfaces, cross-linking, encapsulation, and entrapment for providing bacteria with a free-formed, defined geometrical microenvironment to keep metabolic activity with increasing the production yield to form/degrade of compounds, chemicals, biopolymers, enzymes, and proteins relevant for the food, medical, biotechnology and chemical industries. Naturally, bacteria produce their biofilms in the form of protective gels with very diverse mechanical properties **(Fig 7)**.

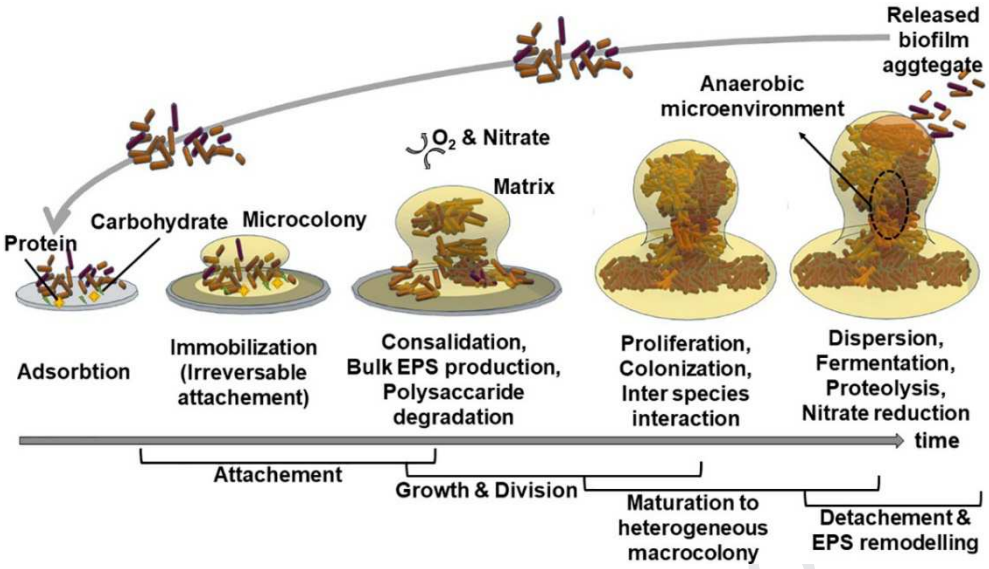


Figure 7. Schematics of the bacterial biofilm formation cycle

Therefore, using bacteria as the programmable biochemical machinery is promising. Researchers try to create "living materials" with controlled self-supporting structures with complex 3D geometries, compositional, and physical attributes of microhabitats and dynamic metabolism *via* 3D bioprinting platforms [181–183] **(see Table 6)**. As a proof of concept, Schaffner and colleagues [174] selected two different species, *A. xylinum* and *Pseudomonas putida* (*P. putida*), to develop living materials for both bioremediation and biomedical applications. *P. putida*, a known phenol degrader, are able to form an interface between air and water by secreting amyloid fibers. To benefit from this feature of bacteria, culture was incubated and embedded in a hydrogel mixture consisting of HA, k-carrageenan, and fumed silica to create a functional bacteria-derived bioink (Flink). In another study, to pattern bacteria communities, *P. aeruginosa* were used similarly. To control EPS and biofilm formations, researchers focused on optogenetic manipulation of second messenger cyclic dimeric guanosine monophosphate (c-di-GMP) levels which regulate intracellular signaling process in numerous bacterial species [184].

Table 6. An overview of 3D bioprinting applications with bacteria-associated bioinks

Purpose	**Bacteria**	**Bioink**	**Bioprinter**	**Ref.**
Biocatalytic process of particulate monooxygenase (pMMO) for selective methane conversion	*Methylococcus capsulatus*	PEG-pMMO	Projection microstereolithography	[185]

Observation of microbial community interactions by patterning bacteria on agar, glass, and paper surfaces	*Paenibacillus dendritiformis T* and *P. dendritiformis C*	Bacteria included carboxymethyl cellulose, xanthan gum and gum arabic	Screen printing	[182]
Demonstration of patterned living materials, combining 3D printing and genetic engineering	*E. coli*	Plasmid containing alginate	Microextrusion	[183]
Bioremediation and biomedical applications	*P. putida* and *A. xylinum*	Functionalized HA	Microextrusion	[174]
Optogenetic manipulation of engineered strains	*P. aeruginosa*	Self-produced EPS matrix	Microprinting	[184]

As the biomaterial-depended *in vitro* cell culture techniques have a vital role in the biomedical field, there is an expanding demand for biocompatible materials to functionalize engineered models. Biocompatible polymers are preferred as they can be designed and synthesized through the desired properties. Although using both natural and synthetic polymers as bioink is common, current studies indicate that synthetic polymers are lack of biocompatibility, biodegradability, and bioactivity, and it is challenging to characterize natural polymer such as alginate, cellulose, chitosan [39]. As it was mentioned at previous section (**see section 3.1.2**), Ulusu *et al.* (2017) [186], generated a new achievement to overcome this problem by using protein as a bioink instead of polymer. They used Caf1 protein which is able to remain stable and bioactive under the extreme conditions like high pH levels, salt, and chemical concentrations and reported that Caf1 protein was a suitable component for 3D cell culture applications. In addition to usage of protein as a part of bioink in biomedical applications, Blanchette *et al.* (2016) [185] widened their perspectives and performed a study in which enzyme, particulate methane monooxygenase, was mixed with polyethylene glycol diacrylate (PEGDA) to construct a biocatalytic polymer to observe conversion of methane to methanol.

6. Bioprinted Tissue Systems Using Bacteria

Bacteria generally grow within structured 3D inhabitants formed of multiple bacterial species in the human body. Organization of each bacteria and bacterial populations as aggregates play critical roles in community characteristics and communication. Accordingly, geometry may affect the pathogenicity and viability of bacteria. 3D printing of bacterial communities provides chemically interactive, native-like physical arrangement with a defined size, shape, and density [173]. There are different approaches to develop new platforms for TE-constructs in particular for skin and bone tissue engineering using live bacteria, bacteria-produced materials, or functionalized materials preventing bacterial infections **(see Table 7)**.

Table 7. An overview of the use of bacteria for bioprinted tissue engineered-models.

Applications	Purpose	Cell Line	Bacteria	Bioink	Bioprinter	Ref.
Innovative approaches to fabricate TE platforms	Decellularization to create patterns in interconnected micropores/microchannels in the scaffold for TE applications	Murine embryonic fibroblasts (NIH-3T3)	Gram negative-bacteria (*E. coli*)	Agarose	Microextrusion (custom-made)	[187]
	Production of miniature drug/antibiotic-screening platforms	Human kidney cell line 293	Green fluorescent protein-expressing *E. coli*	Alginate and Agar	Thermal inkjet bioprinting	[188]
Bacteria-produced materials for TE	Immobilization of bacteria in a 3D matrix to produce bacterial cellulose (BC) scaffolds applicable for	NA	*A. xylinum*	Functionalized HA (Flink)	Microextrusion	[174]

	personalized skin transplants					
	Generation of 3D hierarchical scaffold mimicking natural intervertebral discs (IVD)	Nucleus pulposus cells and annulus fibrosus cells	*A. xylinum*	BC	NA	[162]
	Generation of bacteria-produced Caf1 polymers applicable for 3D cell culture and wound healing	NA	Transformed competent *E. coli* cells with pGEM-T Caf1 plasmid	Caf1	Inkjet printer	[186]
Antibacterial modification of biomaterials with gel film	Generation of Carboxymethylated-Periodate Oxidized Nanocellulose Constructs for wound dressing applications	NA	*P. aeruginosa*	Pinus radiata based-nanocellulose	Microextrusion	[189]
Antibacterial modification of biomaterials with np	Generation of material with antibacterial property and osteogenic capability and a high potential for bone defect therapy and reconstruction	Rabbit bone marrow stromal cells	*E. coli*	β-TCP bioceramic scaffolds coated with Ag Np - GO nanocomposite	3D printer (developed by the Fraunhofer Institute for Materials Research and Beam Technology)	[190]

	Fabrication of interconnected and well-ordered microporous antibacterial scaffolds for bone TE applications	Homo sapiens bone osteosarcoma cells (MG63)	*E. coli*	nMgO modified poly (3-hydroxybutyrate-co-3-hydroxy valerate)	Selective laser sintering	[191]
	Fabrication of anti-infective grafts for bone TE applications	Human mesenchymal stem cells (hMSCs)	*S. aureus, S. epidermidis,* 25 methicillin-resistant *S. aureus*	Quaternized chitosan /PLGA / HA	4th generation 3D BioplotterTM 2	[192]
Functionalization of biomaterials with antibiotics-loading	Fabrication of 3D printed antibiotics-loaded biodegradable polymeric scaffold for regenerating bone tissue	RAW 264.7 Cell Line murine Macrophage	*E. coli* and *S. aureus*	Tobramycin-loaded PCL/PLGA	Multi-head deposition 3D printing system	[193]
	Fabrication of new bioactive glass (BG)-polymer - antibiotic composite films for stainless steel implant coatings	MG63 cells	*E. coli* and *S. aureus*	BG - Polymethyl methacrylate	3D printing with the matrix-assisted pulsed laser evaporation method	[194]

One of the innovative approaches is to use live bacteria as the sacrificial porogens for decellularization to create patterns in a 3D printed scaffold, which is broadly applicable and compatible with tissue-specific applications [187]. In such a study, the bioprinting technology was used to produce miniature drug-screening

platforms, which realistically and inexpensively evaluate biochemical reactions in a picolitre-scale volume at a rapid rate to stimulate drug/antibiotics discovery for developing countries [188].

In another study, *Acetobacter xylinum* (*A. xylinum*) was cultured in a specific hydrogel ink, called Flink, to create a functional 3D matrix for immobilization of bacteria to produce bacterial cellulose (BC) used for personalized biomedical applications [174]. As an example, *A. xylinum*-produced BC was used for the generation of 3D hierarchical structures containing type II collagen-based nucleus pulposus (NP) with NP cells and BC-based annulus fibrosus (AF) with AF cells for mimicking natural intervertebral discs (IVD) to act as a replacement for the therapy of degenerative disc disease [162]. Ulusu *et al.* (2017) [186] used transformed *Escherichia coli* (*E. coli)*-produced capsule-like antigen fraction 1 (Caf1), as a well-defined, bioactive and thermostable 3D scaffold for 3D cell culture and wound healing applications.

Most TE studies generally focused on the production of antimicrobial biomaterials or modification/functionalization of biomaterials utilized for implantation. In majority of the cases, the modification of biomaterials was carried out by forming gel films- or nanoparticle (Np) coating. In a study, plant-based nanocellulose was printed in a 3D porous structure for modifying film surfaces as a bio-responsive, elastic gel to carry/release antimicrobial components for wound dressing applications [189]. Also, Yang *et al.* (2016) [192] created 3D chitosan-based polymeric nanocomposite porous scaffold with high potentials such as less risk of antibiotic-resistance, repairing infected cortical/cancellous bone defects and the restoration of infected bone defects. Moreover, Zhang *et al.* (2017) [190] developed a combination of a 3D-printing method and a layer-by-layer coating technique to prepare antibacterial silver - graphene oxide nanocomposite coated-bioceramic scaffolds for bone defect therapy and reconstruction. Similarly, researchers fabricated nano-magnesium oxide modified polymeric scaffolds with 3D interconnected and well-ordered microporous structures, and evidenced functional advantages such as intense antibacterial activity, cellular adhesion, proliferation, and osteogenic differentiation [191].

On the other hand, antibiotic loading is used to fabricate modified-functional scaffolds to improve the anti-inflammatory, bactericidal effects. As an example, Shim *et al.* (2015) [193] generated 3D printed antibiotic-loaded biodegradable polymeric scaffold which is capable of eradicating chronic osteomyelitis and regenerating bone tissue, which would be a promising solution as a carrier for delivery of antibiotics in orthopedics. In another example, Floroian *et al.* (2016) [194] fabricated antibiotic loaded-bioactive glass-

based polymeric composite films to coat stainless steel implants, and showed an anti-biofilm/antimicrobial activity.

7. Summary and Future directions

Microbial systems have been studied *in vitro* by focusing on the interaction of a microorganism with a single host cell cultivated as 2D monolayers. Although this reductionist approach has advanced our understanding of mechanisms that underlie infection and disease, the correlation of *in vitro* and *in vivo* results has been challenging. Recent technological advancements in the field of bioprinting and 3D cell culture have revealed new approaches to model microbial infections, host-pathogen interactions, niches for microbiota, biofilm formation, and determine microbial resistance to antibiotics. Spheroid/organoid cultures, explant/organotypic cultures, polymeric scaffolds, natural and synthetic hydrogel scaffolds, and microfluidics, programmable and customizable platforms to engineer cell-laden constructs are among the most widely reported techniques for modeling host tissues and studying various diseases. 3D organotypic epithelial raft cultures, gut-on-a-chip, liver-on-a-chip, and dynamic cell culture vessels have been applied for modeling virus infections (i.e., HPV, HIV, HSV, VZV, ADV, CVB1, HBV, HCV, and HEV). 3D bioprinting enables the fabrication of human cell-based scaffolds (i.e., cell-free bioink and bioink-cell mixture) that can be used as surrogates to replace animal models and study *in vivo* like physiologial conditions (including cell migration, adhesion and proliferation). Various 3D printed models were particulary developed for investigating quorum sensing, bacterial biofilm formations (*Geobacillus stearothermophilus*, *Bacillus atrophaeus*, *Bacillus subtilis*, *E. coli*, *P. aeruginosa*, *S. aureus*) and gene therapy. In the future, a remarkable emphasis is expected to be devoted to the design and formulation of various bioinks, where cell viability, cellular distribution, and efficiency of infection will be the key parameters for optimization. We expect the integration of modular infectious units with different organs-on-chips, for modeling viral and bacterial infections, where infectious units can be added or removed for a more realistic assessment of the pathologies and effects on respected organs. With the emergence of 4D printing, “intelligent” 3D constructs will be printed which can respond to external stimuli including pressure, heat, electric current, ultraviolet light, leading to a desired change in the shape or function of the construct. Despite the current achivements, considerable research is still required for the utilization of *in vitro* infection models as a standard approach in preclinical studies and personalized

treatments in clinical settings. But the establishment of numerous start-up companies and the rapid growth in the field of tissue and microbial engineering will expedite the arrival of such systems into real-field applications and thereby advancing efficent tissue models to control and eliminate infectious diseases.

Author Contributions

E.S. and A.A.D.G. contributed equally to draft the article. A.A.D.G designed figures using open-source 3D modelling platform, Tinkercad™, Autodesk®. O.Y.C. and M.S.D. contributed equally for revising the draft critically for important intellectual content. All authors contributed for conceptualization and design of the study, and gave approval for submission.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: