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Print Me An Organ! Why We Are Not There Yet

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Abstract:

Bioprinting offers a highly-automated and advanced manufacturing platform that facilitates the deposition of bio-inks (living cells, biomaterials and growth factors) in a scalable and reproducible manner, a process that is lacking in conventional tissue engineering approaches. Significant improvements in the field of bioprinting have occurred over the last two decades. This reviews provides an in-depth analysis of recent improvements in the bioprinting techniques, progress in bio-ink development, implementation of new bioprinting and tissue maturation strategies. Special attention is given to the role of polymer science and how it complements 3D bioprinting to overcome some of the major impediments in the field of organ printing. A concise overview of the anatomy and physiology of different tissues/organs is provided, followed by important design considerations to better facilitate the fabrication of biomimetic tissues/organs for tissue engineering and regenerative medicine (TERM). Last, a realistic overview of current status in organ bioprinting is presented, including recent accomplishments in bioprinting tissue-engineered constructs, the limitations and challenges, as well as opportunities for future research. We strongly believe that with the advances in polymer sciences, it will be an impending reality for on-demand bioprinting of patient-specific tissues/organs.

Keywords: 3D Bioprinting; 3D Printing; Additive manufacturing; Bio-inks; Bioprinting strategies

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List of Abbreviations

2D	two-dimensional	H&E	hematoxylin and eosin
2PP	two-photon polymerization	HA	hyaluronic acid
3D	three-dimensional	HEK	human embryonic kidney
Ca ₂ Cl	calcium chloride	hiPSC	human induced pluripotent stem cells
Ca ₂ SO ₄	calcium sulfate	HRP	horseradish peroxidase
Ca ₂ CO ₃	calcium carbonate	LSECs	liver sinusoidal endothelial cells
H ₂ O ₂	hydrogen peroxide	MEW	melt electro-writing
AECs	alveolar epithelial cells	MMC	macromolecular crowding
ALI	air-liquid interface	MRI	magnetic resonance imaging
CGTP	current good tissue practice	NaOH	sodium hydroxide
CT	computed tomography	NO	nitric oxide
dECM	decellularized extracellular matrix	PCL	polycaprolactone
DHT	dehydrothermal	PEG	poly(ethylene glycol)
DLP	digital light processing	PEO	poly(ethylene oxide)
DMD	digital micro-mirror device	PF127	pluronic F-127
DOD	drop-on-demand	PPO	poly(propylene oxide)
ECM	extracellular matrix	SLA	stereolithography
ESCs	embryonic stem cells	SMCs	smooth muscle cells
EVE	excluded volume effect	TERM	tissue engineering and regenerative medicine
FDA	Food and Drug Administration	TGase	transglutaminase
FRESH	freeform reversible embedding of suspended hydrogels	UV	ultra-violet
GAGs	glycosaminoglycans	VEGF	vascular endothelial growth factors
GelMA	gelatin methacrylate	VOT	valve opening time
GMP	good manufacturing practices		

1. Motivation

Tissue or organ failure due to natural aging and diseases is a critical medical challenge. Current treatment for organ failure relies mostly on organ transplantation from living or deceased donors. The problem of organ shortage has escalated over the last three decades (Table 1); numerous patients on the waiting list for organ transplantation have died due to the scarcity of suitable donors, whereas those recipients with successful organ transplantation are subjected to constant immunosuppression to prevent acute and chronic graft rejection [1]. This problem could be solved by using autologous cells derived from the patient's own body to create a 3D patient-specific replacement tissue/organ [2]. This would potentially minimize the risk of tissue/organ rejection and eliminate the need for lifelong immunosuppressant.

The conventional tissue engineering approach relies on the use of suitable scaffolds to support cell attachment, proliferation and tissue formation. The scaffold-based approach is advantageous for achieving optimal scaffold's mechanical properties and degradation kinetics, as well as delivering biomolecules and growth factors embedded within the scaffolds [3]. However, the use of scaffolds poses some challenges such as the low initial cell seeding density and poor homogeneity within large 3D porous scaffolds. The field of tissue engineering is poised to meet the unmet clinical needs through the development of new fabrication approaches and complementary strategies. A promising approach to fabrication of 3D patient-specific autologous tissues/organs is using 3D bioprinting technique [4, 5]. It offers a highly automated manufacturing platform for the fabrication of complex bio-engineered constructs; the living cells and biomaterials were precisely deposited via a layer-by-layer bioprinting process to control the spatial arrangement of these functional components in the complex 3D tissue-engineered constructs. Researchers are currently striving to fabricate 3D functional living human constructs with biological and mechanical properties that closely emulate the native tissues/organs. The key challenges are to reproduce the complex micro-architecture of the extracellular matrix (ECM) components and to recapitulate the critical cell-cell and cell-ECM interactions within the 3D bioprinted constructs. Although solid organ bioprinting for human transplantation is still only in the infancy stages, it is a promising approach to scalable and reproducible fabrication of patient-specific tissue-engineered constructs [6]. The implementation of good manufacturing practices (GMP), quality control and legislation are facilitated using automated processes. The goal of this comprehensive review article is to present a comparative evaluation of bioprinting techniques, current trends in bio-ink development, the implementation of novel bioprinting and tissue

maturation strategies, the role of bioprinting in organ bioprinting and lastly to highlight the potential challenges and achievements in organ bioprinting.

Fig. 1. Global statistics on the organ transplantation relative to the number of related deaths in year **2015** (blue) and **2016** (orange) (latest statistics obtained as of January 2019)

2. Evaluation of 3D bioprinting Techniques

The term “3D bioprinting” can be defined as “the use of computer-aided transfer processes for patterning and assembling living and non-living materials with a prescribed 2D or 3D organization in order to produce bioengineered structures serving in regenerative medicine, pharmacokinetic and basic cell biology studies” [10-12]. The 3D bioprinting processes can be classified into five main categories; (i) extrusion bioprinting [13, 14], (ii) stereolithography bioprinting [15], (iii) inkjet bioprinting [16], (iv) laser-assisted bioprinting [17] and (v) microvalve-based bioprinting [18]. The other complex bioprinting processes which are not covered in this review include two-photon polymerization (2PP) [19-21], microfluidic printing [22-24] and acoustic bioprinting [25-27].

2.1. Extrusion

The extrusion-based bioprinter is driven by a pneumatic- or mechanical- (piston or screw) system to extrude cell-laden bio-inks through the nozzle orifice in a continuous manner; the printing process is repeated in a layer-by-layer approach to eventually fabricate 3D complex constructs. The pneumatic system is prone to delays associated with the compressed gas volume, while the piston-driven system may provide more direct control over the flow of the bio-ink and the screw-based deposition provides more spatial control and is capable of dispensing bio-inks for higher viscosities [28]. However, the huge pressure drop generated by the mechanical deposition can be harmful to the encapsulated cells due to possible disruption of the cell membranes which results in cell death. Conversely, the pneumatic deposition can be controlled by the air pressure to deposit a broader range of bio-ink type and viscosities.

It is the most widespread approach for fabrication of 3D cell-laden constructs; the bio-inks are generally loaded in disposable printing cartridges and dispensed either pneumatically or mechanically onto a receiving substrate. The minimum nozzle size of an extrusion-based bioprinting system is generally over 100 μm , as smaller nozzle size would result in a considerable increase in the shear stress and corresponding cell damage and death and increase likelihood of nozzle clogging [13].

Although the bio-ink viscosity has been reported as the determining factor for good shape fidelity, a high bio-ink viscosity does not necessarily confer high mechanical strength or printing accuracy [29]. A recent study has highlighted that the printability of a hydrogel-based bio-ink in

an extrusion-based bioprinting system is highly dependent on the loss tangent ($\tan \delta$) [30]. The loss tangent is the ratio of the loss modulus (G'') to the storage modulus (G'):

$$\tan \delta = \frac{G''}{G'}$$

The printability of each individual bio-ink is defined by its own set of loss tangent “printability window” and a minimum printing pressure is required to overcome the yield stress of the bio-ink prior to extrusion of the printed filament. Furthermore, another study has highlighted the advantages of computational approaches in simulating the 3D bioprinting process by reducing the time and cost in the development and evaluation of bio-inks in extrusion-based bioprinting system [31].

The governing equation of extrusion-based bioprinting process [32] can be defined as:

$$d = D^2 \cdot \sqrt{\frac{1}{32\mu Lv} \cdot \left(\frac{4n}{3n+1}\right) \cdot \Delta P} \quad (1)$$

The above equation correlates the relationship between the printed filament diameter (d), nozzle diameter (D), apparent viscosity (μ), nozzle length (L), stage moving speed (v), power law index (n) and the pressure drop (ΔP). The apparent viscosity (μ) and power law index (n) can be determined from the rheological study of the bio-inks. The recommended range of printing viscosity ranges between 30 mPa.s to 6×10^7 mPa.s [33] and the maximum printable cell density is up to 10^7 cells per ml. However, it is important to note that an increase in the cell density was shown to affect the reproducibility of bioprinting by interfering with the hydrogel formation [34, 35].

The key factor that determines the cell viability is the influence of shear stress acting upon the living cells in the nozzle orifice during the printing process. The shear stress is influenced by bio-ink viscosity, input force, nozzle dimension and geometry. The printed cells in a tapered nozzle only experienced higher shear stress near the nozzle orifice, whereas the printed cells in a straight cylindrical needle experienced high shear stress along the entire nozzle length [36]. A recent study utilized highly sensitive cells such as embryonic stem cells (ESCs), which showed a cell viability $\geq 90\%$ when a low shear stress of 100 Pa was applied [37]; the cell viability also increases with shorter printing time and printing temperature near to physiological temperature. Due to its relatively larger nozzle size, the extrusion-based bioprinting system can facilitate continuous and high throughput printing at a high printing speed. Apart from the extrusion of

cell-laden hydrogels, melt electro-writing (MEW) [38, 39] enables the extrusion of reinforcement PCL fibers in the micro-scale (2 – 50 μm) to potentially fabricate tissue constructs of anatomically-relevant shape, size and structural integrity.

2.2. Stereolithography (SLA)

The SLA bioprinting process is based on the spatial solidification of liquid resin by photopolymerization; the bioprinting process can be categorized into two different types: (i) bottom-up SLA or (ii) top-down SLA. In the bottom-up printing process, the photo-curable resin is cured through a window in the bottom of the vat by a beam spot via a light source from below. The build platform is raised out of the resin vat and a ‘peel’ step is required between each layer. Similarly, the resin is cured by a beam spot from the light source that is positioned above the vat and the build platform is lowered down into the resin vat for the top-down printing process. Conversely, the use of a digital micro-mirror device (DMD) in digital light processing (DLP) enables the instantaneous crosslinking of a single photo-curable layer instead of a single beam spot in SLA [40]. Higher printing speed can be achieved using top-down printing process as continuous light exposure is used to cure the resin without the series of alternating light exposures and ‘peel’ steps. A pre-defined pattern is illuminated onto the surface of the photo-curable resin inside a vat using a computer-controlled laser beam or a digital light projector and the cured resin is then solidified to a defined depth which allows it to adhere to the build platform [15]. Upon curing of the first layer, the build platform will move away from the light source to facilitate the recoating of the liquid resin in the subsequent build layer. The following steps are repeated to eventually fabricate solid 3D complex constructs.

The printability in a SLA system can be represented by the rate of photo-initiated polymerization (R_i), which is given by [41]:

$$R_i = \frac{2IC_i\epsilon\phi f}{N_A h\nu} \quad (2)$$

Here, I is the incident light intensity (units of power/area) and C_i is the photo-initiator concentration. Intrinsic properties of the photo-initiator that influence its utility are ϵ , the molar extinction coefficient (absorptivity); ϕ , the quantum yield or cleavage events that occur per photon absorbed; and f , the photo-initiator efficiency or the ratio of initiation events to radical generation by photolysis. Other parameters include the Avagadro’s number, N_A ; Plank’s constant, h and the frequency of the initiating light, ν . Equation 2 demonstrates how the various

parameters affect the utility and performance of a photo-initiator when exposed to uniform light intensity of a monochromatic wavelength. Support material is always required in SLA system (both bottom-up and top-down) and the support structures are printed in the same material as the part and are removed manually after printing. Structures such as overhangs and bridges (critical overhang angle is usually around 30°) are necessary to support the complex structures in the bottom-up SLA printing process. Some of the other critical criteria include designing a base layer for good adhesion between the printed construct and build platform and minimizing the cross-sectional area of each printed layer to lower the force experienced by the printed construct during the “peeling” step. Similarly, support structures are also required for accurate printing of complex constructs in the top-down SLA system.

The size of voxel can be derived by including the depletion of photoactive molecules into:

$$\frac{\partial R(t)}{\partial t} = (P_o - R(t))\sigma_2\chi N^2 \quad (3)$$

where R is the radial density at the time t and P_o is the initial concentration of initiating molecules. For a time-independent photon flux $N(r, z, const.)$, the spatial photon flux $N(r, z)$ can be written for a Gauss shaped laser beam at $r = 0$ and $z = 0$ as the center of the focus, as:

$$N(r, z) = N_o \left(\frac{\omega_o}{\omega(z)} \right)^2 e^{-2r^2/\omega(z)^2} \quad (4)$$

Where $N_o = N(0,0)$, r , is the radial distance from the center axis of the beam ω_o , is the beam waist and the beam width at position z , $\omega(z) = \omega_o \sqrt{1 + \left(\frac{z}{Z_R}\right)^2}$, with the Rayleigh length Z_R .

The printing resolution (voxel diameter $d(N_o, t)$ and length $l(N_o, t)$) can be calculated by integration of the rate equation over the irradiation time t and the condition $R(r, z) \geq R_{TH}$ to yield [42]:

$$d(N_o, t) = \omega_o \sqrt{\ln\left(\frac{\sigma_2\chi t N_o^2}{\ln\left(\frac{P_o}{P_o - R_{TH}}\right)}\right)} \quad (5)$$

$$l(N_o, t) = Z_R \sqrt{\ln\left(\frac{\sigma_2\chi t N_o^2}{\ln\left(\frac{P_o}{P_o - R_{TH}}\right)}\right) - 1} \quad (6)$$

where ω_o is the beam waist, σ_2 is the two-photon absorption cross section, χ is the electric susceptibility, t is the irradiation time, N_o^2 is the squared photon flux, P_o is the initial

concentration of initiating molecules, R_{TH} is the threshold radial density and Z_R is the Rayleigh length. A minimum radial density is required to overcome the threshold value necessary for occurrence of photochemical reactions. The two most frequently used parameters to influence the voxel size are the average laser power (i.e. the photon flux) and the irradiation time. High-resolution voxel diameters in the range of 80 – 100 nm can be achieved with short irradiation times [43]. Conversely, applying a radial density that is close to the threshold value would potentially result in inadequate laser parameters like pointing stability or inhomogeneity of the material [44]. One of the biggest problems relating to the accuracy of printed constructs using SLA is resin shrinkage when exposed to excessive light. The typical lateral printing resolution is dependent on the laser spot size and it is usually in the range of 20 – 50 μm whereas the typical layer height in SLA system ranges between 25 – 100 μm .

The highest resin viscosity that can be employed in stereolithography for satisfactory layer recoating is approximately up to 5 Pa.s [45] and the maximum printable cell density is up to 10^8 cells per ml. The choice of light wavelength of the SLA system has a huge influence on the cell viability; it has been reported that the short UV rays (UVA: 320 – 400 nm, UVB: 280 – 320 nm, UVC: 200 – 280 nm) have more detrimental effects on the cell's nuclear DNA as compared to the visible light. Furthermore, increased light intensity and increased photo-initiator concentration can also have cytotoxic effects on the living cells [41].

2.3. Laser-assisted

A typical laser-assisted bioprinting system consists of a pulsed laser beam with a focusing device, a donor slide (an energy-absorbing layer coated with subsequent layer of cell-encapsulated hydrogel) and a collector slide facing the ribbon. The laser energy vaporizes the energy-absorbing layer to induce a pressure that ejects the droplets of cell-encapsulated hydrogel towards the collector slide.

It is a nozzle-free printing approach that eliminates clogging issues. A layer of energy-absorbing 'ribbon' (~60 nm thickness) is first coated onto the donor slide, followed by the manual coating of the cell-encapsulated hydrogel layer (~ 3 – 20 μL) using a coater blade [46]. It is important to ensure a ~50 μm homogeneous cell-hydrogel layer (cell homogeneity and constant layer thickness) and multiple 'ribbons' (one ribbon for one single cell type) are utilized to fabricate heterogeneous cellular constructs. A gap of 350 – 500 μm is maintained between the donor and collector slides.

The transient pressure generated by the interaction of short-pulsed laser with the ribbon-hydrogel interface results in bubble growth and propagation that govern the printing regime. The pressure in the ribbon can be derived from the general Bernoulli equation [47]:

$$p(r, t) = p_{\infty} + \frac{R}{r} [p(R) - p_{\infty}] + \frac{1}{2} \rho \frac{R}{r} \dot{R}^2 \left[1 - \left(\frac{R}{r} \right)^3 \right] \quad (7)$$

where p_{∞} is the pressure in the hydrogel flow at the infinite distance from gas bubble, $p(R)$ is the pressure in the liquid at the bubble boundary, R is the current gas radius, $\dot{R}$ is the first order derivative of R , r is the distance from the center of bubble and ρ is the liquid density. The pressure increase resulted in formation of a vapor bubble at the ribbon with three potential printing regimes; a sub-threshold regime, a jetting regime and plume regime. A sub-threshold regime occurs ($r < r_2$, low laser fluence) when there is no material transfer due to weak bubble expansion, whereas the plume regime occurs ($r > r_1$, high laser fluence) when there is liquid splashing due to violent bubble expansion. Jetting regime occurs ($r_2 < r < r_1$) when intermediary values of laser fluences are applied [48].

The printing resolution is highly dependent on the laser pulse energy, gap distance, focused laser spot size, thickness of the energy-absorbing layer, substrate surface properties and cell-encapsulated hydrogel [46, 48]. The amount of ejected bio-ink (V) using an elliptical Gaussian beam with energy E_o can be represented [49]:

$$V(x, y) = \frac{2E_o}{\pi\omega_x\omega_y} e^{-2\left(\frac{x^2}{\omega_x^2} + \frac{y^2}{\omega_y^2}\right)} \quad (8)$$

where x and y are the Cartesian coordinates along the major and minor axes of the beam respectively. The optimal range of printing viscosity ranges between 120 – 300 mPa.s and a minimum of 10^8 cells per ml is necessary to ensure at least one cell was present in each printed droplet [50]. A study has reported that a ribbon thickness of at least 40 μm can improve the cell viability to over 95% [51]. Other important considerations that might affect the cell viability include the fast drying of the thin cell-encapsulated hydrogel layer [52] and possible transfer of harmful residues from the energy-absorbing layer [53]. The current laser-assisted bioprinting process has a relatively high throughput rate of up to 100 kHz [50]; but the incorporation of multiple laser beams for simultaneous scanning can help to improve the throughput rates.

2.4. Inkjet

The inkjet bioprinting system is dependent on the physical properties (viscosity, surface tension and density) of bio-inks for successful deposition of nanoliter droplets onto an underlying substrate layer. The inkjet bioprinting system can be categorized into (i) continuous inkjet bioprinting and (ii) drop-on-demand (DOD) inkjet bioprinting. The DOD inkjet bioprinting is more commonly utilized for tissue engineering applications; the different variations of DOD inkjet bioprinting systems include thermal, piezoelectric, electrostatic and electrohydrodynamic inkjet bioprinters. Each print-head in the DOD inkjet bioprinting system is connected to a fluid reservoir and a varying number of nozzles. Pressure pulses are applied from the actuator to overcome the surface tension of the bio-ink at the orifice and bio-ink droplets are deposited.

For an inkjet-based bioprinting system, the droplet size and the deposition rate are dependent on the fluid's viscosity and surface tension. The vibration frequency (Hz) and the driving voltage waveform determine the droplet deposition process. An optimization of pulse width (p_w) and amplitude is necessary for consistent printing of discrete droplets [16]; no droplet is formed below $p_{w,min}$, while satellite droplets are generated above $p_{w,max}$. An optimal range of pulse width ($p_{w,min}$ and $p_{w,max}$) is required to achieve single droplet deposition for each corresponding pulse amplitude (p_a). The profound relationship and cursory knowledge of these parameters in actuating the piezoelectric printhead compound the difficulty of establishing stable droplets. Hence, it is time-consuming to determine the optimal dispensing parameters for each specific type of bio-ink [54]. Typical inkjet printing nozzle diameters are between 15 to 200 μm .

The four key parameters that influence the printability include viscosity, density and surface tension of the printable bio-inks and the radius of printing orifice [55]. The printability of the bio-inks can be denoted by the Reynolds number (N_{Re} : a ratio of inertial to viscous forces) and the Weber number (N_{We} : a balance between the inertial and capillary forces).

$$N_{Re} = \frac{vrp}{\eta} \quad (9)$$

$$N_{We} = \frac{v^2rp}{\gamma} \quad (10)$$

$$Z = \frac{N_{Re}}{(N_{We})^{1/2}} = \frac{(rpy)^{1/2}}{\eta} \quad (11)$$

where v , ρ , η and γ are the average travel velocity, density, viscosity and surface tension of the bio-inks respectively, and r is a characteristic dimension (radius of the printing orifice). The Z value is the inverse of Ohnesorge number (Oh), which can be represented as a ratio between the Reynolds number and square root of the Weber number and it is independent of bio-ink velocity.

The printing resolution [56] of an inkjet-based bioprinting process can be defined as:

$$D_{max} = D_o \sqrt{\frac{(N_{We}+12)}{3(1-\cos\theta) + 4\frac{N_{We}}{\sqrt{N_{Re}}}}} \quad (12)$$

where D_{max} is the printing resolution, D_o is the diameter of dispensed spherical droplet, N_{We} is the Weber number, N_{Re} is the Reynold number and $\cos\theta$ is the static contact angle between the substrate surface and the bio-ink. A study has demonstrated that the nozzle size is the most significant parameter that influences the printing resolution; the printing resolution is approximately 1.2 – 2 times of the nozzle size. It has a range of printable viscosities between 3 to 30 mPa.s [16] and the maximum printable cell density is approximately in the range of 10^6 cells per ml. The cell viability is dependent on the shear stress experienced by the printed cells and the impact upon contact with the substrate surface. Although the inkjet-based bioprinting has the highest throughput rates of up to 30 kHz, droplet instability is clearly evident at high frequencies (> 0.5 kHz) due to pressure fluctuations within the inkjet print-heads [54].

2.5. Microvalve-based

An applied voltage pulse will induce a magnetic field in the solenoid coil to open the nozzle orifice by pulling the plunger upwards while the pneumatic pressure from each individual gas regulator is required to overcome the bio-ink's viscosity and surface tension at the opened orifice of the print-head to facilitate DOD printing of bio-inks [18]. The microvalve-based bioprinting system is a simple, user-friendly bioprinting system; the printing process is mainly controlled by the valve opening time and printing pressure. Furthermore, it has an easy bio-ink loading process and does not require much trouble-shooting prior to printing [18]. It has a typical printing nozzle diameter that ranges between 100 to 250 μm .

The DOD printing process is highly dependent on the nozzle diameter, valve opening time (VOT), pneumatic pressure and the bio-ink's properties (viscosity and surface tension) [54]. The printability of a bio-ink in a microvalve-based bioprinting system is also governed by the Z values as discussed in the earlier sections. Although a combination of longer VOT and higher printing

pressure is generally used to eject more viscous bio-inks, applying additional pressure will not facilitate droplet generation if the bio-ink exceeds the range of printability. It was shown that an excessive printing pressure would generate undesired satellite droplets during the printing process. The printing resolution in a micro-valve based bioprinting system is typically $\sim 1.5 - 2.5$ times of its nozzle diameter. A variation in the nozzle size is more effective approach to tune the droplet diameter, whereas a variation in the printing pressure does not result in significant change in the droplet diameter. The printable range of viscosities ranges between 1 to 70 mPa.s [18] and the maximum printable cell density is in the range of 10^6 cells per ml. A recent study on micro-valve bioprinting has demonstrated that a low shear stress of ≤ 5 kPa should be used to achieve high cell viability of $> 90\%$ [57]. It has also been reported that a high printing pressure and small nozzle size result in significant low cell viability; most of the cells will undergo a cell recovery phase during the 1st 24 hours post printing [58]. Overall, the micro-valve based bioprinting system is reliable and facilitates high throughput printing of up to 1 kHz.

Table 1 Comparative analysis of different bioprinting techniques

	Extrusion	Stereolithography	Laser-assisted	Inkjet	Microvalve-based
Working principle	Pneumatic-or mechanical extrusion	Photo-polymerization of photo-initiators	Localized vaporization of energy-absorbing layer	Use of actuators to overcome surface tension	Use of actuators to overcome surface tension
Ease of operation	Easy Loading of bio-inks into cartridges Printing pressure	Easy Loading of bio-inks into vat Laser intensity and scanning speed	Medium Coating of homogeneous ribbon layer Laser intensity and scanning speed	Difficult Loading of bio-inks into cartridges Optimization of specific range of pulse width for each pulse amplitude)	Easy Loading of bio-inks into cartridges Valve-open time and printing pressure
Nozzle size	$\geq 100 \mu\text{m}$	N/A	N/A	$15 - 200 \mu\text{m}$	$100 - 250 \mu\text{m}$
Printability	Bio-ink properties (G' and G'')	Rate of photo-polymerization	Vapor pressure	Bio-ink properties (Z values)	Bio-ink properties (Z values)
Printing resolution	$\geq 200 \mu\text{m}$	$20 - 50 \mu\text{m}$	$20 - 80 \mu\text{m}$	$\geq 20 \mu\text{m}$ (1.2 – 2x nozzle size)	$\geq 150 \mu\text{m}$ (1.5 – 2.5x nozzle size)
Viscosity	$30 \text{ mPa.s} - 10^7 \text{ mPa.s}$	$\leq 5,000 \text{ mPa.s}$	$120 - 300 \text{ mPa.s}$	$3 - 30 \text{ mPa.s}$	$1 - 70 \text{ mPa.s}$
Cellular density	Up to 10^6 cells/ml	$> 10^8$ cells/ml	$> 10^8$ cells/ml	Up to 10^6 cells/ml	Up to 10^6 cells/ml
Cellular viability	Shear stress	Wavelength Intensity Curing duration Photo-initiator concentration	Ribbon thickness & viscosity Substrate surface	Shear stress Substrate surface	Shear stress Substrate surface
Throughput rates	High	Medium	Low	Low	Medium

2.6. Trends in bioprinting techniques

Fig. 2. (Top) Number of publications on 3D bioprinting (“bioprinting technique” + “cells” + “biomaterials”) since year 2000. (Bottom) Detailed break-down of the number of publications for each bioprinting technique for each year (from 2000 to 2018) – Inset: the 5-years publication trend (from 2014 – 2018).

It is clearly evident that bioprinting-related research has grown substantially since year 2000; the number of bioprinting-related technical publications per year has increased significantly from only 1 publication in year 2001 to > 50 publications in year 2018 according to Web of Science and Google Scholar using the following three keywords: “bioprinting technique – extrusion,

stereolithography, inkjet, laser-assisted or microvalve-based” + “biomaterials” + “cells”. Furthermore, the booming 3D bioprinting market is forecasted to grow from USD 411.4 million in 2016 to USD 1332.6 million by 2021, at a Compound Annual Growth Rate (CAGR) of 26.5% [59]. The extrusion-based bioprinting technique is still the most commonly used bioprinting approach (**Figure 1**) due to its fast printing speed and wide range of printable bio-inks [60]. The rapid development of novel bio-inks and bioprinting strategies has enable the fabrication of human-scale tissue constructs using extrusion-based bioprinting techniques (to be discussed in subsequent sections). Next, the stereolithography-based bioprinting technique has several advantages such as high printing resolution, wide range of printable viscosities, high printable cell density and potential to print highly complex structures. Furthermore, the development of new photo-initiators facilitates photo-crosslinking in the visible light range (less DNA damage) and rapid crosslinking within shorter time frame [61, 62]. The other bioprinting techniques such as laser-assisted, inkjet and microvalve-based bioprinting techniques are attractive for fabrication of smaller tissues/organs by enabling drop-on-demand (DOD) patterning of different types of cells and biomaterials on the same planar surface to enhance the cell-cell and cell-matrix interactions. Each of the bioprinting techniques has their own unique advantages and limitations; hence the choice of a suitable bioprinting technique is highly dependent on the desired application.

3. Progress in bio-ink development

The bio-ink used in the bioprinting process can be defined as a printable cell-supporting material; different types of bio-inks are used for each specific bioprinting technique. The bio-inks can be in the form of cell-encapsulated hydrogels, cell suspension or even tissue spheroids (whereby the cells are the mandatory component) [63]. The development of bio-inks enables scientists to manipulate biological and biochemical microenvironments to facilitate optimal cell-cell and cell-biomaterial interactions. In the following sections, we provide in-depth evaluation of the different bio-inks and the current trend in bio-ink development.

Hydrogels are referred to a class of crosslinked polymeric substances capable of absorbing and retaining large quantities of water [64, 65]. The hydrogels can be classified into two broad groups: naturally-derived and synthetically-derived hydrogels. The use of hydrogels in tissue engineering and regenerative medicine has numerous advantages such as their hydrophilic nature, tunable physical and chemical properties, biocompatibility and biodegradability.

Nevertheless, both natural and synthetic hydrogels have some limitations. Natural hydrogels are generally weaker in mechanical properties, whereas the synthetic hydrogels are lacking in bioactive molecules for essential cell adhesion and proliferation. Unlike the conventional tissue engineering approaches where cells are seeded on the surface of polymeric scaffolds, the use of hydrogels in the bioprinting process can provide a highly-hydrated microenvironment for multiple types of printed cells and facilitate critical cell-cell and cell-biomaterial interactions. In the following sections, the role of advances in polymer sciences in the development and optimization of different polymers to fulfil the stringent criteria of printable bio-inks is highlighted. Furthermore, the optimal printing parameters of the bio-inks in each bioprinting technique is also presented.

3.1. Natural bio-inks

3.1.1. Alginate

Alginate is the most popular hydrogel used in the bioprinting process due to its good printability, relatively cheap cost and simple crosslinking mechanism. The alginate is typically obtained from brown seaweed and it is a naturally occurring anionic linear copolymers comprising blocks of α -L-guluronate (G) residues and (1,4)-linked β -D-mannuronate (M) [66]. The blocks consist of consecutive G residues (GGGGGG), consecutive M residues (MMMMMM) and alternating M and G residues (GMGMGM). The alginates extracted from different sources differ in M and G compositions as well as the length of each block [67]. The physical properties of the alginate and its resultant hydrogels are highly dependent on the material composition (G/M ratio), sequence, G-block length and molecular weight [68].

The commercially-available alginate is usually sold in powder form; typical molecular weights range between 32,000 and 400,000 g/mol. Increasing the molecular weight of alginate can improve the overall physical properties of the resultant hydrogels. The alginate powder can dissolve easily in water to form an aqueous alginate solution; which is then crosslinked with divalent cations such as Ca^{2+} (CaCl_2 or calcium sulfate, CaSO_4 solutions) to form alginate hydrogel. The G-blocks are solely responsible for the intermolecular crosslinking with divalent cations (e.g. Ca^{2+}); the divalent calcium ions induce formation of junctions (egg-box model) between guluronate blocks of adjacent polymer chains to form alginate hydrogels. Increasing the content of G residues can result in much stiffer hydrogel network as compared to those with low amount

of G residues [69]. The gelation rate, which is an imperative factor that determines the gel uniformity and strength, can be manipulated by the type of ionic crosslinkers and the gelation temperature [70]. A slower gelation rate produces more uniform structures and greater mechanical integrity. The use of calcium chloride (CaCl_2) typically leads to rapid and uncontrolled gelation due to its high solubility in aqueous solution, whereas the use of calcium sulfate (CaSO_4) and calcium carbonate (CaCO_3) slows the gelation rate due to their lower solubilities. The insoluble CaCO_3 is first mixed with the alginate solution, followed by the addition of glucono- δ -lactone to dissociate Ca^{2+} from the CaCO_3 by lowering the pH [71]. This gradual release of Ca^{2+} initiates the gelation of alginate solution to form uniform structure with enhanced mechanical properties. Furthermore, the use of lower temperatures lowers the reactivity of ionic crosslinkers (e.g. Ca^{2+}) to form crosslinked hydrogel network of higher order with improved mechanical properties [72]. The major critical drawback of ionically crosslinked alginate hydrogels is the limited long-term stability under physiological conditions due to the leaching of divalent ions into surrounding media due to exchange reactions with monovalent cations. Hence, this has led to the interest in covalently crosslinked alginate hydrogels with different molecules such as poly(ethylene glycol) (PEG) [73, 74], methacrylate in the presence of photo-initiators such as eosin and tri-ethanol under laser irradiation at 514 nm [75] or even another photo-crosslinking approach using polyallylamine partially modified with α -phenoxycinnamyl diene acetylchloride at 330 nm UV light exposure [76].

The lack of alginase enzyme in mammals results in non-degradable alginate, but the ionically crosslinked alginate hydrogels can dissolve easily due to the leaching of divalent ions under physiological conditions. However, most commercially available alginates have molecular weights that are higher than the renal clearance threshold of the kidneys and they are unlikely to be completely removed from the body [77]. Some of the strategies to make degradable alginate hydrogels include partial oxidation of the alginate chains with sodium periodate [78], oxidation of polyguluronate (solely from G-blocks isolated from alginate) with sodium periodate to form poly(aldehyde guluronate) hydrogels with tunable degradation rates [79], use of binary alginate hydrogels (partially oxidized alginates with low and high molecular weights by either ionic or covalent crosslinking) [80, 81].

The alginate hydrogel has low protein adsorption that hampers cell attachment and the most commonly-used strategy is to modify the alginate with RGD peptides for improved cell adhesion [82]. The number of adherent cells and corresponding proliferation rates is highly dependent on

the bulk RGD density in the alginate hydrogels; the number of RGD peptides per alginate chain and the spacing between clusters of RGD peptides are shown to dramatically impact the response of cells to RGD-modified alginate hydrogels [83, 84].

The alginate hydrogels are commonly printed using the extrusion-based bioprinting approach due to higher storage modulus (G') and ease of crosslinking. The alginate hydrogels used in the extrusion-based bioprinting system can be printed in the precursor or pre-crosslinked form. An alginate concentration of 5 – 8% w/v is typically used for extrusion-based bioprinting of cell-laden constructs with high shape fidelity and cell viability [85, 86]. In laser-assisted bioprinting systems, a typical concentration of 1% w/v cell-laden sodium alginate precursor is usually printed under a laser fluence of 800 mJ/cm² into a 2% w/v CaCl₂ solution [87]. Particularly, the total gelation time in the CaCl₂ solution should not exceed two minutes, as excessive ionic crosslinking in the CaCl₂ solution leads to poor diffusion of oxygen and nutrient to the encapsulated cells. The 1% w/v sodium alginate precursor can also be used as a delivery medium to deposit the cell-laden bio-ink directly onto Matrigel/fibrin substrate [50]. Another feasible printing approach is the inkjet-based bioprinting of alginate precursor (< 2% w/v) [88], the cell-laden alginate precursor solution can either be printed into a CaCl₂ crosslinker pool [89] or used as a receiving substrate that is subsequently crosslinked by the crosslinker (CaCl₂) solution [90].

3.1.2. Collagen

Collagens are a superfamily of proteins that consist of up to 28 different types of collagen with distinct properties, functionalities and distribution within native human tissues (~30% of total protein mass) [91]. Collagen is one of the most abundant proteins and it can be easily extracted from bovine skin and tendons, porcine skin, rat tail and even fish skin via enzymatic digestion and/or dissolution in acid [92]. The selection of collagen isolation process is highly dependent on the species and type of tissue [93-96]. The collagen-rich tissues are typically immersed in either enzymatic and/or acidic solution over several days to first degrade the tissue and release the collagen proteins. The solubilized collagen is then maintained at low pH and temperature to prevent the annealing of the dissolved peptide fragments. Collagens comprise of three polypeptide α -chains (contain repeating motifs of three amino acids which include glycine, proline and hydroxyproline) that form the helical quaternary structure called triple helix, which is a common structural motif in all types of collagens. The triple helix structure is stabilized by the

presence of glycine at every third interval (Gly–X–Y repeats), high content of proline and hydroxyproline, inter-chain hydrogen bonds and electrostatic interactions [97].

The commercially-available collagen is usually sold in the solution form; they have varying collagen concentrations that range between 3 - 35 mg/ml. A small volume of sodium hydroxide (NaOH, 1M) solution is generally mixed with cold collagen solution before incubation at 37°C to initiate the collagen fibrillogenesis process to eventually form a collagen hydrogel over a period of thirty minutes. The assembly of a collagen solution into a fibrillar collagen hydrogel is governed by diffusion-limited aggregation [98]; this fibrillogenesis process can be evaluated via turbidimetry methods at 313 nm absorbance [99]. The solubilized collagen molecules undergo fibrillogenesis process when the conditions are adjusted near physiological values (*i.e.* pH of 6.5 – 8.5 and temperature of 20 – 37°C) [100]; some important factors that influence the collagen fibril dimension and microstructure of the collagen gels are pH, temperature and ionic strength [101]. Nevertheless, alteration of collagen microstructure by inducing a change in pH, temperature or ionic strength has detrimental effects on the encapsulated cells. Some novel strategies to manipulate the fibrillogenesis process within cell-encapsulated collagen hydrogels include non-enzymatic glycosylation of collagen hydrogels using reducing sugars [102, 103] and macromolecular crowding (MMC) of collagen matrices using macromolecules [104, 105]. An increase in the reducing sugar concentration induced slower collagen fibril formation and thicker fibril diameter, which resulted in an increase in the compressive modulus [102, 103]. Conversely, an increase in the macromolecule concentration induced faster collagen fibril formation and formation of more porous collagen architecture with thicker collagen fibrils [104].

Although the reconstituted collagen hydrogels are stable in aqueous media, they are typically less robust than native collagenous tissues due to incomplete inter- and intramolecular crosslinking and reduced protein density. As such, different crosslinking approaches (physical, chemical and enzymatic) have been implemented to increase the mechanical properties of collagen matrices. The principle of crosslinking reaction is dependent on the modification of the amine and carboxyl groups within the collagen molecules to facilitate the formation of covalent bonds. Physical crosslinking of collagen can be performed by either ultra-violet (UV) irradiation (~ 15 mins) or dehydrothermal (DHT) (~3 – 5 days) treatment approaches [106]; both techniques were shown to improve the overall mechanical properties of the collagen hydrogels. Although crosslinking via UV irradiation approach can be performed within a short time frame, it is only effective for thin collagen hydrogels due to its limited UV penetration depth. Chemical

crosslinking of collagen can be performed using aldehydes (*i.e.* glutaraldehyde/formaldehyde), isocyanates (*i.e.* hexamethylene diisocyanate) or carbodiimides (*i.e.* 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide hydrochloride). However, all these chemical crosslinking approaches are not suitable for preparation of cell-encapsulated collagen-based hydrogels. Notably, the use of genipin (a compound extracted from the fruit of *Gardenia Jasminoides*) at a low concentration of 1 mM facilitates crosslinking of cell-encapsulated collagen hydrogels with no significant cell death and also emits red fluorescence for easy visualization and quantification of crosslinking [107]. Other biocompatible crosslinking approaches involve the use of enzymes such as transglutaminase [108, 109] and lysyl oxidase [110, 111] to improve the mechanical properties and resist cell-mediated degradation of cell-encapsulated collagen hydrogels.

The collagens represent major components of our native extracellular matrices (ECM) that provide the overall tissue stiffness and integrity; it is a widely used biomaterial due to its high biocompatibility and *in vivo* degradation by human collagenases. The matrix metalloproteinases (MMPs) are the main contributors to tissue remodeling and collagen degradation; these MMPs first bind to the collagen molecules, next unwind the three α -chains and lastly cleave each strand of the triple helix to eventually degrade the collagen fibrils *in vivo* [112]. The degradation peptides from collagen type I to III were shown to function as chemotactic stimuli for fibroblasts *in vivo* and recruit neighboring fibroblasts to repair damaged tissues [113]. Collagen is widely utilized for tissue engineering applications due to its high affinity for adherent cells; direct cell-collagen interactions involve the recognition of specific peptide sequences (*i.e.* GPO motif (Gly-Pro-Hyp) [114], GFO motif (Gly-Phe-Hyp) [115] within collagen molecules by the cell receptors, whereas indirect cell-collagen interactions involve the binding of proteins of fibronectin [116] decorin or laminin [117].

The collagen precursor (rat tail origin, at a concentration of 0.2 – 0.3% w/v) is commonly printed using the microvalve-based [118, 119] bioprinting systems with multiple print-heads in a drop-on-demand approach due to its relatively low printing viscosity and fibrous micro-architecture [120]. A layer of collagen precursor (made up of nanoliter collagen droplets) from one printing cartridge is then crosslinked using sodium bicarbonate (NaCO_3 , 0.8M in distilled water) solution from another printing cartridge to facilitate pH-dependent collagen fibrillogenesis process. The final printed collagen constructs are then incubated at 37°C to further accelerate the collagen fibrillogenesis process. This printing approach ensures good printability of the uncrosslinked collagen precursor, high cell viability and uniform pH-dependent collagen

crosslinking in a nanoliter scale [118, 119]. A novel 3D bioprinting strategy has incorporated the printing of macromolecules within layers of collagen precursor to manipulate the collagen microstructure using MMC [121]. As for the extrusion-based bioprinting system, the collagen precursor (rat tail origin, at a concentration of 0.3% w/v) is first neutralized using 1M NaOH and then maintained at 10°C prior to printing [122]. The main objective of collagen crosslinking is to increase the viscosity for improved printability, but this approach has several limitations such as extremely poor cell viability that is close to 0% (if printed constructs are not incubated in culture medium after 2 minutes of printing time) and slow collagen crosslinking (the printed cells are washed to the edges if medium is added too vigorously or before complete collagen crosslinking) [122]. Hence, the collagen bio-ink is not suitable for extrusion-based bioprinting. In order to achieve cell printing at microscale resolution in the laser-assisted bioprinting system, a minimum bio-ink viscosity of at least 120 mPa.s is required [50]. Hence, the cell-encapsulated collagen precursor needs to be crosslinked using 1M NaOH prior to transfer [123, 124].

3.1.3. Gelatin

Gelatin is a soluble protein compound obtained by partial hydrolysis of collagen; the most abundant sources of gelatin are derived from pig skin (46%), bovine hide (29.4%), pork and cattle bones (23.1%) and fish gelatin (1.5%) [125]. The conversion of insoluble native collagen into gelatin requires two main processes; a chemical pre-treatment process will first destroy the non-covalent bonds to destabilize the protein structure and induce collagen solubilization and swelling. Subsequent heat treatment (> 45°C) cleaves the hydrogen and covalent bonds to destabilize the triple helix structure, resulting in a helix-to-coil transition and conversion into soluble gelatin [126]. The degree of collagen conversion into gelatin is dependent on pH, temperature and extraction duration in both chemical pre-treatment and heat treatment extraction process.

The commercially-available gelatin is usually sold in the powder form; the 2 different types of gelatin are type-A (isoelectric point at pH ~ 8–9) and type-B (isoelectric point at pH ~ 4–5) gelatin, which can be obtained under acidic or alkaline pre-treatment conditions respectively. In contrast to collagen, gelatin is water soluble which is a significant advantage in terms of handling gelatin solutions. The gelatin is an attractive hydrogel due to its relatively low cost, good biocompatibility and lower antigenicity. The main attributes of gelatin are the gel strength and thermal stability (gelling and melting temperatures); both gel strength and thermal stability are

largely dependent on the amino acid composition (species-specific) and the molecular weight distribution (processing conditions) [127]. The gel strength can be defined by the normalized “bloom value”; the commercially available pig or bovine gelatin have gel strength values in the range of 200 – 300 g, whereas cold-water and warm-water fish species have a typical gel strength values in the range of ≤ 100 g and ≥ 200 g respectively [125]. As such, warm-water fish species are the preferred source of fish gelatin due to its similar rheological properties and thermal stability as mammal gelatins. The fundamental crosslinking mechanism of gelatin is temperature-dependent; the coil-to-helix transition occurs at temperatures below 30°C, whereas the random coil conformation occurs at temperatures above 30°C. The weight of unmodified gelatin hydrogels decreases by 50% after 10 hours of incubation and it completely dissolves within 24 hours, hence further modifications to the gelatin hydrogels are necessary for tissue engineering applications [128]. Some common crosslinking approaches involve the use of glutaraldehyde or transglutaminase to improve the overall structural integrity for long-term cell culture. Furthermore, gelatin can be also used to achieve polyelectrolyte complexes for bioprinting applications [129-131]. A novel strategy incorporated phenolic hydroxyl (Ph) group into gelatin using aqueous-phase carbodiimide activation chemistry to obtain cell-laden gelatin-Ph hydrogels that crosslinked rapidly within 10 seconds with horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) [128].

A pioneering work on extrusion-based bioprinting, a 20% w/v gelatin bio-ink was utilized to fabricate gelatin/hepatocyte constructs at $< 20^\circ\text{C}$, followed by a post-process treatment in 2.5% glutaraldehyde solution for 5 seconds crosslinking [132]. The low temperature of $< 20^\circ\text{C}$ facilitates the printing of stable cell-laden gelatin constructs, while optimal crosslinking with 2.5% glutaraldehyde over 5 seconds results in stable gelatin hydrogels for more than 2 months. In another study, 3% w/v gelatin bio-ink supplemented with 2% w/v polyethylene oxide (acts as viscosity additive) was first printed and then crosslinked with 3U/ml microbial transglutaminase (TGase) for 3 minutes at 37°C . The printed human embryonic kidney (HEK) 293 cells were able to proliferate and spread within the crosslinked gelatin hydrogels [133]. The TGase crosslinking is more efficient in type A gelatin as compared to type B gelatin due to presence of more glutamic and aspartic residues. The TGase enzyme catalyzes the acyl transfer reaction between the γ -carboxamide group of glutamine residues and the ϵ -amine group of lysine residues of peptide chains [134]. Interestingly, the addition of TGase before or during/after gelatin has a huge influence on the gel strength. A decrease in gel strength was observed when crosslinking is

performed before cooling; the prior introduction of intra- and intermolecular covalent crosslinks caused by the presence of TGase makes it more difficult for the treated gelatin molecules to form physical gelatin crosslinking upon cooling. Conversely, the TGase-mediated crosslinking after formation of physical gelatin hydrogels at low temperatures leads to a reinforcement of the hydrogen-bonded triple helix network that improves gel strength [135]. A 20% w/v gelatin concentration is typically used for ribbon preparation in laser-assisted bioprinting systems, it has been used to pattern human dermal fibroblasts (with high post-transfer viability of $91 \pm 3\%$) [136] and mouse embryonic stem cells (with high pluripotency) [137]. Although inkjet printing of gelatin at high resolution (up to 6 μm) is possible at 37°C [138], further crosslinking is necessary to ensure the structural integrity of the printed constructs.

3.1.4. Hyaluronic acid (HA)

HA is a linear, non-sulfated glycosaminoglycan (GAG) with repeating disaccharide units of β -1,4-D-glucuronic acid and β -1,3-N-acetyl-D-glucosamine; it is a major macromolecular component that are highly abundant in almost all connective tissues. HA can be extracted from different animal species and tissues such as rooster comb, bovine cartilage, human umbilical cord, synovial fluid and vitreous body or even from genetically-modified bacterial strain [139, 140]. As HA is usually present as a complex linked to other biopolymers, several separation (protease digestion, HA ion-pair precipitation) and purification (centrifugation, ethanol precipitation) steps are required to obtain a pure HA [141]. HA molecules are negatively-charged, and the high hydrophilic nature enables the absorption of large amount of water.

The commercially-available HA is usually sold in the powder form as sodium hyaluronate (10 kDa to 1.8 MDa); the powder can be easily dissolved in deionized water and stirred gently to obtain HA solutions. Factors influencing HA hydrogel gelation are temperature, HA molecular weight, HA crosslinker concentration and crosslinking chemistry [142]. The conformation of HA polymer is dependent on its molecular interactions; the hydrogen bonding and hydrophobic interactions resist the electrostatic repulsion between the negative charges and constrict the coil dimensions to stabilize the expanded molecular interactions. HA chains are generally flexible coils in dilute solutions but entangle with each other and form a temporary network in concentrated solutions. The critical concentration for “coil overlap” is approximately 1.4 mg/ml for HA molecules of 1 MDa, and it is highly-dependent on the ionic strength and molecular weight [143]. This “coil overlap” occurs when the chains in the solution are forced to interpenetrate each other

and its specific viscosity begins to dramatically increase at critical concentration. As such, HA solution exhibits relatively high viscosity even at low concentration (1% w/v) and it demonstrates interesting viscoelastic properties at different ionic strength and pH [144]. HA exhibits a shear-thinning behavior with increasing shear rates when dissolved in aqueous solution but the same solution exhibits shear-thickening behavior upon addition of NaCl (lower than 0.15M) [145]. The temporary network between the HA molecules in aqueous solutions are disrupted by the high shear rates; hence it leads to shear-thinning behavior. Conversely, the sodium ions suppress the electrostatic repulsion between the carboxyl groups of HA molecules and contract the extended coil dimensions. As a result, the HA chains in aqueous solutions with NaCl exhibit a transition from “random coil” to “stretched coil” at critical extensional rate (can be defined as strain rate at which extensional viscosity begins to exhibit the strain thickening) [145].

Degradation of HA *in vivo* occurs through three separate pathways; local cellular turnover, endocytosis at tissue level and scission under oxidative conditions [146]. The use of HA hydrogels is largely hindered by its rapid removal by drainage or degradation pathways, slow gelation rates and poor mechanical properties; hence further modification and crosslinking is necessary to create more versatile HA-based hydrogels [147]. The three major functional groups that govern the chemical activity of HA are glucuronic carboxylic acid groups, the primary and secondary hydroxyl groups and the *N*-acetyl groups. Some of the biocompatible crosslinking mechanisms include enzyme-mediated coupling of tyramine to HA carboxylic groups [148], *N*-hydroxysuccinimide (NHS)-modified HA [149], Cu(I)-catalyzed cycloaddition reaction between an azide derivative and an alkyne derivative of HA [150], host-guest interactions between cucurbit[6]uril-conjugated HA (CB[6]-HA) and diaminohexane-conjugated HA (DAH-HA) in the presence of tags-CB[6] [151], thermo-crosslinking of HA-based copolymer hydrogels [152], crosslinking of thiol-modified HA via oxidation to form disulfate linkages [153, 154], crosslinking of thiol-modified HA using PEG derivatives [142, 155], crosslinking of furan-modified HA derivatives with dimaleimide PEG [156], gold nanoparticles (AuNPs of 24 nm) [157], photo-crosslinking of tyramine-substituted HA hydrogels [158] or even photo-crosslinking of allyl-functionalized poly(glycidols) [159]. Although HA is well-known for its non-cell-adherent properties due to its highly negative charge and hydrophilicity; it can still bind to proteins and cells through cell surface receptors such as CD44, RHAMM and ICAM-1 [160]. Functional groups such as thiols, acrylates, amines can be conjugated to the HA backbone through mild chemistries to form biocompatible cell-laden HA hydrogels. Common approaches to improve the

cytocompatibility of HA hydrogels involve the incorporation of Cys-modified RGD peptides [161, 162], functional fibronectin domains [163], growth factors [164-167] and other cell-adhesive biomaterials [168-171].

The use of unmodified HA alone is not suitable for 3D bioprinting as it exists as a highly viscous solution that has poor structural fidelity and fast degradation rates. Modified HA are commonly printed using the extrusion-based bioprinting approach; some of the reported work included extrusion printing of dynamically-crosslinkable gold nanoparticle-hyaluronan hydrogels (2% w/v) [157], HA (2% w/v) crosslinked with tetrahedral PEG derivatives [172], tyramine-substituted HA (2.5% w/v) that is crosslinked using enzymatic and photo crosslinking [173] and extrusion of single- and double-network HA-based hydrogels (1.5% w/v) containing dynamic covalent crosslinks [174]. Other bioprinting approaches include two-photon polymerization of 3.5% w/v enzymatically pre-crosslinked tyramine-substituted HA [158], laser-assisted bioprinting of 1% w/v HA as a hydrogel delivery matrix [175] and microvalve-based bioprinting of tyramine-HA bio-ink (2.5% w/v) blended with dry nanocellulose that is crosslinked in 0.001% H₂O₂ post-printing for 5 minutes [176].

3.2. Synthetic bio-inks/biomaterial-inks

3.2.1. Gelatin methacrylate (GelMA)

GelMA is a photopolymerizable hydrogel comprising of modified natural ECM components that make it an attractive biomaterial for tissue engineering applications [177]. GelMA is prepared from water-soluble gelatin that forms thermo-reversible physical hydrogel, which is not stable under physiological conditions. As such, one of the most commonly used strategies is to perform chemical functionalization with unsaturated methacryloyl groups to obtain GelMA hydrogels, which forms covalently crosslinked hydrogels by photo-initiated polymerization under mild conditions that enables cell encapsulation with high cell viability [178].

GelMA hydrogel are typically synthesized [179] by adding 10% w/v gelatin from porcine skin (Type A of gel strength 300 bloom) in deionized water/PBS solution and stirring at 50°C to ensure that the gelatin is fully dissolved to obtain a clear solution. Methacrylic anhydride (MA) is typically slowly added at 0.6 g per 1 g of dissolved gelatin under continuous stirring for 60 minutes to achieve a homogeneously opaque solution with a high degree of methacrylation (75 ± 9% as assessed using ninhydrin or fluoraldehyde assay). The degree of methacrylation can be tuned by

varying mass ratio of MA to gelatin, reaction time and temperature. The opaque solution is then centrifuged at 3,500g for 3 minutes at room temperature to remove the unreacted methacrylic anhydride and then diluted with four volumes of pre-heated (40°C) deionized water/PBS solution to stop any further reaction. The mixture is then dialyzed against distilled water using 12 – 14 kDa cutoff dialysis tubing for approximately 1 week at 40°C with daily change of water to eventually obtain a clear GelMA solution without any odor of residual methacrylic anhydride or acid by-products. The GelMA solution is then filter-sterilized using 0.2 µm syringe filter. The GelMA solution is aliquoted into 50 ml tubes and froze overnight in -80 freezer or snap-frozen in liquid nitrogen. The frozen GelMA solution is then lyophilized in freeze-dryer over a week to obtain white porous GelMA foam. The lyophilized GelMA is then stored at -20°C and must be protected from light and moisture until use.

The lyophilized GelMA is usually dissolved in PBS solution containing a photo-initiator to form photo-crosslinkable GelMA hydrogels. The photo-initiator (PI) plays an important role in determining the rate of polymerization, the resulting properties of the printed constructs and overall fabrication time. The mechanical properties of GelMA-based hydrogels can be tailored by varying the following factors: the degree of methacrylation, the polymer and PI concentration, as well as photo-crosslinking time. Some of the PIs include water-soluble 2-hydroxy-1-[4-(2-hydroxyethoxy) phenyl]-2-methyl-1-propanone (Irgacure 2959) [180-183], lithium phenyl-2,4,6-trimethylbenzoylphosphine oxide (LAP) [184-186], 2,2'-azobis[2-methyl-*n*-(2-hydroxyethyl)propionamide] (VA-086) [187-189], eosin Y [190, 191], ruthenium (Ru) and sodium persulfate (SPS) [192] and benzylidene cycloketone-based PIs G2CK and P2CK (for two-photon polymerization) [193]. Although the use of UV-curable PIs (such as Irgacure 2959) might be detrimental to the encapsulated cells, it was reported that there is no significant differences in the cell viability when the UV exposure duration is varied from 15 – 60 seconds [194]. The development of new visible-light curable PIs such as LAP (405 – 490 nm) [184, 185], VA-086 (440 nm) [189], eosin Y (450 – 550 nm) [190, 191] and Ru + SPS (400 – 450 nm) [192] enables the prolonged visible-light photo-crosslinking of hydrogels with high cell viability.

GelMA is a synthetic hydrogel that contains both cell-binding and cell-degrading motifs; only approximately 5% of the amino acid residues in gelatin is chemically modified by MA [177] and the lack of reaction between the RGD motifs and MA ensures good cell-adhesion properties [178]. The use of type I (MMP-1) and type II (MMP-8) collagenases results in accelerated *in-vitro* degradation of GelMA hydrogels, indicating the presence of MMP-sensitive motifs [195]. The

degradation rate of GelMA hydrogels in collagenase solution decreased with increasing GelMA concentration; complete degradation for 5% and 20% w/v GelMA was observed within 3 days and after 8 weeks respectively [195]. Nevertheless, high GelMA concentration ($\geq 7\%$ w/v) usually leads to limited cell activities due to the highly-crosslinked hydrogel network [196]. Hence, it is critical to select an appropriate GelMA concentration and crosslinking density for long-term cell culture studies.

One of the commonly-used bioprinting approaches for GelMA bio-ink is extrusion-based bioprinting followed by photo-crosslinking; a typical concentration of cell-laden GelMA bio-ink ranges from 7% to 15% w/v [34]. Several studies have demonstrated extrusion-based bioprinting of GelMA bio-ink encapsulated with chondrocytes [197, 198], liver hepatic cells [199], osteoblasts [200] and bone mesenchymal stem cells [201]. However, the printability of uncrosslinked GelMA bio-ink during extrusion-based bioprinting process relies heavily on the inherent temperature-dependent sol-gel transition of gelatin. As such, the incorporation of HA [197] or gellan gum [202] to GelMA bio-ink was performed to improve the rheological properties and overall printability prior to photo-crosslinking. In another study, printer modifications were made to precisely control the printing nozzle and substrate platform temperature to improve printability [187]. Other bioprinting approaches include SLA-based bioprinting (using a typical 10 – 15% w/v GelMA concentration [203, 204]), two-photon polymerization (using 20% w/v GelMA concentration [193]) and inkjet-based bioprinting (using two-fold functionalized gelatin – methacrylated and acetylated gelatin at 5 – 15% w/v concentration [205]). In the inkjet-based bioprinting study, the GelMA bio-ink was further modified through acetylation of free amino acid groups to achieve a low printable viscosity that facilitates stable and good printability using piezoelectric inkjet bioprinter at 25°C.

3.2.2. Polycaprolactone (PCL)

PCL is a biodegradable synthetic polymer that has been FDA-approved for applications in maxillofacial surgery [206]. PCL is a hydrophobic, semi-crystalline aliphatic polyester with hexanoate repeat units, which can be prepared via ring-opening polymerization (ROP) of ϵ -CL or condensation of 6-hydroxycaproic (6-hydroxyhexanoic) acid [207]. Various mechanisms such as anionic, cationic, monomer-activated or coordination-insertion ROP can be used to manipulate the polymerization of PCL to achieve varying molecular weight and its distribution, chemical structure and group composition [208].

PCL is usually sold in the pellet form; it has a number average molecular weight that ranges between 3,000 to 80,000 g/mol and can be graded according to its molecular weight [209]. PCL can be readily dissolved in benzene, carbon tetrachloride, chloroform, cyclohexanone, dichloromethane, 2-nitropropane and toluene at room temperature, whereas it has poor solubility in acetone, acetonitrile, 2-butanone, dimethylformamide, ethyl acetate and is insoluble in alcohol, petroleum ether and diethyl ether [210]. It has a glass transition temperature (T_g) of -60°C and its melting point ranges from 59 to 64°C.

PCLs are generally considered non-biodegradable *in-vivo* due to absence of relevant enzymes [211]. Nevertheless, hydrolytic degradation of poly(α -hydroxy) esters occurs slowly via surface or bulk degradation resulting in polymer thinning over time. The degradation kinetics is highly dependent on the molecular weight of the polymer; with higher molecular weight taking longer to degrade due to the increased number of ester bonds. Complete degradation of the homopolymer PCL usually occurs over a period of 2 – 4 years (depending on the initial molecular weight) [212]. The PCL are reported to undergo a two-stage degradation process: non-enzymatic hydrolytic cleavage of ester groups, followed by intracellular degradation when the polymer is more highly crystalline and of low molecular weight [213]. The rate of hydrolysis can be controlled by co-polymerization with other lactones or glycolides/lactides [214, 215]. Studies have shown that PCL and its composites generally have no adverse biocompatibility effects for short- (15 weeks) and long-term (up to 2 years) *in vivo* implantations [206]. As PCL is an inert biomaterial without cell-adhesive binding sites, surface modifications are required to improve the biocompatibility. Some of the approaches include coating with amine layers [216, 217], NaOH treatment [218, 219] and immobilization of RGD peptides [220, 221].

Although PCL-based scaffolds have been fabricated using SLA-based [222] or inkjet-based [223] printing approaches, cells were usually seeded onto the fabricated scaffolds. The PCL filaments/fibers were usually printed within the cell-laden constructs using extrusion-based bioprinting approach to improve the overall mechanical integrity of the printed cell-laden constructs. The PCL polymer ($M_w = 43,000 - 90,000$) is melt-extruded at a high-temperature of 80 – 92.5°C and rapid cooling of the molten PCL occurs quickly in the air before reaching the printing platform [224]. The rapid cooling properties of PCL enables the printing of adjacent cell-laden hydrogels without significant damage to neighboring cells while improving the structural fidelity of the printed constructs.

3.2.3. Polyethylene glycol (PEG)

PEG, also known as polyoxyethylene or poly(ethylene oxide) (PEO), is a linear synthetic polyether which can be conjugated with proteins, enzymes, liposomes and other biomolecules [225]. PEG is non-toxic and is approved by FDA for use as excipients for different pharmaceutical formulations [225]. PEG can be easily synthesized by anionic ring open polymerization of ethylene oxide to achieve a range of molecular weight and various functional groups. It has been widely used for tissue engineering applications due to its hydrophilicity, low antigenicity and immunogenicity. The basic chemical structure is PEG diol with two hydroxyl end groups, which can be modified in a facile and reproducible manner into other functional groups such as acetylene, acrylate, amine, azide, carboxyl, methyloxy, thiol or vinyl sulfone [226].

This polymer is usually sold in the powder form; materials with molecular weight $< 100,000$ are usually classified as PEGs, while higher molecular weight polymers are categorized as PEOs. The amphiphilic PEGs are soluble in water and many organic solvents; the PEGs with low molecular weight ($M_w < 1,000$) form colorless and viscous solution while PEGs with higher molecular weight forms white and waxy solid. The most common crosslinking approach for PEG-based hydrogels is via photo-polymerization; the PEG macromolecules can be easily functionalized through the hydroxyl end groups to yield a variety of hydrogel precursors as such PEG diacrylate (PEGDA), PEG dimethacrylate (PEGDMA) and multi-arm PEG acrylates (n-PEG). Synthesis of PEGDA involves the following steps; adding acryloyl chloride in a drop-wise manner to a solution of PEG diol (10 kDa) and trimethylamine (TEA) in anhydrous dichloromethane (DCM) under mechanical stirring in a nitrogen chamber for 24 hours, followed by washing the reaction solution with 8 molar equivalents of 2M potassium bicarbonate and drying with anhydrous sodium sulfate and lastly precipitating and filtering the end-product in cold diethyl ether for drying under vacuum overnight [227]. PEGDMA can be synthesized by first reacting PEG with excess methacrylic anhydride (MA) (up to 5-fold) in a capped scintillation vial and heating inside a commercial domestic microwave for 5 minutes, followed by addition of ethyl ether upon cooling to form precipitation, which is then filtrated and dried in a vacuum oven [228]. Some of the reported PIs for PEG-based printing include Irgacure 2959 [229-232], LAP [233], VA086 [234], eosin Y [235] and riboflavin (vitamin B2) with triethanolamine (TEA) co-initiator [236]. Although it is highly desirable to achieve a controlled degradation of the PEG-based scaffolds; hydrolytic degradation of the ester bonds in photo-crosslinked synthetic PEG-based scaffolds is relatively slow for both *in vitro* and *in vivo* [226]. Hence, the incorporation of enzyme-sensitive peptides

facilitates proteolytic degradation of PEG-based scaffolds [237-239]. Most PEGs with $M_w < 1,000$ are rapidly removed from the body unaltered with clearance rates inversely proportional to polymer molecular weight. PEG is usually referred as a “blank slate” material due to the absence of cell-adhesion domains, lack of protein binding sites and low degradability [240]. However, it was also reported that encapsulated osteoblasts were able to survive well inside PEG-based scaffolds over a period of 3 weeks without any additional biological cues [241]. To overcome the lack of cell-specific adhesion, cell adhesive peptides (obtained from collagen, fibronectin, laminin) can be incorporated within the PEG-based scaffolds [242].

The most commonly used bioprinting approach for PEG-based bio-ink is SLA-based bioprinting; a typical PEG-based bio-ink concentration is 10% w/v. Particularly, a recent study has highlighted the need to incorporate Percoll (at a concentration of 37.5% v/v) to mitigate cell sedimentation during the SLA-based bioprinting process [233]. Other bioprinting techniques for PEG-based bio-inks include extrusion-based bioprinting of PEG-based bio-ink (20% w/v PEGDA (M_w : 700) blended with 12.5% w/v alginate) using a modified extrusion-based bioprinter with built-in UV-LED crosslinking module [243] and inkjet-based bioprinting of 10% w/v PEGDMA with continuous irradiation from a UV lamp directly above the printing platform (25 cm distance) [229, 230].

3.2.4. Pluronic F-127 (PF127)

PF127 is a FDA-approved synthetic poloxamer-based polymeric compound, which comprises two hydrophilic poly(ethylene oxide) (PEO) blocks between a central hydrophobic poly(propylene oxide) (PPO) block (PEO – PPO – PEO) [244]. They can be easily synthesized by sequential polymerization of PO and EO monomers in the presence of alkaline catalyst such as sodium hydroxide or potassium hydroxide and further purified using chromatographic fractionation [245].

They are usually sold in the powder form with a molecular weight of 12.6 KDa; they can be easily dissolved in cold water to form a thermo-reversible hydrogel which is characterized by a sol-gel transition temperature (which increases with decreasing PF127 concentration from 15°C at 27.5% w/v to 35°C at 15% w/v – inverse thermo-gelling properties) [246]. Cell encapsulation within 20-30% w/w PF127 can be performed at a low temperature of 4-5°C to obtain a homogeneous cell-laden solution prior to physical-crosslinking at sol-gel transition temperature. Although high concentrations of PF127 ($\geq 20\%$ w/v) are necessary to achieve a good printability

for extrusion-based bioprinting, it has been reported that the cell viability within >5% PF127 hydrogels is low during long-term cell culture [247, 248]. Another major limitation of PF127 is its rapid degradation upon immersion; a common strategy to improve the overall structural integrity is by modifying the terminal hydroxyl moieties of PF127 with photo-crosslinkable groups [249-252]. PF127 is commonly used as a “fugitive” bio-ink due to its high printability and easy removal; it exhibits a strong shear-thinning behavior during printing and good thixotropic properties after printing and PF127 bio-ink liquefies at 4°C to facilitate easy removal from printed tissue constructs. The most common bioprinting approach for PF127 is the extrusion-based bioprinting approach; a typical PF127 concentration of 40% w/v is commonly used as a “fugitive” bio-ink [253]. Its high intrinsic viscosity and thermo-sensitive properties make it challenging to print using the droplet-based bioprinting approaches. Furthermore, the non-viscoelastic properties of PF127 hindered its application in laser-based bioprinting as the PF127 hydrogel layer is unable to transfer thermal energy into kinetic energy for jet formation and remains as a solid coating on the quartz support [120].

Table 2 Analysis of different natural and synthetic bio-inks

Natural Bio-inks	Preparation steps	Biocompatible crosslinking mechanisms	Bio-activeness	Optimal printing conditions
Alginate Bio-inks	Alginate powder can dissolve easily in water to form aqueous alginate solution	<p>Ionic crosslinking with divalent ions [70]</p> <p>Covalent crosslinking with PEG [73, 74]</p> <p>Photo-crosslinking with methacrylate [75]</p>	<p>Inert</p> <p>(Requires RGD modification for improved cell adhesion)</p>	<p>Extrusion:</p> <p>5 – 8% w/v [85, 86]</p> <p>Laser-assisted:</p> <p>1% w/v [87]</p> <p>Inkjet:</p> <p><2% w/v [88]</p>
Collagen Bio-inks	Ready-to-use collagen solution	<p>Physical pH- and temperature- mediated crosslinking [100]</p> <p>Chemical crosslinking with genipin/ transglutaminase/ lysyl oxidase</p> <p>[107-111]</p>	Bioactive	<p>Laser-assisted:</p> <p>0.2% w/v (crosslinked) [123, 124]</p> <p>Microvalve-based:</p> <p>0.2 – 0.3% w/v collagen precursor [118, 119]</p>
Gelatin Bio-inks	Gelatin powder can dissolve easily in hot water (60°C) to form gelatin solution	<p>Physical crosslinking via temperature-dependent (reversible) [128]/ opposite-charged polymers (polyelectrolyte complex) [129-131]</p> <p>Chemical crosslinking with horseradish peroxidase and hydrogen peroxide after modification with phenolic hydroxyl group [128]</p>	Bioactive	<p>Extrusion:</p> <p>20% w/v [132]</p> <p>Laser-assisted:</p> <p>20% w/v [136]</p>
Hyaluronic acid (HA) Bio-inks	HA powder can dissolve easily to form viscous HA solution	<p>Chemical crosslinking via coupling of tyramine [148]/</p> <p><i>N</i>-hydroxysuccinimide (NHS) [149]/</p>	<p>Inert</p> <p>(Requires RGD modification for improved cell adhesion)</p>	<p>Extrusion:</p> <p>1.5 – 2.5% w/v (modified HA) [157, 172-174]</p>

		<p>Cu(I)-catalyzed cycloaddition reaction [150]/</p> <p>host-guest interactions [151]/ HA-based copolymer hydrogels [152]/ oxidation of thiol-modified HA [153, 154]/ thiol-modified HA using PEG derivatives [142, 155]/ furan-modified HA derivatives with dimaleimide PEG [156]/ gold nanoparticles (AuNPs) [157]/ photo-crosslinking of tyramine-substituted HA hydrogels [158]</p>		<p>Laser-assisted:</p> <p>1% w/v [175]</p> <p>Microvalve-based:</p> <p>2.5% w/v [176]</p> <p>Two-photon polymerization:</p> <p>3.5% w/v [158]</p>
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Synthetic Bio-inks/ Biomaterial-inks	Preparation steps	Biocompatible crosslinking mechanisms	Bio-activeness	Optimal printing conditions
Gelatin Methacrylate (GelMA) <u>Bio-inks</u>	Lyophilized GelMA foam is dissolved in PBS solution containing photo-initiators	Chemical photo-crosslinking with different photo-initiators: Irgacure 2959 (365 nm) [180-183] LAP (405 – 490 nm) [184-186] VA-086 (440 nm) [187-189] Eosin Y (450 – 550 nm) [190, 191] Ruthenium and sodium persulfate (400 – 450 nm) [192]	Bioactive	Extrusion: 7 – 15% w/v [34] Stereolithography: 10 – 15% w/v [203, 204] Inkjet: 5 – 15% w/v at 25°C [205]
Polycaprolactone (PCL) <u>Biomaterial-inks</u>	PCL can be melted above 65°C	-	Inert (Coating with amine layers, NaOH treatment, immobilization with RGD peptides)	Extrusion: PCL polymer is melt-extruded at 80 – 92.5°C [224]
Polyethylene Glycol (PEG) <u>Bio-inks</u>	PEG powder can be dissolved easily in water and many organic solvents	Functionalized through hydroxyl end groups to form PEGDA, PEGDMA, n-PEG photo-crosslinkable hydrogels with different photo-initiators: Irgacure 2959 (365 nm) [229-232] LAP (visible light) [233] VA086 (405 nm) [234]	Inert (Requires RGD modification for improved cell adhesion)	Stereolithography: 10% w/v [233] Extrusion: 20% w/v [243] Inkjet: 10% w/v [229, 230]

		Eosin Y (visible light) [235] Riboflavin (vitamin B2) with triethanolamine (TEA) co-initiator (405 nm) [236]		
Pluronic F-127 (PF127) <u>Bio-inks</u>	PF127 powder can be dissolved easily in cold water to form thermo- reversible hydrogels	Temperature-dependent physical crosslinking (crosslink at high temperatures) Modification of terminal hydroxyl moieties to form photo-crosslinkable hydrogels [249-252]	Inert (Requires RGD modification for improved cell adhesion)	Extrusion: 40% w/v [253]

3.3. Decellularized extracellular matrices (dECM)

dECM refers to the natural extracellular matrices derived from various parts of native tissues and/or organs via a decellularization process, which recapitulates the complexity of extracellular matrices that plays imperative role in regulation of the cell-cell and cell-biomaterial interactions [254]. The composition and material properties of dECM can vary depending on the tissue source and processing method [255]. Some key considerations for dECM bio-ink preparation are sustainable source of tissues and low antigenic responses. Although human-derived tissues are the ideal dECM source, numerous animals have been used as tissue sources for preparation of dECM bio-inks due to the limited source of human-derived tissues. Over the years, various dECM derived from various tissues and/or organs such as adipose [256], cardiac [257], cornea [258], dentin [259], liver [260], muscle [261], pancreas [262], skin [263, 264], tendon [265], tracheal mucosa [266], vascular [267] have been employed as dECM bio-inks. The dECM composition includes common proteins/macromolecules such as collagen, laminin, fibronectin, elastin and their tissue-specific GAGs, cytokines and growth factors.

The dECM bio-inks for each type of tissue can be obtained by various decellularization protocols using different detergents and delivery methods; some of the decellularization process includes the physical, chemical and perfusion methods [268]. The decellularized tissues are then typically sterilized using 0.1% peracetic acid in 4% ethanol prior to lyophilization and storage at -20°C. The three-key criteria for decellularization efficacy comprise (1) no visible nuclei upon histological evaluation using hematoxylin and eosin and DAPI stains, (2) residual double-stranded

DNA (dsDNA) content should not exceed 200 base pairs in length and (3) the amount of dsDNA should not exceed 50 ng per mg dry weight of the material [269, 270]. To utilize the lyophilized dECM as a bio-ink, it must be crushed into a powder form and solubilized by partial enzymatic digestion in an acidic environment (pepsin with acid) for at least 48 hours, followed by a neutralization process in a drop-wise manner using NaOH and finally storage of the pH-adjusted dECM bio-ink at 4°C prior to printing. The dECM bio-inks (typical concentration of 1 – 4% w/v [271]) are usually printed using the extrusion-based bioprinting approach; a low printing temperature of $\leq 15^{\circ}\text{C}$ is required to prevent pre-gelation of dECM prior to extrusion and the printed constructs are incubated at 37°C for 30 minutes to facilitate temperature-dependent crosslinking. A recent study has investigated the use of additives such as riboflavin to manipulate the mechanical properties of dECM via vitamin B2-induced photo-crosslinking [272]. Due to its weak mechanical properties; PCL reinforcements are usually printed to serve as polymer supporting framework for fabrication of 3D hybrid constructs [273].

3.4. Tissue spheroids

Tissue spheroids are multi-cellular aggregates constituted through forced cell-cell adhesion or spontaneous self-assembly without scaffolds[274]. Previous studies have highlighted that the 3D tissue spheroids exhibit improved biological and regenerative properties as compared to 2D cell monolayers due to enhanced cell-cell interactions and diffusive mass transport [275]. The typical range of spheroid diameter is from about 50-700 μm and it contains about 15 cells/spheroid up to about 42875 cells/spheroid [276]. The maximum diameter of tissue spheroids that are grown to saturation is about 1-3 mm as it is limited by the degradation of the dead cells in the necrotic core [277]. Furthermore, the number of proliferating cells is reduced over time and cell necrosis in the core mainly occurs due to the accumulation of metabolic wastes and reduction in diffused glucose, oxygen and other nutrients [277].

The components of 3D tissue spheroids can be prepared by a single type of cells (homocellular) or several types of cells (heterocellular) [278]. The self-assembly of 3D tissue spheroids is highly-dependent on the cell type and it is typically completed within 24 hours [279]. The different approaches for fabrication of 3D tissue spheroids include hanging drop [280], microfluidics [281], liquid overlay system [282], rotating flask [283], spinner flask [284], micro-molded non-adhesive hydrogels [285, 286] and magnetic assembly [287] (**Table 3**). Although

various methods have been applied for the preparation of 3D tissue spheroids, there is a need to standardize the process for spheroid fabrication [288].

The different approaches to utilize the 3D tissue spheroids as bio-inks include loading of tissue spheroids into capillary micropipettes (500 μm diameter) [289], manipulating external magnetic field to assemble tissue spheroids infused with magnetic nanoparticles [290], piercing and assembling 3D tissue spheroids with stainless steel microneedles [291], transferring of tissue spheroids using vacuum grippers from 3D mold to desired positions [292]. Although the use of tissue spheroids can facilitate the bioprinting of highly-complex and more biomimetic 3D tissues/organs, some key challenges for bioprinting of 3D tissue spheroids include achieving uniform spheroid size and shape, loading of spheroids into printing cartridges, mitigating spheroid fusion in cartridge and designing of optimal printing nozzle to minimize clogging [293].

Table 3 Description of various methods for making 3D tissue spheroids

Method	Description	Ref
Hanging drop	This method helps the cells to self-assemble by gravitational forces to produce spheroids by inverting the lid of the tissue culture plate after adding ~15 – 30 μL of cell suspension (containing about 300 – 3000 cells) onto the inner surface of the lid. The surface tension and gravitational forces cause the cells to aggregate on the lid to form tissue spheroids.	[280, 294-296]
Microfluidics	This method uses a hydrogel-based U-shaped microfluidic chip to form cell aggregates. The fluidic flow and gravitation forces cause the cells to be trapped within the pockets of the chip. The cell capture is achieved by applying a fluidic flow against gravitational forces and the spheroid size can be manipulated by the size of the microstructures. The U-shaped structure also prevents cell damage caused by shear forces while allowing free diffusion of nutrients and waste.	[281, 297-299]
Liquid overlay system	This method is to prevent the attachment of cells to tissue culture plates and promote the cell-cell aggregation. The key to achieving tissue spheroid formation is to use of non- or low-adhesive substrates and culture medium with more nutrient factors.	[282, 300-302]
Rotating flask	This method is dependent on the microgravity environment created by rotating flasks. The rotating motion facilitates the cellular aggregation to form spheroids; it begins with a very low rotation rate (~15 rpm) and a gradual increase in rotation rate (~25 rpm) is required to maintain the suspension of the spheroids.	[283]
Spinner flask	This method is a technique commonly used to culture a large number of spheroids. The impeller or magnetic stir bar in the spinner flask causes the convection forces and a magnetic spinner is added to prevent cellular adhesion. It is critical to maintain the rotation speed below a threshold value; A slow rotation helps to stabilize the spheroids, while excessive high rotational speeds can lead to cell damage caused by shear stress.	[284, 303-305]
Micro-molded non-adhesive hydrogels	This method uses micro-molding of hydrogels to form spheroids. The micro-molds with rounded tops are first designed by the computer-aided design software and fabricated by 3D printing. The array of micro-wells is then cast in non-adhesive hydrogels (polyacrylamide or agarose) for cell seeding. The seeded cell suspension aggregates into cell spheroids and settles into the grooves of the micro-wells.	[285, 286, 306, 307]

Magnetic assembly	This method requires the incubation of cells with magnetic nanoparticles overnight to form magnetic cells. An external magnetic field is then applied to aggregate the magnetic cells into tissue spheroids at the air-liquid interface.	[287]
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3.5. Trend in bio-inks

Fig. 3. Trend in bio-inks over the last decade (year 2009 – 2018). An analysis on bio-ink trend over the last decade (year 2009 – year 2018) has been performed; the number of publications (based on Web of Science) on the top four materials from naturally-derived (Alginate, Collagen, Gelatin and HA) and synthetic bio-inks (GelMA, PCL, PEG and PF127), together with dECM and tissue spheroids.

The top five bio-inks based on the number of publications over the last decade are as follows: alginate > collagen > gelatin > GelMA > tissue spheroids. The alginate-based bio-ink remains the most popular due to its good printability, rapid crosslinking mechanisms and relatively cheap cost [66]. Furthermore, various strategies can be implemented to design cell-interactive alginate-based bio-inks that can be crosslinked rapidly for bioprinting applications. A novel bioprinting strategy has utilized the rapid-crosslinkable alginate bio-ink in a two-component hydrogel blend to facilitate the bioprinting of slow-gelling hydrogels that maintained droplet shape during the gelation process and easy removal of the alginate-based bio-ink via chelation process (**Fig. 4A**) [308]. The collagen is the main structural protein found in most native tissues; hence collagen-based bio-inks such as collagen, gelatin and GelMA are widely utilized due to its excellent biocompatibility and wide availability [125]. These structural proteins form highly-complex hierarchical porous structures in the native tissues that provide essential cues for regulation of cellular behavior and functions [309]. A recent study has demonstrated the use of

macromolecule-based bio-inks that manipulate the porosity within 3D bioprinted collagen matrix to create highly-complex hierarchical porous structures using drop-on-demand bioprinting approach (**Fig. 4B**) [121]. Gelatin, the denatured form of collagen, is highly-unstable at physiological temperature of 37°C due to its thermo-reversible crosslinking mechanism. As such, the gelatin-based bio-ink is mostly used as sacrificial material in the bioprinting process. A recent study has employed the enzymatic crosslinking of gelatin-based bio-inks using microbial transglutaminase to facilitate the printing of cell-laden hydrogels with high accuracy and structural fidelity (**Fig. 4C – 4E**) [133]. The use of tissue spheroids is attractive due to its improved biological and regenerative properties as compared to single cells for fabrication of human-scale tissues/organs with high cell density. The bioprinted tissue spheroids can undergo tissue maturation and fusion within 2 – 4 days to form uniform tubular constructs that were sufficiently sturdy for handling (**Fig. 4G – 4N**) [310].

Notably, there is prevalent use of dECM bio-inks since the first publication in year 2014 [273]. Although the dECM bio-ink is a concoction of various ECM proteins, it still offers some close resemblance to the extracellular matrices found in native tissues. Ultimately, the selection and combination of different bio-inks should be highly-dependent on the fabricated tissues/organs to closely emulate the highly-spatial arrangement and localization of each unique protein within the native tissues.

Fig. 4. (A) A novel bioprinting strategy for two-component hydrogel blends with a removable rapidly-gelling alginate bio-ink. [308], Copyright 2012. Reproduced with permission from John Wiley & Son Inc. (B) Modulation of collagen matrix via macromolecular crowding (MMC) at different fractional volume occupancy (FVO – 0, 18, 36 and 54%) using macromolecule-based bio-inks. [121], Copyright 2018. Reproduced with permission from the Royal Society of Chemistry. (C, D, E) Crosslinking of cell-laden gelatin-based bio-ink with microbial transglutaminase to achieve high structural fidelity, scale bar = 2 mm. [133], Copyright 2015. Reproduced with permission from Springer Nature. (F) Scanning electronic micrographs of multicellular spheroids of different cells. (G, H) Bioprinting a double-layered vascular wall using tissue spheroids; (I, L) H&E staining, (J, M) smooth muscle α -actin (brown) and (K, N) Caspase-3 (brown) after 3 days of fusion. [310], Copyright 2009. Reproduced with permission from Elsevier Inc.

4. Major impediments in tissue/organ printing

The vast advancement in the field of 3D bioprinting over last two decades has evolved from deposition of living cell droplets to fabrication of 3D complex tissues/organs that showed some resemblance to the native human tissues/organs. Nevertheless, more intensive research work is still required to eventually fabricate living tissue constructs that are suitable for human transplantation. Some important design considerations to overcome the major impediments in tissue/organ printing include achieving biomimicry, vascularization and 3D anatomically-relevant

biological structures. In the following sections, the novel bioprinting strategies that can potentially overcome these major impediments are highlighted.

4.1. Biomimicry

The main goal of tissue engineering is to re-engineer a biomimetic microenvironment that provides complex site-specific combination of biochemical and mechanical cues to guide cell adhesion, proliferation and differentiation during tissue maturation process [311]. The highly-automated 3D bioprinting platform facilitates multi-material bioprinting of living cells, biomaterials and growth factors in a layer-by-layer printing process with high precision and repeatability and it holds great potential in achieving the envisioned goal of mimicking the tissue-specific composition and localization of cellular and extracellular components.

Although bio-inks provide critical biochemical cues that regulate cellular behavior, most existing bio-inks are not able to completely emulate the complexity of the native ECM. Even the dECM bio-inks, a concoction of various ECM proteins, is unable to fully replicate the complex ECM as the highly-specific spatial positioning of each unique structural protein within the native tissue is disrupted during the bio-ink preparation step. The recent advances in tissue decellularization techniques facilitate the preservation of intact dECM scaffolds for detailed analysis of ECM composition, localization and biological functions; the tissues should be sectioned into specific regions prior to decellularization process based on the understanding of naturally occurring tissue-specific composition and localization of ECM components. This would allow the extraction of specific ECM components to re-create a biomimetic microenvironment using the bioprinting process.

Apart from an in-depth knowledge on the composition and localization of living cells and ECM within tissue constructs, the development of more advanced bioprinting systems would be advantageous in achieving the envisioned biomimicry using multi-material bioprinting approach. A recent study has demonstrated a multi-material extrusion-based bioprinting platform that utilized up to seven different controllable valves to command the dispensing pattern through a single nozzle in a rapid and continuous manner [312]. It enables the deposition of individual or a blend of multiple ECM components at pre-defined spatial positions to emulate the highly-specific biological and physiological functions in native tissues.

Fig. 5. Design of a digitally tunable continuous multi-material extrusion bioprinter with multiple printheads that are individually actuated by programmable pneumatic valves; the printed microfiber contains seven individually segmented bio-inks. [312], Copyright 2017. Reproduced with permission from John Wiley & Son Inc.

The microenvironment of tissue constructs imparts important mechanical cues (microstructure or stiffness) that control cell fate. One of the key dilemmas for bioprinting process includes the selection of an optimal bio-ink concentration without comprising the print fidelity (requires high bio-ink concentration) or long-term cell viability (requires low bio-ink concentration). The advances in polymer chemistry enables the implementation of novel bioprinting strategies to manipulate the microenvironment by (1) controlling the reversible crosslinking mechanisms of composite polymer bio-inks and (2) hybrid bioprinting of both cell-laden hydrogels with synthetic biodegradable polymers with different volumes of supporting polymers to manipulate the mechanical properties of overall bioprinted tissue constructs. Several strategies such as nanostructuring [313-315], macromolecular crowding [121] and reinforced thermoplastics polymer printing [316] can be implemented within the 3D bioprinting process to manipulate the microenvironment of bioprinted constructs.

Different bioprinting strategies such as nanostructuring and macromolecular crowding were employed to create micro-sized pores within the bioprinted tissue constructs. Nanostructuring refers to the formation of micro-sized pores within composite hydrogel constructs after elution; the composite hydrogel bio-ink facilitates good printability to achieve good structural integrity while providing a porous and softer microenvironment for long-term cell viability after elution process. Some of the commonly eluted hydrogels include PF127 [313-315], gelatin [317, 318] and alginate [308, 319]. Macromolecular crowding (MMC) is a concept that has been developed to describe intra- and inter-cell biochemistry such as protein folding or nucleic acid interactions [104, 105]. Collagen, the most abundant protein within most native tissues, forms highly-complex hierarchical structures within our native tissues [320]. The use of macromolecules manipulates collagen fibrillogenesis process; a novel bioprinting strategy has been implemented to tune the degree of MMC within each printed region to create 3D bioprinted hierarchical porous collagen-based constructs [121].

Lastly, reinforced thermoplastics polymer printing can be used to increase the overall tissue stiffness [273]. PCL is selected as the reinforcement thermoplastics polymer due to its good biocompatibility, relatively long degradation time (~2 years) and low melting temperature of 60°C [321]. Its rapid cooling property enables co-printing with cell-laden hydrogel immediately after extrusion without significant damage to surrounding cells. Different types of PCL framework can be implemented to tune the mechanical properties of the printed constructs without affecting the viability of encapsulated cells within hydrogel network.

Fig. 6. Manipulating microenvironment of bioprinted constructs. A) Schematic drawing of nanostructuring process (the creation of micro-pores within bioprinted constructs after elution). B) FE-SEM images of representative bioprinted hierarchical porous collagen-based constructs creating by manipulating degree of macromolecular crowding within different printed regions. [121], Copyright 2018. Reproduced with permission from the Royal Society of Chemistry. C) Reinforcement with thermoplastics polymer printing to tune the overall tissue stiffness; cell-A (red), cell-B (green) and PCL (gray). [316], Copyright 2016. Reproduced with permission from the Nature Group.

4.2. Vascularization

Vascularization remains one of the key bottle-necks in the field of tissue engineering; the presence of complex vascular networks within human-scale tissue-engineered constructs is important for diffusion-based exchange of nutrients and oxygen [322, 323]. The general strategy for engineering of vascularized tissue constructs is by fabricating a complex network of micro-channels or using cell-based strategies to induce angiogenesis [324-328]. The fabrication of 3D bioprinted micro-channels ($\sim 10^2$ µm diameter) within the tissue constructs is rapid while the formation of micro-capillary networks ($\sim 10^1$ µm diameter) using cell-based strategies is slow and time-consuming.

A common bioprinting strategy to create 3D-printed perfusable micro-channels is via co-axial extrusion printing [329-331]. The development of rapid crosslinkable dual-component bio-inks is integral towards the successful fabrication of perfusable micro-channels using the co-axial bioprinting strategy. The most commonly used bio-ink for co-axial bioprinting is the alginate-based bio-ink facilitates rapid ionic crosslinking with divalent cations; a typical alginate concentration of 2-5% w/v is utilized to create micro-channels ($\geq 700 \mu\text{m}$ diameter) in co-axial bioprinting strategy.

Another bioprinting strategy involves the fabrication of sacrificial micro-channels using “fugitive” bio-inks such as PF127 [332-334] or gelatin [335-337] by manipulating its reversible temperature-dependent crosslinking mechanism. The use of PF127 bio-ink resulted in significantly smaller micro-channels ($\geq 100 \mu\text{m}$ diameter) as compared to gelatin micro-channels ($\geq 700 \mu\text{m}$ diameter) due to its higher printability. Although the existing 3D bioprinting techniques are unable to closely mimic the complexity of vascular networks ($\sim 10 \mu\text{m}$ diameter) found in native human body, it has been demonstrated that the incorporation of these micro-channels within 3D printed tissue constructs improves the overall cell viability. The combination of 3D bioprinting techniques with cell-based strategies complement each other; the human umbilical vein endothelial cells (HUVECs) can be perfused to cover the inner walls of the 3D bioprinted micro-channels. The 3D bioprinted micro-channels maintain the initial cell viability of large printed cell-laden constructs, while the perfused HUVECs form functional multi-scale vascular networks over time [337].

Fig. 7. (Top) Co-axial nozzle assembly that facilitates the crosslinking of hollow alginate micro-channels. [329], Copyright 2013. Reproduced with permission from the ASME Press. (Bottom) Schematic drawing of fabrication of 3D microvascular networks within a hydrogel reservoir, a) deposition of a fugitive ink into a physical gel reservoir that facilitates patterning of branching networks, b) voids induced by nozzle translation are filled with liquid that migrates from the fluid capping layer, c) subsequent photopolymerization of the reservoir yields a chemically crosslinked hydrogel matrix, d, e) Removal of liquified fugitive ink under a modest vacuum to form the microvascular channels, f) fluorescent image of a 3D microvascular network fabricated via omnidirectional printing of a fugitive ink (dyed red) within a photopolymerized PF127-diacrylate matrix (scale bar = 10mm). [332], Copyright 2011. Reproduced with permission of John Wiley & Son Inc.

4.3. 3D anatomically-relevant biological structures

One of the key advantages of 3D bioprinting techniques is the precise control of material and structure properties in three-dimensional planes to fabricate patient-specific 3D anatomically representative tissue-engineered constructs based on medical imaging data from computed tomography (CT) and magnetic resonance imaging (MRI). Interestingly, the native tissues and/or organs have unique anatomical structures that perform highly-complex and specific functions [338].

Most bio-inks utilized in 3D bioprinting are usually soft and lack self-supporting properties for printing of 3D complex structures; hence there is a high probability of material deformation under its own weight. The commonly-used approach to eliminate this issue is to utilize support materials to improve the quality and fidelity of printing process [339, 340]. Notably, a novel strategy termed freeform reversible embedding of suspended hydrogels (FRESH) can overcome this challenge by printing bio-inks within a gelatin slurry support bath that maintains and improves the structural fidelity of 3D complex structures during the printing process [341]. The FRESH bioprinting strategy facilitates the deposition and embedding of printed bio-inks within a thermo-reversible support bath that maintains the intended structure during the print process and significantly improves print fidelity. The support bath exhibits Bingham plastic behavior during the printing process; it behaves as a rigid material at low shear stresses but becomes a viscous fluid at higher shear stress. This unique behavior enables the extruded bio-inks from the moving needle-like nozzle to be held firmly in place within the bath and the gelatin support bath can be easily removed in a non-destructive manner at a physiological temperature of 37°C. The study has demonstrated the use of multiple types of bio-inks (alginate, collagen, fibrin or Matrigel) in FRESH bioprinting technique.

Another interesting approach is the addition of Laponite nanoclay to desired bio-ink to obtain a hydrogel composite that can retain its complex 3D structures with self-supporting properties before crosslinking. The investigation of the thixotropy behavior (time-dependent shear thinning property) of the Laponite colloids showed that the viscosities increase rapidly (~ 0.08 s) upon removal of shear stress to impart good structural fidelity of the bioprinted constructs [342]. Similarly, the Laponite nanoclay can be incorporated into different types of bio-inks without affecting the crosslinking mechanisms (UV crosslinking, ionic crosslinking and temperature crosslinking).

Fig. 8. A) 3D printing of complex biological structures via freeform reversible embedding of suspended hydrogels (FRESH) printing strategy; a hydrogel precursor ink is deposited within the thermo-reversible support bath consisting of gelatin microparticles (scale bar, 1cm). [341], Copyright 2015. Reproduced with permission from the AAAS. B) The incorporation of self-supporting Laponite nanoclay as internal scaffold biomaterial for fabrication of complex structures via i) UV light crosslinking, ii) ionic crosslinking, iii) Temperature-dependent crosslinking. [342], Copyright 2017. Reproduced with permission from ACS Publications.

5. Role of 3D bioprinting in fabrication of specific tissues/organs

5.1. Critical aspects of each type of tissues/organs

Different 3D bioprinting strategies can be implemented to achieve biomimicry and vascularization within 3D anatomically-relevant biological structures that can potentially mature

to form functional 3D tissues. This would offer a potential solution to organ shortage and induce a paradigm shift in medical treatments. In the following sections, a concise analysis of the anatomy and physiology of the targeted tissue/organ in terms of composition and spatial arrangement of both living cells and ECM is first presented. The important design considerations in terms of cellular and microenvironment requirements and the role of polymer science in 3D bioprinting of tissue/organ fabrication will also be highlighted. Next, an evaluation of prior work on the subject of bioprinted tissue/organ will be presented along with an in-depth analysis on the morphological, biochemical and physiological properties. Lastly, a comparison between the bioprinted tissue constructs and native tissue/organ will be performed to determine the current state of organ printing and the future research needed to eventually achieve functional tissue/organ suitable for human transplantation.

5.1.1. Skin

Fig. 9. A) Histological and morphological characterization of the bioprinted skin and native human skin. B) Fluorescence microscopy images of epidermal differentiation and dermal markers' profiles of bioprinted skin in comparison to normal human skin from healthy donors. [343], Copyright 2016. Reproduced with permission from John Wiley & Son Inc.

The human skin is the largest organ and has an average thickness of 2.5 mm; some of its main functions are to provide the barrier function and protect against harmful UV rays [344-346]. The human skin can be categorized into two distinct regions (upper epidermal and lower dermal) which is separated by a basement membrane layer at the epidermal-dermal junction. The upper

epidermal region is characterized by densely-packed keratinocytes that form stratified cell layers with increasing degree of differentiation near skin surface; the proliferating keratinocytes residing near the epidermal-dermal junction undergo sequential differentiation to form fully stratified keratinocyte layers over a period of two weeks. The deeper dermal region can be further classified as upper papillary and lower reticular regions; the upper papillary dermal region comprises densely-packed collagen fibers that are randomly oriented while the lower reticular dermal region comprises porous and thicker collagen fibers that are orderly oriented. The unique 3D hierarchical porous collagen-based microenvironment regulates the fibroblast proliferation and its ECM secretion [309, 347, 348]. The basement membrane at the epidermal-dermal junction is a macromolecular structure with highly-complex ultrastructural and biochemical cues and it is critical for positional orientation of the melanocytes [349]. The basement membrane layer, which includes collagen type IV, VII, laminins and nidogens, is mainly produced by keratinocyte-fibroblast interactions in an organotypic co-culture [350]. The melanocytes and proliferative keratinocytes residing on the basement membrane layer form a functional complex unit called the epidermal melanin unit (EMU); the melanocytes synthesize the melanin pigment (eumelanin, pheomelanin or neuromelanin) that provides the distinctive skin color in each individual and offers protection against the ultraviolet radiation. These synthesized melanin pigments are stored within melanosomes, which are transferred to overlying suprabasal keratinocytes via elongated dendrites; this melanosome uptake is regulated by the degree of keratinocyte differentiation [351].

Although the fabrication of tissue-engineered skin constructs may seem like a straightforward process, the native skin has a highly-complex 3D structure that requires the precise patterning of cells and biomaterials to facilitate important cell-cell and cell-biomaterial interactions. The different types of skin cells (keratinocytes, melanocytes and fibroblasts) can be isolated from skin biopsies or purchased commercially and cultured *ex-vivo*. Collagen type I bio-ink is commonly used for skin bioprinting as it is the major structural protein of native skin [352, 353]. The dermal region is characterized by the presence of collagen fibers with relatively low fibroblast density ($0.2 - 2.0 \times 10^5$ fibroblasts/cm³) [354]; these dermal fibroblasts are responsible for the secretion of important ECM proteins (collagen I and IV, laminin, fibronectin and elastin) and growth factors that facilitates important cell-ECM and cell-cell interactions. The epidermal-dermal junction consists of melanocytes and basal keratinocytes at an approximate ratio of 1:20 and a minimum melanocyte density of 1.0×10^4 cells/cm² is required to restore the skin

pigmentation in tissue-engineered skin constructs [355]. Notably, supplementing the culture medium with macromolecules resulted in significant increase in ECM deposition by fibroblasts [356, 357]. Furthermore, the use of macromolecule-based bio-inks can be advantageous for skin tissue engineering as the macromolecules can be utilized to manipulate the collagen fibrillogenesis process and enhance the ECM deposition by fibroblasts to fabricate biomimetic hierarchical porous collagen-based dermal skin constructs.

Numerous studies on skin bioprinting have been published over the years [345, 358]; the morphological analysis of the 3D bioprinted skin constructs exhibited close resemblance to the native human skin along with presence of important skin biomarkers found in native human skin (**Fig. 9.**) [343]. Although the bioprinted skin constructs consist of stratified epidermis region, more in-depth permeability tests are necessary to benchmark its skin barrier functions against the native human skin. Another study has demonstrated the 3D bioprinting of pigmented human skin construct with uniform skin pigmentation [355]. As of now, the use of droplet-based or laser-assisted bioprinting approach is prevalent for the patterning of the keratinocytes and melanocytes on the dermal skin constructs to recapitulate the natural compartmentalization of epidermal skin cells at high degree of specificity. Although the bioprinted skin constructs can restore partial barrier function, the native human skin serves several other key functions such as temperature regulation (hair follicles and sweat glands), touch sensation (sensory nerves) and skin hydration (sebaceous glands).

5.1.2. Cornea

Fig. 10. A) 3D bioprinted cornea fabricated on PET substrate with moderate transparency exhibited significant shrinkage, B) 3D bioprinted cornea on non-transparent Matrigel® substrate to avoid shrinkage during culture; scale bar = 10 mm, C) Immunofluorescence staining of bioprinted cornea 2 days after printing and human cornea; scale bar = 200 μ m, D) Immunofluorescence staining of 3D bioprinted cornea 7 days after printing against human collagen I (Col I, green) and Von Willebrandt factor (VWF, red) with the human cornea stromal as a control; scale bar = 200 μ m. [359], Copyright 2018. Reproduced with permission from Elsevier, Inc.

The cornea of the eye provides high transparency for light transmission and refraction. Particularly, its high transparency is a result of constant refractive indices in all of its constituent cells [360]. The human cornea (~550 μ m in thickness with a horizontal diameter of ~12 mm) comprises of five distinct transparent layers; the outermost layer of corneal epithelium (~10% thickness), Bowman's layer (~1.5% thickness), stroma (~85% thickness), Descemet's membrane

(~1.5% thickness) and lastly a single layer of endothelial cells (~2% thickness) that lines the posterior cornea [361].

The surface of corneal epithelium is covered by an overlying thin layer of tear film (made up of meibomian lipids [362, 363]) which provides the first barrier to external environment. The corneal epithelium consists of stratified squamous epithelium that is 4 – 6 cell layers thick (40 – 50 μm) [364] and undergo routine proliferation, apoptosis and desquamation [365]. The corneal epithelium consists of superficial corneal epithelial cells (2 – 3 cell layers of flat polygonal cells [364]), intermediate suprabasal or wing cells (single cell layer) and the deepest basal epithelium layer (single cell layer of columnar epithelium ~ 20 μm tall [366]). The basal cells are attached to an underlying basement membrane (0.05 μm thickness) that comprises type VI collagen, laminin and fibronectin secreted by the basal cells and possesses lateral intercellular junctions (gap junctions and zonulae adherens) to facilitate strong attachment between the epithelium and underlying corneal layers [367]. The Bowman's layer is a thin, transparent collagen layer that lies beneath the epithelium region; it has an approximate thickness of 8 – 12 μm and decreases in thickness over time [368]. It acts as a physiological barrier to maintain corneal homeostasis between the epithelial-stroma cells [369]. Upon injury, the Bowman's layer does not regenerate and form a permanent scar [370]. The corneal stroma occupies approximately 80 – 85% of the corneal thickness and is predominantly made up of type I collagen fibrils organized into uniformly-spaced fibrils that form larger fiber bundle termed "corneal lamellae" that are typically 0.2 mm broad and 2 μm thick [371]. The stroma consists of 200 lamellae that are made up of collagen bundles with higher collagen packing density in the anterior as compared to posterior lamella [372]. There is a unique lamella arrangement at different stroma depths; the anterior stroma consist of interweaving bow spring-like arrangement in and out of the Bowman's layer at an insertion angle of $\sim 19^\circ$ [373], the lamella are arranged regularly at orthogonal directions in the middle region of stroma, while the posterior stroma also contains interwoven collagen fibrils (rich in collagen type VI) at the Descemet-stroma interface [374]. Furthermore, the presence of crimps within the collagen fibrils in combination with the elastic fibers makes the cornea more pliable and absorbs intraocular pressure fluctuations [375, 376]. These unique features within the corneal stroma provide structural support to maintain the shape of the anterior corneal surface [377]. The Descemet's membrane is made up of collagen type IV and VIII secreted by the underlying endothelial cells [378]. The Descemet's membrane, the basement membrane for the corneal endothelium, assists in the maintenance of corneal dehydration [368]. It increases in

thickness over time (eventually accumulate to form $\sim 10 \mu\text{m}$ thickness) and it can undergo self-regeneration after injury [370]. The corneal endothelium is the thin, innermost layer of cornea; the endothelial cells flatten over time to form a mono-layer of honeycomb-like mosaic ($\sim 4 \mu\text{m}$ in thickness) and the basal surface of the endothelium contains numerous hemi-desmosomes that facilitates adhesion to the Descemet's membrane [370]. The humans are born with a significant reserve of endothelial cells, as they have no mitotic activity *in vivo* [370]. The primary function of the endothelium layer is to maintain the stroma in a relatively deturgescenced state (78% water content) by pumping excess fluid out of the stroma [379]. This pump function is highly dependent on the endothelial cell morphology (size and shape) [380].

The different cells (corneal epithelial, stroma and endothelial cells) can be isolated or purchased commercially. The most challenging obstacle in corneal tissue engineering lies in the fabrication of the corneal stroma with unique lamella arrangement; complex structures such as the interweaving fibrils at both the anterior and posterior stroma and the high uniformity of the nanoscale fibrils in the alternating collagen lamellae arranged in orthogonal directions. The thin corneal collagen fibrils ($\sim 30 \text{ nm}$ diameter) serve as the principal load-bearing constituents of the lamellae in corneal stroma and confer high transparency to the cornea. The flattened corneal keratinocytes reside in the stroma region at an average density of $2.0 - 2.4 \text{ million cells/cm}^3$ [367, 381]; an interconnected keratinocyte network is formed via direct contact of dendrites between neighboring cells with a higher cell density in upper stroma region. The quiescent human keratinocytes in the stroma region contain corneal crystallins (ALDH1A1, ALDH3A1, TKT, α -enolase, LDH and G3PDH) that maintains the high transparency and optimal refraction in the corneal [382]. These crystallins are UV-absorbing proteins that regulate the refractive index of cell cytoplasm to match the extracellular matrix and thus eliminate light scattering [383]. Upon injury, the activated keratinocytes start to synthesize collagen type I, V and VI and keratan sulfate (that maintains corneal hydration) [384].

Most of the bioprinting work focus on the fabrication of the cornea stroma ($\sim 85\%$ corneal thickness). Although there is some success in creating anatomically-relevant 3D bioprinted corneal stroma, none of the bioprinted corneal stroma is able to achieve the high transparency in the corneal tissue [359]. The intensive research work over the past decades on corneal transparency has indicated that the thin and highly-ordered corneal collagen fibrils ($\sim 30 \text{ nm}$ diameter) along with the embedded corneal crystallins confer high transparency to the cornea

and serve as the principal load-bearing constituents of the lamellae in corneal stroma. The morphological appearance of the bioprinted corneal stroma (loosely-packed matrix) differs significantly from the native corneal stroma (densely-packed matrix) as it is highly challenging to achieve the high uniformity of the nano-scale fibrils (~30 nm diameter) found in alternating collagen lamellae arranged in orthogonal directions (**Fig. 10.**). Some prior work has demonstrated the use of collagen type I (major structural protein of the corneal stroma) for multi-photon excitation [385, 386] and the two-photon polymerization (2PP) bioprinting system is an attractive fabrication platform that can be used to potentially achieve the nanoscale resolution required for the tissue engineering of corneal stroma [20, 387, 388] with varying cornea curvature of high transparency. Alternative research approaches would include different strategies to facilitate the compaction and self-organization of collagen fibers into thin and highly-ordered collagen fibrils or development of new, printable bio-inks of high transparency and stability to facilitate the realization of clinically relevant corneal grafts.

5.1.3.

Cartilage

Fig. 11. A) Histological sections of the 3D bioprinted cartilage-like tissue constructs at week 0, week 1 and week 2 of differentiation, which is followed by 2 weeks of proliferation in the iPSC maintenance medium (stained with Alcian blue can Geison for proteoglycans/GAGs (blue) and nuclei (brown); scale bar = 100 μm, B) 3D bioprinted chondrocyte-derived iPSCs at week 5 of differentiation, (top row) magnified view and (bottom row) whole section stained for GAGs, Saffranin O for cartilage (with nuclear counterstain) and hematoxylin and eosin (H&E) for extracellular matrix (with nuclear counterstain); scale bar = 100 μm (top row) and 500 μm (bottom row). [389], Copyright 2017. Reproduced with permission from Springer Nature.

The articular cartilage, located at the joint surfaces of two bones, is usually translucent with an average thickness of approximately 2.4 mm. The articular cartilage is mainly made up of structural components with limited supply of nerves and blood vessels; the structural anatomy is uniquely designed to protect its surrounding structures. The articular cartilage has a relatively low metabolic activity; the lack of precursor and stem cells severely limit its self-regeneration ability [390]. The chondrocytes, a derivative of mesenchymal stem cells, occupy only about 1.65% of the total cartilage tissue volume with approximately 104 cells per mm³ of tissue. The

chondrocytes have an average cell diameter of $\sim 13 \mu\text{m}$ and the main function is to withstand friction and compressive stress and forces [391, 392]. The chondrocytes secrete and maintain a hydrated extracellular matrix (ECM) comprising of type II collagen, GAGs, proteoglycans (PG) and glycoproteins [393]. Interestingly, the chondrocytes from different zonal regions not only differ in quantities and shapes, they also secrete different components of cartilage which are then arranged in their own specific manner for each individual layer [394]. Therefore, it is a major challenge and hurdle to mimic specific cartilage tissues with different layers, structures and components using bioprinting.

The articular cartilage at different locations of our body varies slightly with the thickness and compositions of different cartilage layers [395]; it is generally made up of four layers (**Table 4**) ; the superficial, intermediate, deep and calcified regions. The superficial layer serves to protect the articular cartilage and takes up $\sim 20\%$ of the cartilage volume, whereas the middle zone serves to act as a bridge between the superficial and deep zones and it takes up approximately 60% of the cartilage volume. The deep and calcified areas are separated by a thin basophilic line called the tidemark. This tidemark serves to distinguish between the mineralized and non-mineralized layers [396]. The tidemark junction is characterized by its high calcium concentration and studies using both light and electron microscopy has revealed that the tidemark interface is a highly variable and irregular feature of cartilage in term of both its structure and related mechanical function. Furthermore, continuity between the superficial and deep layers is achieved via the tidemark by a continuum of collagen fibers between it. It is also shown in previous studies that the tidemark has well aligned and densely packed collagen fibers which allows it to withstand releated and direct compression [397]. The deepest calcified layer serves to anchor the cartilage to the subchondral bone. The type II collagen is responsible for establishing the cartilage frame and tensile strength, while prostaglandins contribute to its hydrophilic behavior. These components work together to help preserve water within the ECM, which further preserves the unique physical properties of the ECM and adds to its stress enduring nature. Each layer has its own unique compositions percentages and the layers work together to achieve its function.

Table 4 Layers of articular cartilage and their composition

Layers	Composition	Arrangements of fibers	Cells
Superficial	Mainly collagen II and IX	Packed tightly with parallel fibers to articular surface	High number of flattened chondrocytes
Intermediate	Proteoglycans and thicker collagen fibrils	Fibers are arranged in an oblique manner	Low number of spherical chondrocytes

Deep	Highest proteoglycan content, largest collagen fibrils (diameter), lowest water concentration	Fibers arranged perpendicular to surface	Chondrocytes arranged in columnar orientation parallel to collagen fibers
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It is worthy to note that collagen alone can only elongate no more than 10% of its original length [398, 399] and the incorporation of GAGs form complex structures to withstand external compressive forces. The unique physicochemical properties of GAGs make proteoglycans highly hydrophilic and provides additional expansion properties to make cartilages more resistant to compression [400]. The aggrecan (most abundant and largest) interacts with hyaluronic acid via connexin to form large proteoglycan aggregates that allow the cartilage to resist external stress and also to retain water molecules. The cartilage microenvironment (such as pore size, fiber size, type of structural proteins) and external stimuli (cyclical compressive forces, hypoxia) play important role in influencing cellular behavior [401].

Previous studies have reported cartilage tissue constructs fabricated by different 3D bioprinting techniques [402-405]. Although both natural and synthetic biomaterials have been applied to 3D printing technology to manufacture cartilage tissue constructs, the use of natural and synthetic bio-inks has its respective advantages and limitations [406]. Hence, it would be fruitful to combine the positive effects of different natural and synthetic bio-inks to achieve a synergistic effect with the hybrid cartilage constructs. The synthetic polymer serves as a structural framework for improved mechanical properties, while the natural hydrogel serves as a delivery carrier for cell encapsulation. Previous studies have shown that cell viability and metabolism are significantly increased using hybrid tissue. Compared with pure poly(lactic-co-glycolic acid) (PLGA) scaffold, the hybrid PLGA constructs with cell-encapsulated alginate has significantly enhanced GAG levels than control group [407]. Furthermore, studies using hybrid PLGA constructs with cell-encapsulated gelatin showed that cells retained their natural morphology even after implantation [408]. Multi-layer hybrid tissue constructs were fabricated using dual print-head extrusion-based 3D printing system (an extrusion printhead for PCL/PLGA deposition and the other extrusion printhead for hydrogel deposition) [409]. Notably, the pore size plays a major role in regulating the cellular activity and ECM secretion; the cells grown in scaffolds with large pore sizes as high as 400 μm showed higher GAG secretion [410] compared to scaffolds with smaller pore sizes (100–200 μm). An optimal pore size of 200 – 500 μm enhances chondrocyte activities and ECM production. Furthermore, the fabrication of hierarchical porous

tissue constructs (gradient in pore size and distribution across the constructs) would closely mimic the native articular cartilage and enhance cellular differentiation and ECM secretion. Furthermore, the incorporation of hyaluronic acid (HA) has been found to upregulate type II collagen and GAG production [411]. A recent study demonstrated the 3D bioprinting of human-derived induced pluripotent stem cells in a nanocellulose/alginate bio-ink for cartilage tissue engineering; sections of the 3D bioprinted tissue constructs stained for GAGs, Safranin O (with nuclear counterstain), and hematoxylin and eosin (H&E) showed great resemblance to native cartilage tissues [389]. The current bioprinting techniques facilitate the fabrication of complex structures at high printing resolution, and together with an in-depth knowledge on the histological and anatomical structure of the articular cartilage, it is highly possible to fabricate biomimetic cartilage tissue constructs that closely resemble native tissues.

5.1.4. Cardiac

Fig. 12. A) CAD model of cardiac patch, B) Schematic drawing of the printing concept and the distinct cellular bio-inks, C) A bioprinted vascularized cardiac patch, D) Transplantation of the printed patch in between two layers of rat omentum (Dashed, white line represents the patch border) and sections from the explanted patch stained with sarcomeric actinin (red) and nuclei (blue); scale bar from left to right = 100 μ m, 50 μ m and 25 μ m, E) CAD model of human heart model, F, G) A bioprinted heart within a support bath, H) Perfusion of bioprinted heart construct with red and blue dyes at the left and right ventricles respectively, I) 3D confocal images of printed heart with cardiomyocytes (CMs) in purple and endothelial cells (ECs) in orange; scale bar = 1 mm J, K) Cross-sectional of the heart immunostained against sarcomeric actinin (green); scale bars = 1 mm and 50 μ m respectively. [412], Copyright 2019. Reproduced with permission from John Wiley & Son Inc.

The heart is a muscular organ that pumps blood to facilitate the transport of oxygen, nutrients and removal of metabolic wastes through the blood vessels of the circulatory system. The heart consists of four distinct layers; beginning with the outermost pericardium (protective sac) and the heart wall in the following order: epicardium (outer), myocardium and lastly endocardium (inner) [413]. The cardiovascular disease is one of the leading cause of deaths worldwide due to the limited regenerative capacity of the myocardium [414]; hence the motivation for cardiac

tissue engineering is to fabricate human engineered cardiac tissues that closely emulate the native myocardium tissues. The myocardium consists of three main types of cardiac cells: myocytes, fibroblasts and the endothelial cells. The cardiomyocytes are the main building blocks of the heart (10^8 cells/cm³) [415]; they exhibit cylindrical morphology with an average diameter of 20 – 30 μ m and length of $\sim$ 120 μ m [416]. These cardiomyocytes are composed of bundles of myofibrils that form distinct, repeating units of sarcomeres that regulate the contractile function of the myocardium. The cardiac fibroblasts are the primary cell type responsible for the homeostatic maintenance of the heart ECM and serve as mediators of inflammatory and myocardial remodeling upon injury [417]. Furthermore, the presence of vascular endothelial cells is critical for formation of capillary network within the cardiac tissue.

The myocardium has a unique structural matrix composed mainly of fibrillar collagen type I ($\sim$ 80%) and collagen type III ($\sim$ 10%), which is responsible for the support and alignment of the cardiomyocytes and the capillaries [418-420]. These fibrillar collagen fibers can be classified as epimysial, perimysial and endomysial fiber bundles [420]. The epimysial fiber bundle is loose weave of fibrils surrounding and tethering individual cardiomyocytes, while the perimysial fiber bundle is the collagen sheath that surrounds the bundles of cardiomyocytes and lastly the endomysial fiber bundle is the collagenous fascia that encloses groups of perimysial bundles. Furthermore, tiny capillaries are closely positioned to the cardiomyocytes within the cardiac ECM to facilitate rapid diffusion-based exchange. The myocardium consists of highly-organized sheets with an average transmural fiber angle of $14.9 \pm 6.9^\circ$ per mm depth and exhibits a non-linear relationship between the fiber angle rotation and transmural depth throughout the ventricular myocardium ($\sim$ 20°/mm near epicardium, $\sim$ 15°/mm at mid wall and $\sim$ 13°/mm near endocardium) [421]. The measured equibiaxial Cauchy stresses of human left ventricle (LV) myocardium at 15% stretch showed an average of 18.9 ± 6.3 kPa in the mean-fiber direction (MFD) and 10.5 ± 4.0 kPa in the cross-fiber direction (CFD) [421]. Furthermore, the adult myocardium has a reported Young's modulus of 0.2 – 0.5 MPa [422] and a contractile force in the range of 15 mN/mm² [423]. Particularly, advanced maturation of the tissue-engineered cardiac tissues (increased expression of connexin43) from the early-stage hiPSC-derived cardiomyocytes can be achieved by subjecting the tissues to electromechanical conditioning with increasing intensity (from 2 Hz to 6 Hz) over time soon after the initiation of spontaneous contractions (period of high cell plasticity) [424].

The myocardium has highly-complex tubular structures in the micro- and nanoscale; the highly-aligned collagen fiber bundles are critical for cardiomyocyte alignment and the presence

of capillary networks within the engineered myocardium is necessary for survival for the densely-populated cardiomyocytes. The mature cardiomyocytes exhibit limited self-renewal potential [425] and the human induced pluripotent stem cells (hiPSCs) are an attractive cell source due to its wide availability and possibility to differentiate into cardiomyocyte cell lineages. The addition of vascular endothelial cells and fibroblast cells to cardiomyocytes can also induce a synergistic effect that facilitates the formation of vessel-like networks and the deposition of provisional ECM that improves the survival rate and mechanical integrity of the human engineered cardiac tissues [426, 427]. Furthermore, the selection of an optimal bio-ink material is also critical towards improved functional maturation of the cardiomyocytes [428, 429]. A recent study has demonstrated the 3D bioprinting of small-scale cellularized human hearts with major blood vessels that showed good structural integrity and facilitated perfusion of colored dyes within the embedded blood vessels [412]. Nevertheless, the printed blood vessels in the study is limited and more intensive work is necessary to fully comprehend the complexity and branching of blood vessels within the human heart. Finally, more advanced technologies are required to precisely print small diameter blood vessels within thick, vascularized human heart constructs.

5.1.5. Lung

Fig. 13. Laser scanning micrographs of two cell-layers composed of first layer of Matrigel™ layer with endothelial cells, second layer of Matrigel™ layer with epithelial cells by A) manual and B) bioprinting approaches after 3 days, scale bar = 50 μm . All the cells were stained for F-actin (red) and nuclei (white), C) Brightfield images of conventionally seeded and D) bioprinted paraffin embedded histological cross sections stained with Masson-Goldner trichrome coloration, scale bar = 100 μm . Cytoplasm is stained red, collagen fibers of the ECM Matrigel™ green and cell nuclei dark brown. The thick ECM layers in manual approach are hardly visible in the bioprinted samples. [430], Copyright 2015. Reproduced with permission from Springer Nature.

The lungs are conical in shape with a narrow-rounded apex at the top and a broad convex surface of the diaphragm. The lung can be visualized as having two distinct parts: the central airways (trachea, bronchi and terminal bronchioles) and the peripheral parenchyma (respiratory bronchioles, alveolar ducts and alveoli). During breathing, the air first enters the body through the nose or mouth, and then travels down the throat through the larynx and trachea before entering the lungs through the bronchi. The bronchi further divide into smaller bronchi (secondary and tertiary) and then into even smaller bronchioles and lastly into tiny air sacs called alveoli [431]. A typical pair of human lungs contain ~ 300 million alveoli and possess a surface area of $\sim 140 \text{ m}^2$ [432]. The alveoli ($\sim 40 \mu\text{m}$ in radius, $\sim 48 \mu\text{m}$ in length [433]) are the primary functional and structural units of the lung parenchyma where a thin tissue barrier separates the surface of

the airspace from the luminal surface of capillaries; the main function of this extremely thin air-blood barrier is to facilitate diffusion-based gas exchange (uptake of oxygen and excretion of carbon dioxide). The integrity of the alveolar wall is maintained by the interstitial connective tissues comprising of collagen and elastin fibers that interweave around the capillaries, forming “thin” and “thick” regions within the alveolar wall [434]. The thin portions of the alveolar walls represent the air-blood barrier, whereas the thicker portions of the alveolar walls contain collagen-elastin ECM intermixed with interstitial cells such as fibroblasts or myofibroblasts [434].

Gaseous exchange occurs in the alveoli across an extremely thin air-blood barrier of $\sim 0.6 - 2$ μm in thickness. The major cell types of the air-blood barrier are type I and type II alveolar epithelial cells (AECs), macrophages and pulmonary endothelial cells. The type I AECs are thin, flattened cells that line the alveolar walls and cover about 95% of the alveolar surface; they form functional tight junctions that protects against inhaled toxins, particles or micro-organisms and regulates gaseous exchange [435]. The type II AECs are cuboidal cells that synthesize, secrete pulmonary surfactant that regulates alveolar surface tension and also serve as progenitor cells for type I AECs upon injury [436]. The macrophages play important role in clearing apoptotic cells and cellular debris as well as participating in immunological responses [437]. The endothelial cells form the lining of the pulmonary artery and its branches (~ 500 μm diameter) and play important role in vascular homeostasis [438]. The ECMs in the lung are typically found in the basement membranes and the interstitial spaces; the alveolar fibroblasts found in the interstitial spaces are mainly responsible for ECM production and also serve as effector cells during injury [439].

The native human lung is a highly-complex organ that is an integral part of the bronchial, pulmonary circulation systems and autonomic nervous system; it is a large organ with numerous highly-complex structures that range from the trachea (macro-structures) to the alveoli (micro-structures) and current research on lung bioprinting is primarily focused on the air-blood barrier (which is the main site for diffusion-based gas exchange) [430]. The air-blood barrier is a relatively simple planar structure and the use of drop-on-demand bioprinting technique demonstrated the deposition of thin, homogeneous cellular (type II AECs and endothelial cells) and ECM layers (Matrigel) at specific regions to create simplistic air-blood barrier models in a reproducible and fully-automated manner [430]. The incorporation of more lung cells (such as type I AECs, macrophages and alveolar fibroblasts) within the bioprinted constructs would lead to more biomimetic air-blood barrier models. The formation of functional tight intercellular junctions is

imperative towards the barrier function of the air-blood barrier; a study has demonstrated that applying cyclic stretching (emulate breathing) to the air-blood barrier tissue models helps to improve the barrier function [440].

5.1.6. Liver

Fig. 14. A) A macroscopic image of a 3D liver tissue, B) H&E staining of a tissue cross-section; compartmentalization between the parenchymal and non-parenchymal fractions, C) ECM deposition assessed by Masson's trichrome staining, D) IHC staining of the parenchymal compartment for E-cadherin (green) and albumin (red), E) IHC staining for CD31 (red) and desmin (green) to assess organization of the endothelial cells and the presence of quiescent hepatic stellates, F) IHC staining for desmin (green) and α -SMA (red) to assess stellate cell activation, G) Oil-red O staining of 3D liver tissue cryosections to measure lipid storage, H) PAS staining to identify glycogen granules. Scale bar = 25 μ m (B-D, G-H), 50 μ m (E, F), I) Evaluation of toxicity test using Trovafloxacin in 3D bioprinted liver tissues; histological analysis shows loss of cellular adhesion (arrow) and increased hepatocyte necrosis (arrowheads) in Trovafloxacin treated tissues. [441], Copyright 2016. Reproduced with permission from PLOS.

The liver is the largest gland in the human body and the only organ capable of self-regeneration. A normal healthy liver weighs approximately 1.5 kg and it plays numerous key functions such as detoxification of various metabolites, regulation of metabolism and production of biochemicals for digestion. The liver has a dual blood supply; the portal veins provide ~70% of the hepatic blood flow (deoxygenated blood with high nutrient content from the digestive system) while the hepatic arteries supply the remaining ~30% (oxygenated blood from the heart)

[442]. The liver consists of a rich population of high-specialized cells that perform complex functions; they can be characterized as “parenchymal cells” such as hepatocytes (~60%) and “non-parenchymal cells” such as liver sinusoidal endothelial cells (LSECs) (~20%), Kupffer cells (~15%), hepatic stellate cells (~5%) and dendritic cells (< 1%) [443, 444]. The entire liver is covered by a thin, fibrillar connective tissue layer (mainly type I and III collagen fibers) termed as Glisson’s capsule and millions of hepatocytes (main cells that perform the detoxification and metabolic functions) form the functional units of the liver called lobules. The liver sinusoids (fenestrated capillaries) found within the lobules serve as a junction for mixing of nutrient-rich blood from the portal vein and oxygen-rich blood from the hepatic artery [445]. The sinusoids has a lining of specialized cells known as liver sinusoidal endothelial cells [446] and Kupffer cells [447]; the sinusoidal endothelial cells are fenestrated cells that play a critical role in the regulation of blood flow in the sinusoids by expressing endothelial nitric oxide synthase (eNOS) and releasing nitric oxide (NO) [448] while the Kupffer cells are macrophages that protect the systemic circulation from toxins [449]. The hepatic stellate cells located in the perisinusoidal space of Disse (mainly collagen type IV basement membrane) have several important functions such as storage of vitamin A and fats [450], regulation of the blood flow [451] and generation of scar tissue upon injury [452]. These hepatic stellate cells remain in a quiescent state in normal healthy liver but become activated upon injury to transform into collagen-secreting myofibroblasts with contractile properties [452].

The liver tissue is a densely-packed cellular structure with sinusoids (fenestrated capillaries of ~100-200 μm diameter) [453]. The crosstalk interactions between the hepatocytes, hepatic stellate cells and liver sinusoidal endothelial cells are critical for a healthy liver; the hepatocytes and hepatic stellate cells secrete vascular endothelial growth factor (VEGF) [454] to maintain the LSEC phenotype, while the healthy LSECs prevent the activation of excessive hepatic stellate cells that can potentially leads to liver fibrosis [455]. A recent study has also highlighted the importance of primary human Kupffer cells in recapitulating the temporal dynamics of fibrogenic responses to different cytokine and drug stimuli [456]. All these four types of liver cells can be isolated from human tissues or purchased commercially [441, 456, 457] and the replacement of lost hepatic mass can be mediated through proliferation of these liver cells (hepatocytes, hepatic stellate cells, LSECs and Kupffer cells) to create biomimetic liver tissue constructs [458]. Furthermore, the prevalent view is that the LSECs attracted by the VEGF can penetrate the clusters of newly proliferated hepatocytes and re-establish the sinusoidal networks [458].

Interestingly, the liver also contains an intrinsic “hepatostat” control system that regulates its own weight (a bigger liver tends to decrease in size after transplantation and *vice versa*) for performance of its homeostatic functions [459].

Prior bioprinting studies have demonstrated the extrusion-based bioprinting of biomimetic liver tissues using bio-inks of extremely high cell density (1.5×10^8 cells/ml [310, 460]) by printing the parenchymal cells in the core of hexagonal compartments surrounded by non-parenchymal cells at the peripheral [441, 456]. Histological analysis of the bioprinted human liver tissue exhibited distinct intercellular hepatocyte junctions, CD31+ endothelial networks and biochemical characterization of the bioprinted liver constructs exhibited sustained expression of ATP, albumin and cytochrome P450s over 4 weeks of culture. Furthermore, Trovafloxacin induced significant, dose-dependent toxicity to the bioprinted human liver tissue constructs at clinically relevant doses, demonstrating its potential in predictive toxicology for drug induced liver injury. The current bioprinted human liver constructs are mostly simplified 3D tissue models that are suitable for drug development, it is still challenging to create the complex structures found in functional liver human constructs for human transplantation.

5.1.7. Kidney

Fig. 15. (Top) 3D printed convoluted proximal tubule acquired by confocal microscopy, (Middle) 3D proximal tubule morphology and molecular markers, (Bottom) 3D proximal tubules form a tissue-like polarized epithelium. [463], Copyright 2016. Reproduced with permission from Springer Nature.

The human kidney is a bean-shaped organ (~10 – 13cm wide, ~ 120 – 170g weight) found on both sides of the human body; its key functions include filtration of blood and regulation of electrolytes, pH and fluid balance. Each kidney is enclosed by a fibrous renal capsule, beneath which the renal parenchyma is divided into an outer cortex and inner medulla (can be divided laterally into a number of conical lobes called the renal pyramids [461]). The blood enters the

kidney via the renal artery which branches to form numerous interlobular arteries that reach the glomeruli of the nephrons. Each nephron (~30 – 55 mm long) consists of a renal corpuscle (glomerulus of ~200 µm diameter and Bowman's capsule) and a renal tubule (proximal convoluted tubule ~65 µm diameter, loop of Henle ~35 µm diameter – descending limb of loop of Henle and the ascending limb of loop of Henle, distal convoluted tubule and the connecting tubule [462]). The filtered waste is then excreted from the collecting tubules at the base of each lobe to the renal pelvis; the renal papillae (ranges from 4 to 18 per kidney [463]) located at the base of these renal pyramids are connected to individual minor calyces that converge into major calyces that eventually merge into renal pelvis.

The nephrons are the primary structural and functional units of the kidney, with up to 2 million nephrons per kidney. The kidney nephrons are derived from the ureteric bud and meta-nephrogenic mesenchyme progenitor cells that can differentiate into more than 26 types of renal cells in the adult kidney [464]. The ureteric bud contains the precursor of the epithelial cells of the collecting duct, whereas the meta-nephrogenic mesenchyme contains the precursor for the epithelial for the rest of the nephron, endothelial cell precursors and stroma cells [464]. Furthermore, the liver comprises a plethora of different ECM proteins such as collagens type I, III, IV, V, XVII, heparin sulfate proteoglycans, fibronectins, laminins and nidogens [465].

The current research on kidney bioprinting is primarily focused on small-scale renal structures such as the proximal convoluted tubules [466, 467]. The fabrication of proximal tubule is of tremendous interest for studies on nephrotoxicity because the active clearance, reabsorption, intracellular concentration and local interstitial accumulation of drugs occur primarily at this site in the kidney [468, 469]. These bioprinted proximal convoluted tubules can serve as potential tissue models via epithelial-interstitial interactions for assessment of nephrotoxicity and pathogenic responses. Although the vast variety of cells and ECM proteins constituting the numerous nephrons of the kidney has proven to be extremely challenging for bioprinting of functional kidneys at the present stage, extensive research have been conducted to elucidate the mechanisms of stem/progenitor cell differentiation into multiple renal cell lineages under appropriate *in-vitro* culture conditions [470-472]. Using these knowledge, hiPSCs undergo progressive maturation over a period of 20 days under optimal culture conditions to form self-organizing kidney organoids containing nephrons (with proximal and distal tubules, early loops of Henle and glomeruli containing podocytes) with collecting duct network [472].

5.1.8. Bone

Fig. 16. (Top) A, B, C) 3D bioprinting of mandible bone defect from human CT image data, D, E) Osteogenic differentiation of bioprinted cells confirmed by Alizarin Red S staining, indicating calcium deposition. (Bottom) A, B, C) Images of bioprinted calvarial bone construct, D-L) Histological and immunohistological images of constructs after implantation. [316], Copyright 2016. Reproduced with permission from the Nature Group.

The human bone has a highly-complex hierarchical structure that performs diverse mechanical, biological and chemical functions. The bone has two distinct regions: outer compact bone (cortical bone; elastic modulus: 3 – 30 GPa) and inner trabecular bone (cancellous bone; elastic modulus: 0.02 – 2 GPa) [473]. The cortical bone forms the hard exterior of bones and gives

the bone its smooth, white appearance; its main function is to provide structural support and protection for the human body. The cortical bone is a highly-dense structure made up of building units called osteons (~10 – 500 μm) and it is scarcely populated by osteocytes, canaliculi and blood vessels found in only 3 – 5% of its volume. The inner trabecular bone is a highly-porous trabeculae network (~50 – 90% porosity) filled with bone marrow [473], where the production of blood cells (hematopoiesis) occurs. The bone is a unique organ with self-regenerative capability for controlled growth and remodeling in response to stimuli upon injury. However, the self-regenerative ability is inadequate when there is a large bone defect and there is a need for bone implants (autografts, allografts or engineered substitutes) [474]. The bone contains three major types of cells, two of mesenchymal origin (osteoblasts and osteocytes) and one of hematopoietic origin (osteoclasts); the bone activity is well coordinated through autocrine, endocrine, paracrine and mechano-sensing signals to regulate the systemic balance of the calcium-phosphate metabolism while maintaining the homeostasis and microarchitecture of bone adapted to its biomechanical needs [475]. Generally, the osteoblasts form bone, the osteoclasts resorb bone and the osteocytes maintain bone and regulate the activities of osteoblasts and osteoclasts during bone remodeling [475]. The osteoblasts arise from a common pluripotent mesenchymal stem cell (MSC) following timely programmed steps with expression of specific genes under the pro-osteogenic pathways [476], they synthesize a protein mixture known as osteoid (unmineralized, organic portion of bone – predominately made up of 90% collagen type I); a portion of the osteoblasts are embedded within the osteoid to form osteocytes while the remaining osteoblasts form a layer of lining cells above the newly-synthesized bone matrix [477]. Furthermore, the osteoblasts play important role in regulating hematopoietic stem cell homeostasis, energy metabolism and male fertility [478-480]. The osteoclasts, mostly found on the bone surfaces (particularly in *Howship's lacunae*), are large multi-nucleate cells responsible for bone resorption and calcium homeostasis [481]. The osteocytes are the most abundant cells in the bone (90 – 95% of total population); they originate from embedded osteoblasts during bone formation and these osteocytes can secrete soluble proteins that regulate surrounding osteoblast and osteoclast behavior [482].

The human bone has a highly-complex hierarchical structure that comprises both organic components (type I, III, IV collagen and fibrillin) and inorganic crystalline mineral (hydroxyapatite) in a highly-ordered network [483, 484]. The hydroxyapatite nano-crystals (plate-shaped, 50 x 25 x 4 nm) are preferentially oriented with their *c* axis parallel to fibers and arranged in a periodic,

staggered array along the collagen fibrils (~300 nm long, ~1.5 nm diameter) [485]. The highly-complex nanoscale structures of the native human bone provide a multitude of deformation and toughening mechanisms that make the bone resistance to fracture. These reinforced collagen fibers form a tough yet light-weight material that is the universal building element for both compact and trabecular bones.

The design and fabrication of biomimetic bone scaffolds have been investigated extensively over the last two decades; some of the important design considerations include biodegradability, biocompatibility, mechanical similarity and pore size (200 – 350 μm) [486, 487]. Notably, metals [473] and biomaterials (polymeric/ceramic) [474] are prevalently used to fabricate these bone scaffolds. The main strategy is to develop mechanically stable bone scaffolds with controlled degradation rate and sustained release of biomolecules (for angiogenesis and osteogenesis) as temporary bone implants. A recent bioprinting strategy using integrated tissue-organ printer (ITOP) has demonstrated the ability to create vascularized cell-laden bone constructs with tunable mechanical properties [316]. There are three key components in the ITOP printing strategy; the thermoplastics PCL (can be printed at different fill ratios) confers the mechanical strength, the crosslinked hydrogel provides a biocompatible microenvironment for cell proliferation and differentiation and lastly the removal of uncrosslinked sacrificial components provides hollow channels for potential vascularization.

5.1.9. Blood vessels

Fig. 17. Fluorescence images of a vascular-like channel in cross section. A) A schematic cross section of a single layer of endothelial cells and B) an additional layer of SMC. C) The Immunofluorescence staining images of EC at the channel wall after 14 days of flow in cross section (CD31 in green, DAPI in blue, actin-phalloidin in red). D) The images of the combination of SMC layer and the EC layer after 7 days of flow (CD31 in green, DAPI in blue, prelabelled fibroblasts in red, prelabelled SMCs in yellow). E) The images of the whole architecture of a multi-layered vessel model after 4 days of dynamic cultivation (phalloidin in green, DAPI in blue, prelabelled fibroblasts in red, prelabelled SMCs in yellow). [488], Copyright 2018. Reproduced with permission from Springer Nature.

The wall of a native blood vessel is generally comprised of three layers with the innermost tunica intima layer, followed by the middle tunica media layer and the outermost tunica adventitia layer [489]. Although all the arteries, veins and capillaries have three distinct layers, the wall thickness of each layer differs for each type of blood vessel. The arteries (carry blood from heart to the rest of the body) have thicker lumen walls to withstand higher amount of

pressure, whereas the veins (carry blood from the rest of the body back to the heart) have thinner walls due to a much lower pressure from the flowing blood and the blood capillaries (smallest of the body's blood vessels) mainly serve as sites for nutrient exchange. The tunica intima consists of a single layer of endothelial cell arranged parallel to the blood vessels and serves to protect the blood vessels and prevent blood from leaking out into the interstitial tissues. The tunica media is mainly made up of smooth muscle cells, elastin, reticular fibers and proteoglycans and provides the elasticity of the blood vessels. The tunica adventitia comprises mainly of longitudinal collagenous tissue embedded with fibroblasts. The high collagen content prevents vascular rupture under intense pressures and provides additional mechanical support by anchoring the blood vessel to the surrounding tissues [490].

Table 5 Comparison of tunica in both arteries and veins.

	Arteries	Veins
General appearance	Small round lumens with relatively thicker walls	Large flattened lumens with relatively thinner walls
Tunica intima	Wavy endothelium due to thick smooth muscular layer Presence of internal elastic membrane: consist of fibrillin, microfibrils and collagen fibers (secreted by endothelial cells)	Smooth endothelium Absence of internal elastic membrane
Tunica media	Thickest amongst all three layers with mainly smooth muscle cells and elastic fibers Presence of external elastic membrane	Thinner than externa with mainly smooth muscle cells and collagen fibers Absence of external elastic membrane
Tunica adventitia	Thinner than tunica media with collagen and elastic fibers Nerves and vasa vasorum present	Thickest layer amongst all three layers with collagen, smooth fibers and elastic fibers Nerves and vasa vasorum present

An ideal tissue-engineered blood vessel should consist of the three layers (tunica intima, tunica media and tunica adventitia), good biocompatibility and compatible compliance and visco-elastic capabilities. Previous reports have shown that mechanical properties of vessels are dependent on several factors such as macromolecular volume fraction, molecular orientation and number of smooth muscle cells [491]. Native blood vessels are often characterized as a non-linear stress-strain channel but in reality, they are found to have two distinct regions of distinct linear behavior which is similar to the loading mechanism of elastic fibers and collagenous fibers. It has a lower range of 0.2 – 0.6 MPa and an upper range of 2 – 6 MPa. It is important for the fabricated vessels to possess similar mechanical behavior in order to withstand the blood

pressure, as the constant shear stress, transmural pressure and axial extension experienced by the engineered vessels can also further influence downstream cellular behaviors [492]. Another important consideration is to facilitate anastomosis and integration of fabricated vessels with host vessels; a common strategy is to increase density of fibroblasts within the engineered vascular structures prior to implantations. A report showed that a higher density of 2 million cells/mL of fibroblasts, as compared to 0.2 million cells/mL, demonstrated superiority in in-vivo anastomosis and perfusion as compared and results were consistent with trends observed in in-vitro cultures [493]. Another study highlighted that implanted vascular networks anastomose with host vessels through a previously unidentified process termed "tapping and wrapping" between grafted vascular channels and host vessels [494].

A variety of bioprinting techniques have since emerged to fabricate vessels. Such techniques can be broadly categorized into extrusion-based, droplet-based and laser-based techniques. Each technique has its own advantages and limitations. For example, extrusion-based techniques facilitate fabrication of complex structures with the aid of supportive materials; however, one main disadvantage of such techniques is the a lack of specificity in cell seeding due to cellular encapsulation in bio-inks [332]. Conversely, droplet-based techniques facilitates fabrication of high-resolution tissue constructs with patterning of multiple types of cells. However, the main disadvantage for this techniques is that hydrogels with high viscosity could easily clog up the nozzle and provides high shear stress for cells which could negatively influence cellular behaviors [495]. Recent clinical studies have shown that engineered vessels are a viable treatment option for cardiovascular diseases [496]; recent advances in engineered vessels include the fabrication of high-aspect ratio tubular structures such as tubular bifurcated structures [497], vascular channels with a Young Modulus of 8 – 28 MPa, as well as tiny vascular stuctures of 10 – 100 μm [498]. A droplet-based bioprinting technique was used to create *in-vitro* vascular models, including continuous endothelium that mimics the tunica intima, a layer of elastic smooth muscle cells (SMC) that mimic the tunica media, and surrounding fibroblast fibers and collagen matrices imitating the tunica adventitia (**Fig. 17.**) [488]. The results showed that there is a SMC distribution around the endometrium of the thickness of 425 μm , elongated healthy cells, mimicking the middle membrane and having a wall thickness similar to that of small arteries and veins in the human body. However, in order to be able to design the structure of the resulting vessel wall more freely, the reduction in wall thickness is a goal of future efforts. Hence, it is highly likely to fabricate biomimetic 3D-bioprinted vascular graft in the near future.

6. Complementary strategies to achieve functional tissues/organs

6.1. Dynamic co-culture perfusion system

The bioprinted tissues/organs is not suitable for immediate tissue transplantation and post-processing step is one of the key essential steps for the living cells within the printed constructs to interact, remodel and mature into functional tissue constructs [499]. Most of the tissue-engineered constructs are only subjected to static culture conditions; whereas the cells in the native human body are constantly experiencing the dynamic laminar flow from the blood capillaries. The mass-transfer limitation is evident in the *in-vitro* culture of large 3D tissue constructs under static culture conditions and the use of a bioreactor system can be utilized for improved mass transport, delivery of soluble growth factors and mechanical conditioning of the perfused tissue constructs [500].

The next critical challenge is the co-culture of multiple types of cells within the bioprinted constructs [500, 501]; it becomes a daunting task to define a common co-culture medium for multiple cell types within a single bioprinted construct. Although there is no fixed formula for the development of optimal co-culture medium, the more sensitive cell type should generally have a greater weight in the formulation of the final co-culture medium via a “trial and error” process. It requires a whole series of experiments beginning with the characterization of each individual cell type in isolation to the co-culture of multiple cell types using the co-culture medium. It is important to ensure the maintenance of cell phenotype in all the cell types within the bioprinted constructs by evaluating the expression of specific phenotypic markers of all the cell types at different time points of data collection.

6.2. Macromolecular crowding

One of the critical challenges of tissue engineering is to emulate the highly-complex ECM found in the native tissues. The living cells are mainly responsible for creating such complex hierarchical structures found in ECM of the native human body via ECM production and remodeling process; functional regeneration can be achieved by leveraging on the innate capacity of the cells to create their own tissue-specific ECM. Hence, a biophysical approach termed macromolecular crowding (MMC) can be applied to increase the thermodynamic activities and biological processes by several orders of magnitude to create ECM-rich tissue

constructs [105]. Macromolecular crowding is a concept that describes the inter- and intra-cellular space; it is a phenomenon that drives cell biochemistry via the “excluded volume effect” (EVE) [502]. A surrogate marker known as fractional volume occupancy (FVO) is dependent on the hydrodynamic radii of the crowders and it is used to assess the effectiveness of crowders in solutions. The FVO of the human blood lies within the physiological range of 9% v/v to 54% v/v; which includes the total FVO of albumin, fibrinogen and globulin in blood plasma and interstitial fluid [503]. A recent study has demonstrated that the use of PVP 360kDa crowders enables enhanced ECM deposition up to 54% v/v which is not achievable with other crowders such as Ficoll mixture (Ficoll 70 and Ficoll 400) or PVP 40kDa [503]. The viscosity is known to play a major role in protein association; the higher the viscosity, the slower the diffusion rate and this would in turn reduce the kinetics of diffusion-controlled reactions. Notably, PVP 360kDa exhibits significantly lower viscosities at high FVOs and makes it an attractive source of macromolecular crowders for droplet-based bioprinting applications [121].

A recent study has utilized culture medium supplemented with macromolecules to enhance the ECM deposition to create ECM-rich tissue constructs within 48 hours under optimal culture conditions and the macromolecular polydispersity is the key modulator of ECM deposition [357]. Furthermore, different macromolecules have been used to tune the microstructure of the collagen type I fibrils (a major ECM protein found in most tissues) during its fibrillogenesis process [504, 505]. Hence, the incorporation of different macromolecules within the co-culture medium can potentially help to accelerate tissue maturation.

7. Transplantation using 3D printed tissues/organs

Although the bioprinted organs can be fabricated using autologous primary or stem cells to eliminate the need for lifelong immunosuppressant drugs in allogeneic organ transplantations, the transplantation of bioprinted organs still face similar challenges as allogenic organs such as the potential onset of cellular ischemia or edema [506]. The conventional organ preservation technique involves the use of hypothermic static storage which resulted in a series of potential anatomic, cellular and metabolic injuries to the preserved organs due to ischemia [507]. Notably, the use of portable *ex-vivo* machine perfusion improves the microvasculature patency and integrity, prolongs preservation time and holds potential for organ resuscitation and reconditioning to improve the quality and quantity of donor organs [508]. A recent study has demonstrated extended organ preservation for up to 4 days using a novel technique that

combines subzero supercooling preservation with machine perfusion [509]. The advancement in new preservation techniques can potentially help to ease logistical burdens and facilitate transportation of bioprinted organs worldwide [510].

Next, it is also crucial to ensure long-term survival and functions of bioprinted organs after transplantation. The surgical transplantation procedure is always accompanied by the onset of innate inflammatory responses; the proteins from the blood and interstitial fluid adsorb to the surface of transplanted organs to form a transient provisional matrix that triggers inflammatory responses [511]. Hence, different strategies have been implemented to modulate the inflammatory responses elicited by the cells and ECM in the bioprinted tissues/organs for improved tissue integration and performance [512, 513].

Some of the pioneering work on human transplantation of tissue-engineered tissues include autologous bladder [514], autologous urethra [515] and autologous vaginal organs [516]. The follow-up urodynamic studies (22 – 61 months) on the transplanted autologous bladder tissues in 7 patients (age 4 – 19) showed improved bladder compliance and urinary continence and the histological and immunohistochemical evaluation of tissue biopsies from the engineered bladder segments also showed adequate structural architecture and phenotype [514]. The tissue-engineered urethras (tubular structure of 4 – 6 cm in length, 0.5 cm outer diameter) in 5 male patients (age 10 – 14) exhibited normal patency with no fistulae or urinary tract infections during follow-up studies (36 – 76 months) and the urethral biopsies showed a normal appearing architecture by 3 months after implantation [515]. The tissue-engineered vaginal tissues were fabricated by seeding autologous epithelial and smooth muscle cells over intestinal submucosa segments (7 x 10 cm each, from Surgisis); the follow-up studies (up to 8 years) on the transplanted vaginal organs in 4 female patients (age 13 – 18) showed complete integration of the tissue-engineered vaginal matrix with surrounding native tissues after 6 months and the yearly serial deep-tissue biopsy samples showed good healing results without morbidity [516]. Prior work demonstrated that successful use of tissue-engineered organs to replace ailing biological counterparts. The paradigm shift from a one-size-fits-all model to a patient-specific model of healthcare leads to the use of 3D printing techniques that can leverage the image acquisitions from different imaging modalities to potentially fabricate patient-specific tissues/organs on demand [517]. One of the pioneering investigations on human transplantation of 3D-printed PCL-based airway splint demonstrated the 3D printing of anatomically-specific implantable devices for an infant with tracheobronchomalacia; there was no further complication after 21 days and

full resorption of the 3D-printed PCL splint in the human infant is expected to occur within 3 years [518].

As highlighted in the earlier sections, it is no longer a daunting task to fabricate 3D anatomically-relevant biological structures using the recently developed bioprinting strategies. With the rapid advancement in the 3D bioprinting and tissue maturation techniques, the bioprinted tissue constructs can exhibit some resemblance to the native tissues/organs after tissue maturation. The human skin is considered as a relatively simple tissue/organ but it is a highly-complex organ that serves several functions such as the skin barrier function (formation of stratified keratinocyte layers), skin pigmentation for protection against harmful ultraviolet rays (accumulation of melanin granules above the keratinocytes' nuclei), touch sensation (presence of sensory nerves), temperature regulation (presence of hair follicles and sweat glands) and skin hydration (presence of sebaceous glands). Although it is highly challenging to fabricate fully-functional tissues/organs, the key focus is to determine a degree of tissue functionality deemed suitable for human transplantation. The tissue/organ that predominantly serves as structural support (cartilage, bone) will likely be the first bioprinted tissue construct to be transplanted in humans. A recent study has demonstrated the feasibility of printing stable, human-scale tissue constructs (such as cartilage and bone) using an integrated tissue-organ printer (ITOP) that can mature into vascularized functional tissues *in vivo* (in animal models) [316]. The results indicated potential utility of 3D bioprinted tissues/organs in translational applications. The difficulty of tissue printing increases with tissues/organs that consist of multiple types of cells and highly-complex micro- and nano-scale structures that offer numerous important tissue-specific functionalities (liver, kidney). It imposes increasingly difficult obstacles that encompass stringent cell and material requirements, adequate vascularization and innervation and finally tissue maturation and functionality.

8. Outlook

8.1. Commercialization and regulatory aspects

The successful commercialization of bioprinted tissues/organs is strongly dependent on the regulatory and funding approval [519]. The key challenges that hinder the development of tissue engineering and regenerative medicine (TERM) are the regulatory processes and diversities [520]. The bio-inks (living cells and printable biomaterials) used in bioprinting processes should

be manufactured using a strict regulatory guideline; the implementation of Current Good Tissue Practice (CGTP) for human cell, tissue, and cellular and tissue-based product (HCT/P) by The Food and Drug Administration (FDA) in year 2004 aims to prevent the introduction or transmission of potential communicable diseases [521]. Although other critical criteria such as quality control and process validation procedures can also be implemented along with good manufacturing practices, the bioprinted tissues/organs (comprise different living cells, biomaterials and growth factors) still create difficulties in regulatory approval due to the ever-increasing complexity of clinical studies. As of now, there are no manufacturing standards for bioprinting process [522] and this current lack of regulatory actions needs to be rectified prior to the utilization of 3D bioprinting on a clinically-relevant scale.

8.2. In-situ bioprinting

The status of bioprinting primarily focuses on the pre-fabrication of tissues/organs and further *in-vitro* maturation prior to implementation. Particularly, *in-situ* bioprinting can be implemented to directly deposit living cells and biomaterials over a defective site in the human body [523]. The concept of *in-situ* bioprinting (non-invasive *in-vivo* printing) is largely restricted to easily accessible tissues/organs (such as skin, cornea or cartilage) and the human body functions as the “*in-vivo* bioreactor” to facilitate maturation of the bioprinted tissues/organs in a more physiological environment. Furthermore, the integration of scanning modalities with *in-situ* bioprinting system facilitates real-time non-invasive tissue repair in a highly-automated approach; this essentially eliminates the need for difficult handling and fixation of fragile bioprinted constructs at the defective site. Although *in-situ* bioprinting is still in its infancy stage, some studies have demonstrated successful delivery of living cells and biomaterials over the defective sites [524-526]. Some important considerations for *in-situ* bioprinting include development of novel rapid crosslinkable bio-inks, sterilization and safety of the *in-situ* bioprinting systems.

8.3. Tissue-electronics interface: Cyborg

The term “cyborg” refers to a human being with both organic and bio-mechatronic body parts. The innate capability of living cells to generate and react to electrical stimuli has spurred scientists to develop technologies to electrically stimulate and evaluate biological tissues [527, 528]. A 3D printed bionic ear was fabricated using chondrocyte-encapsulated alginate hydrogel and electrically conductive silver nanoparticles (AgNPs) to achieve a seamless interface between

the electronic components and biological tissues. The bionic ears are matured over a period of 10 weeks and are capable of receiving electromagnetic signals over an expansive frequency range from hertz to gigahertz (normal human hearing range is only up to kilohertz) [529]. Furthermore, customizable 3D-printed limb prostheses can help to restore the functions of a missing limb due to genital limb deficiency or amputation [530].

Over the last two decades, the brain-computer interface (BCI) technology has evolved rapidly and it can be a potential radical solution for those with neuromuscular impairments [531-534]. The BCI technology serves as a bridging technology that can help to unlock the immense potential of tissue-electronics interface. The increasing scientific interest would eventually lead to the design and implementation of more bionic organs that can potentially enhance human capabilities [535].

Fig. 18. Electrical characterization of the bionic ear. (A) Image of the 3D bioprinted bionic ear (scale bar = 1 cm), (B) The response of bionic ear to radio frequencies in terms of S21, the forward power transmission coefficient. (C) Schematic representation of the radio signal reception of two complementary (left and right) bionic ears. [529], Copyright 2013. Reproduced with permission from the American Chemical Society.

9. Concluding remarks

The field of bioprinting has evolved rapidly over the last two decades; it has already succeeded in fabricating human-scale tissues/organs that can matured into vascularized and functional tissues/organs *in vivo* (in animal models). Despite the advancement in bioprinting

systems, a key dilemma of bioprinting lies in the need to strike a balance between achieving the nano-scale resolution that closely emulate the native ECM found in human tissues/organs and improving the fabrication speed for fabrication of human-scale tissues/organs. Furthermore, the stringent printing conditions have resulted in a limited choice of bio-inks mainly based on its rheological properties and crosslinking mechanisms. Although the recent development of dECM bio-inks provides an important step forward for bioprinting of functional tissues, it is a mere concoction of various ECM proteins. The dECM bio-ink is unable to fully replicate the complex ECM as the highly-specific spatial position of each unique protein within the native tissue is disrupted during the bio-ink preparation step. Hence, it is important to improve the understanding of the native ECM by analyzing the localization and composition of various ECM proteins in the decellularized tissue scaffolds. An in-depth understanding of the composition and spatial arrangement of living cells and ECM within tissue constructs along with the development of advanced bioprinting strategies is also necessary to achieve the envisioned biomimicry within anatomically-relevant tissue constructs using multi-material bioprinting approach. In addition, the use of complementary strategies is critical to ensure the maturation and assembly of bioprinting tissue constructs into vascularized, functional tissues/organs that can be potentially used for transplantation. Particularly, the incorporation of scalable automated robotic technologies into a biofabrication production line (bioreactor for cell propagation, bioprinter for organ printing and perfusion apparatus for tissue maturation) is important for regulation and commercialization of bioprinted tissues/organs. Other interesting concepts include the direct *in-situ* bioprinting of cell-encapsulated bio-inks as a form of minimally-invasive repair of easily accessible tissues/organs and the possibility of integrating tissue-electronics interface with the native human body to potentially enhance human capabilities. The human body contains a plethora of cells that work in tandem to perform highly-specific tissue functions; the current state-of-the-arts tissue culture technique is limited to only triple co-culture (3 types of cells). Advancement in tissue culture techniques is necessary to address the bottleneck of maturing bioprinted multi-cellular 3D tissue constructs into functional tissues. The potential of 3D bioprinting along with advances in polymer sciences and tissue culture techniques would make bioprinting tissues/organs an impending reality.

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