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► **To cite this version:**

Wissam Farhat, François Chatelain, Auriane Marret, Lionel Faivre, Lousineh Arakelian, et al.. Trends in 3D bioprinting for esophageal tissue repair and reconstruction. *Biomaterials*, 2021, 267, pp.120465. 10.1016/j.biomaterials.2020.120465 . hal-03419042

**HAL Id: hal-03419042**

**<https://hal.science/hal-03419042>**

Submitted on 7 Nov 2022

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# Trends in 3D bioprinting for esophageal tissue repair and reconstruction

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## Abstract

In esophageal pathologies, such as esophageal atresia, cancers, caustic burns, or post-operative stenosis, esophageal replacement is performed by using parts of the gastrointestinal tract to restore nutritional autonomy. However, this surgical procedure most often does not lead to complete functional recovery and is instead associated with many complications resulting in a decrease in the quality of life and survival rate. Esophageal tissue engineering (ETE) aims at repairing the defective esophagus and is considered as a promising therapeutic alternative. Noteworthy progress has recently been made in the ETE research area but strong challenges remain to replicate the structural and functional integrity of the esophagus with the approaches currently being developed. Within this context, 3D bioprinting is emerging as a new technology to facilitate the patterning of both cellular and acellular bioinks into well-organized 3D functional structures. Here, we present a comprehensive overview of the recent advances in tissue engineering for esophageal reconstruction with a specific focus on 3D bioprinting approaches in ETE. Current biofabrication techniques and bioink features are highlighted, and these are discussed in view of the complexity of the native esophagus that the designed substitute needs to replace. Finally, perspectives on recent strategies for fabricating other tubular organ substitutes via 3D bioprinting are discussed briefly for their potential in ETE applications.

**Keywords:** *3D bioprinting; Tissue engineering; Esophagus substitute; Regenerative medicine; Esophageal repair*

## 1. Introduction

3D bioprinting is a sophisticated approach that enables the fabrication of well-designed tissue constructs and organs from 3D simulated models [1][2]. In 2004, the term ‘*bioprinting*’ was introduced to the scientific community during a workshop that was held at the University of Manchester, UK [3]. Since then, bioprinting has been used as a pioneering strategy in designing a variety of complex tissues and functional organs [4][5][6]. The notable reputation of bioprinting in the bioengineering sector stems from its relative low cost, ease of use, precision, and the ability to print cell-laden bioinks [7]. Advances in 3D bioprinting are at the forefront of new strategies in the research areas of tissue engineering (TE) and regenerative medicine. TE is an interdisciplinary field that requires collaboration and knowledge from various disciplines including material science, chemistry, biology, biophysics and biomechanics, pharmaceutical science, and medicine to develop therapeutic solutions for repairing or replacing damaged tissues and organs. Conventional TE methods rely mostly on bulk materials, random distribution of cells, matrices, bioactive cues, and other biological components that allow the tissue to repair itself [8]. In contrast, 3D bioprinting technologies, as illustrated in the context of this review, offer the control over the 3D architecture of biological components (living cells, polymers, metals, biochemicals, etc.) to mimic the complex nature of native tissues [8][9][10][11]. 3D bioprinting has thus contributed to significant advances in the biomedical field, encompassing disease modeling [12], tumor models [13][14], drug and toxicology screening [14][15][16], high-throughput assays [17], tissue engineering and regenerative medicine [18] (Figure 1). It is worth noting that

substantial efforts have been devoted to bioprinting for transplantation applications, where bioprinted constructs have been implanted *in vivo* [19]. In fact, bioprinting of functional organs at clinically relevant sizes remains a huge challenge due to a number of issues such as vascularization, cell incorporation, mechanical performance, and structural integrity [19][20]. Nonetheless, several approaches to bioprint complex tissues and organs, comprising bone and cartilage, lung tissue, blood vessels, esophagus, and other thin and hollow tissues have been explored with reasonable degrees of success [9][21].

The esophagus is a hollow organ which transports the food bolus from the pharynx to the stomach [22]. It consists of four layers (mucosa, submucosa, muscularis propria, and adventitia) encompassing a mosaic of different cell types (epithelial, glandular, and muscle cells) [22][23]. A number of congenital and acquired diseases may lead to esophagectomy [24], a surgical resection procedure whereby the diseased esophagus is replaced with other digestive tract organs (stomach, jejunum or colon) [25][26][27]. According to the National Cancer Institute, esophageal cancer is considered to be the eighth most common cancer worldwide and is associated with a poor prognosis, and with estimated deaths of 4 per 100,000 persons per year [28][29]. In fact, esophagectomy is only feasible in one out of five patients suffering from advanced gastroesophageal cancer, which represents 96 000 procedures each year, i.e. 20% of the 480,000 new cases worldwide [30]. Besides esophageal cancer, esophageal replacement is often the only treatment available for other esophageal disorders. These include long gap esophageal atresia, esophageal stricture refractory to endoscopic dilation, mega-esophagus related to dysfunctional esophageal disorders, esophageal perforation, caustic injury, aggressive sclerotherapy, and systemic sclerosis, among others [26].

Esophagectomies often lead to serious complications (transplant necrosis, anastomotic stricture, etc.) and are associated with impaired quality of life and substantial mortality [31][32]. An alternative approach currently being investigated by a number of teams is the use of decellularized scaffolds to substitute and repair the damaged esophagus [23][33][34]. This approach uses esophageal matrices from human and animal tissues which are subjected to an appropriate treatment to remove cells and immunogenic materials [35][36]. These matrices usually retain their initial macro- and micro-architecture and preserve the molecular constituents of their extracellular matrix [37][38][39]. It is proposed that these matrices could be a reliable approach for esophageal regeneration by promoting cellular proliferation, migration, and differentiation [23][34]. In spite of the satisfactory outcomes of these strategies in certain *in vitro* and *in vivo* trials, issues such as donor shortage as well as biological risks associated with donor tissue, inadequate mechanical strength, inflammatory responses, inadequate size, and sometimes accelerated degradation have hindered the broader use of such grafts. Hence, 3D bioprinting-based tissue engineering could provide an ideal solution and unlimited supply of substitutes for esophageal regeneration, where each esophageal substitute could be exquisitely tailored to the patient's morphology and pathology.

In the current review, we offer a comprehensive overview of the recent attempts to exploit 3D bioprinting to design artificial esophageal substitutes. We will first introduce 3D bioprinting as a new biofabrication tool in the TE sector. The critical characteristics of each 3D

bioprinting technology and the recent advances within this field will be described. We will then present the current approaches to design an artificial esophagus with their advantages and their drawbacks. Based on the recent developments in 3D bioprinting technologies, as well as on parallel approaches to build other tubular organ substitutes, we will elaborate on promising strategies that could yield significant progress in ETE in the near future.

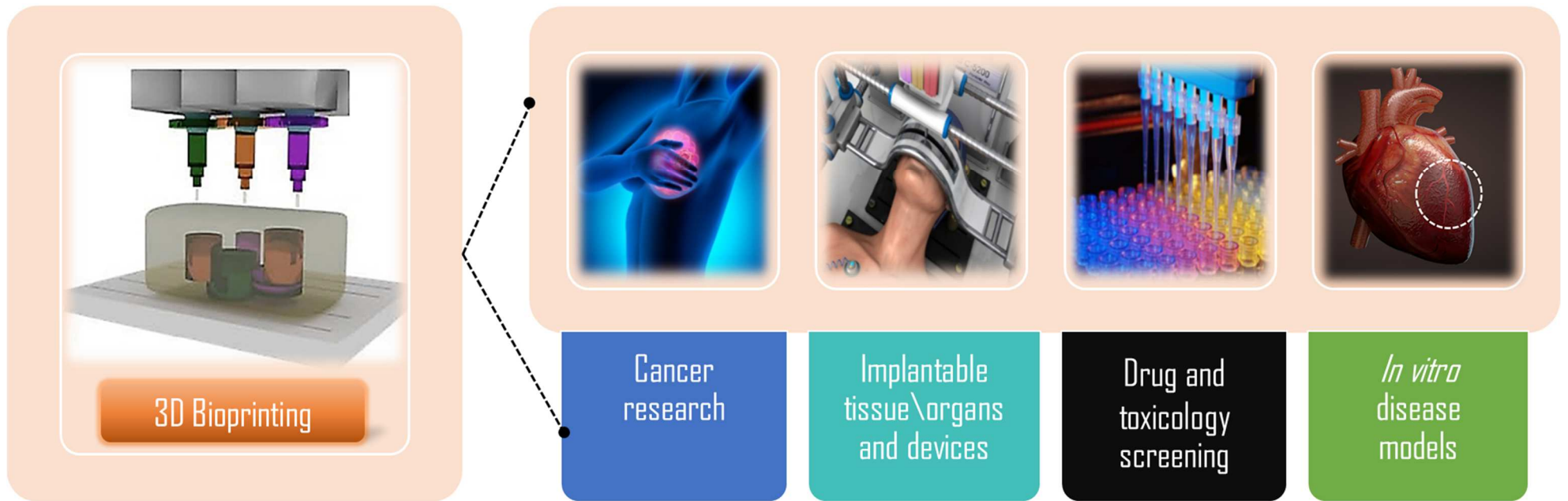


Figure 1. Schematic illustration of the concept of 3D bioprinting technology. This illustration shows the contribution of 3D bioprinting in biomedical research, including cancer models, tissue engineering and regenerative medicine for implantable materials, drug and toxicology screening, and *in vitro* disease models

## 2. 3D bioprinting: A biofabrication technique

3D bioprinting, otherwise known as robotic additive biomanufacturing, is a cutting-edge technology that enables the fabrication of multifaceted 3D biological scaffolds by patterning and assembling living and non-living components, referred to as “bioinks”, in a predetermined 3D architecture [40]. Bioinks include cell aggregates, cells loaded in hydrogels or viscous fluids, cell-seeded micro-carriers, and cell-free polymers (synthetic or natural) that provide mechanical support to the designed scaffold [41]. In 3D bioprinting, the ultimate goal is to replicate the sophisticated architecture of tissues and organs with prior knowledge of the composition and arrangement of its constituents [42][43]. In order to gain a decent understanding of the complex nature of native tissues, various imaging techniques are used to provide a visual illustration of the precise 3D structures, in particular in the design of patient-specific tissues [44]. Medical imaging techniques, such as magnetic resonance imaging (MRI), computerized tomography (CT) and radiological diagnosis, as well as computer-aided design and manufacturing (CAD/CAM) technologies and mathematical modeling are non-invasive tools used to collect and digitalize the complex topographic and architectural information of the body’s tissues [4][43]. Electronic microscopy (TEM/SEM) is useful to gain insight about microstructure and porosity, while immunohistochemistry helps to identify the composition and microorganization of ECM proteins. Ideally, in order to generate an analog of the tissue/organ of interest, the information from all these imaging techniques would be fed into a 3D bioprinter to pattern layer-by-layer the selected bioinks.

### 2.1. Bioprinting techniques: pros and cons

The contributing technologies to 3D bioprinting to create living tissue constructs encompass three complementary technologies: inkjet, microextrusion, and light-assisted bioprinting (Figure 2A). Currently, bioprinting processes are typically conducted in laboratory clean rooms to manufacture the desired scaffold. For some indications, it is envisioned that the process of bioprinting could be performed directly *in situ* on the human body during surgery, which would provide the patients with their own personalized grafted tissue in a short timeframe (Figure 2B&C). The quantitative and qualitative characteristics of each bioprinting technology are summarized in Table 1.

*Inkjet bioprinting:* Inkjet bioprinting is based on a drop-on-demand (DOD) process, whereby droplets of bioink are ejected from a nozzle at very high frequency, induced by a thermal or a piezoelectric actuator [45][46]. The term drop-on-demand refers to the aspect of dispensing fluidic bioink only upon request (or actuation), thus minimizing the loss of bioinks. DOD biofabrication can handle and print small volumes of bioink (1–100 picoliters) at a remarkable resolution, precision, and speed [47][48][49][50]. However, this technique is associated with several drawbacks: it is limited to very low viscosity bioinks and is very sensitive to nozzle clogging by cell or protein aggregates [45][51]. In addition, rapid evaporation of the tiny droplets may require operation in a confined high humidity enclosure.

*Microextrusion bioprinting:* This medium-cost technology is arguably the most widespread system used for bioprinting. It uses either pneumatic (e.g. compressed air) or mechanical (e.g. piston/screw) forces to extrude a continuous filament of bioink [52][53]. Microextrusion

bioprinting is considered as a preferred approach to bioprint nearly all kinds of hydrogels with a wide range of viscosities, as well as high-density cellular aggregates including multicellular tissue spheroids that further self-assemble into the anticipated 3D structure [54][55]. Despite the unifying features of this system, some drawbacks must be taken into consideration, including its relatively low printing speed and low resolution, as well as high cell death induced by shear stress in particular when cells are extruded in viscous fluids [56][57].

*Light-assisted bioprinting technologies:* This category covers two types of nozzle free bioprinting technologies using light. The first is called “Laser-assisted bioprinting” (LAB) where a pulsed laser is used to locally heat a donor surface, which creates a bubble and ejects a droplet of the bioink from the donor film onto a receiving substrate placed parallel to it [58][59][60][61][60][62]. LAB is not as widespread as microextrusion and inkjet systems, but is gaining traction as it offers a number of advantages: the absence of a nozzle enables the use of higher viscosity bioinks and bypasses the clogging issues seen with other technologies [63][64][65]. Moreover, LAB is a high-resolution system which can be fine-tuned by modifying a wide set of parameters including the laser fluence (energy delivered per unit area), the surface tension, the wettability of the substrate, and the thickness and viscosity of the bioink [4][66]. However, some limitations of this system have been reported: the heat produced by the laser can induce cellular stress or aggregation in the construct [60][66]. Furthermore, sedimentation and random distribution of cells in the bioink as well as lengthy fabrication times are other drawbacks of LAB [60].

The second light-assisted bioprinting approach has two main variants, referred to as *SLA (Stereolithography)* and *DLP (Digital Light Processing)*. It is also a nozzle-free system for layer-by-layer biofabrication. In this approach, a UV light source is focused on the surface of a liquid photopolymerizable bioink containing a photoinitiator, inducing its polymerization and layer-by-layer assembly which converts it into a 3D scaffold [67]. In SLA, a scanning laser is used to draw the pattern of each layer. In DLP, a digital micro-mirror device is used to project the full image of each layer onto the photopolymerizable bioink [37]. This technology allows the incorporation of cells embedded in the 3D-patterned hydrogel with a wide range of printable polymers and control over microarchitecture and scaffold features [40]. This technology has demonstrated high resolution, and speed [43]. Still, certain limitations in using this technology are observed [68][69], mainly due to the use of UV light in the 360 nm range, which can result in some cytotoxicity [68][69], although huge progress has been made recently with biocompatible photoinitiators working in the visible light spectrum [70][71][72], which is considerably less cytotoxic.



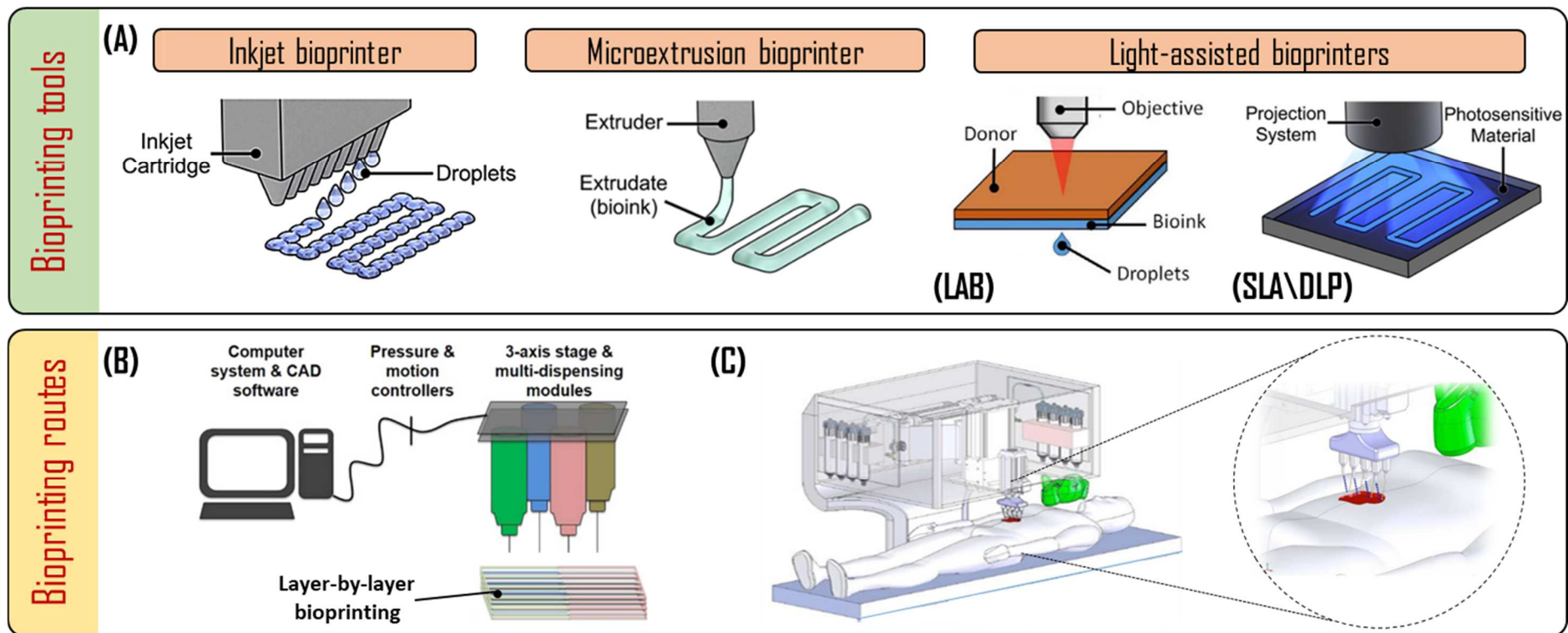


Figure 2. Schematic illustration of 3D bioprinting tools and processes. (A) Technologies used in 3D bioprinting for the biofabrication of tissue constructs and organs. Adapted with permission from [73], copyright 2016, American Chemical Society. (B) 3D bioprinting of the targeted tissue is typically carried out in a laboratory clean room and implanted in the animal/patient after fabrication/maturation. Adapted with permission from [74], copyright 2015, International Society of Biofabrication. (C) For some clinical applications, the envisioned 3D bioprinting process would involve bioprinting *in situ* directly onto the human body during surgery. Adapted with permission from [75], copyright 2019, Nature Publishing Group.

Table 1. Comparison of the features of the different bioprinting technologies

<i>Bioprinting tool</i>					
	<b>Inkjet</b>	<b>Microextrusion</b>	<b>LAB</b>	<b>SLA /DLP</b>	<b>Refs</b>
<b>Print speed</b>	Fast	Slow-Moderate	Moderate -Fast	Fast	[4][5][21][10][76][77][78][79]
<b>Quality and integrity of 'Z' dimension</b>	Low	High	Low-Moderate	Moderate-High	[5][80]
<b>Resolution in xy</b>	Moderate (10-100µm droplets)	Low ( 100-300 µm filaments)	Moderate (~ 10 µm droplets)	High (~ 1.2 µm voxels)	[59][76][77][79]
<b>Cell density</b>	Low (10 <sup>6</sup> cell/ml)	High (cell spheroids 10 <sup>8</sup> cells/ml)	Moderate - High (10 <sup>8</sup> cells/ml)	Low - Moderate (10 <sup>6</sup> cell/ml)	[59][81][82]
<b>Cell viability</b>	High (80 – 90 %)	Low –Moderate – High (40-90%)	High (~ 95%)	Moderate - High (~ 85%)	[5][76] [81]
<b>Bioink viscosity</b>	Very low (≤ 12 mPa/s)	Very high (30-10 <sup>7</sup> mPa/s)	Low (1–300 mPa/s)	Moderate (≤ 2000 mPa/s)	[5][76][81][82]
<b>Process principles and key features</b>	Ejection and deposition of liquid droplets through a pulsed-actuated nozzle. The deposited bioink requires further post-processing to stabilize its structure via chemical or photo-crosslinking curing	A continuous filament is extruded from a nozzle under mechanical or pneumatic pressure. Structures often need to be stabilized further by post-processing (chemical or photo-crosslinking curing). Biocompatible thermoplastics are printed hot and solidify at room temperature.	Nozzle-free system, a laser locally creates a gas bubble that ejects bioink droplets from a donor film onto a substrate and the construct requires further post-processing via chemical or photo-crosslinking.	Nozzle-free system, patterned UV light induces layer-by-layer polymerization of a liquid photoactivable bioink, creating a 3D structure, no post-processing is required.	[45][52][58][69]
<b>Pros</b>	Multiple bioinks can be used simultaneously (in as many nozzles). The equipment is relatively inexpensive	Compatible with the majority of hydrogel bioinks and other highly viscous inks (e.g. cell spheroids, and thermoplastic polymers) and with a wide range of resulting feature dimensions.	Nozzle free system (no clogging), and no thermal or shear stress involved.	Nozzle free system (no clogging), precise multi-layer fabrication. No post-processing needed	[76][80][83][84][85][86]
<b>Cons</b>	Risk of thermal and mechanical stress to cells, requires a rapidly gelling bioink to post-stabilize the structure, and prone to nozzle clogging	Nozzle clogging, high shear stress may lead to reduced cell survival, and the bioink requires post-processing (curing\ solidification) to maintain its shape. Multiple bioinks can only be deposited sequentially.	Expensive hardware, metallic particle contamination, time-consuming, and fabrication limited to a single bioink.	Cumulative UV exposure may induce cell toxicity, Fabrication limited to a single bioink.	[68][69][76][84][85]

## 2.2. The suitable bioink

Bioink designates the mix of biological molecules (for example, proteins or carbohydrates of the extracellular matrix (ECM), growth factors, chemical agonists, etc.) and other natural or artificial hydrogels/polymers with or without cells used in the production of 3D printed constructs. Once printed, the composite material should ideally resemble the extracellular environment of the tissue of interest to enable cell proliferation and differentiation [87]. The development of a bioink formulation is far from trivial and is often a major hurdle prior to bioprinting [88]. Currently, the majority of the commercially-available bioinks are based on native or amended biomaterials used in current TE applications, and there is no universal material particularly intended for bioprinting purposes [53][89]. The selection of a biomaterial for a particular bioprinting application, apart from mimicking the native tissue, depends on several parameters and mainly include printability, rheological properties, and gelation/polymerization mechanisms [90][91][92]. Bioink printability is by far the most important feature. Printability comprises several subparameters including solution viscosity, surface tension, and crosslinking behavior [93]. The viscosity and crosslinking mechanism of the bioink have significant impacts on the mechanical fidelity, cell encapsulation efficacy, resolution, and shape maintenance of the bioprinted construct [93]. High viscosity bioinks are typically processed by extrusion-based bioprinting and they possess a moderate flowing capability enabling the printed materials to maintain their structure for a long time post-printing [94][95]. Yet, they necessitate high pressure to be extruded, which limits the size of the nozzle and can harm the encapsulated cells. Low viscosity bioinks are usually deposited by inkjet bioprinting, as these liquids can be ejected through thinner nozzles and break down into droplets at high frequency which leads to higher printing resolution. However, low viscosity bioinks limit the thickness of the printed object and eventually the structural fidelity. Therefore, just after exiting the nozzle, the bioink needs to be further processed to gelate and/or solidify in order to maintain the printed shape and architecture [96]. This step can be difficult to control and often leads to clogging of the bioprinter nozzle [97][98]. Moreover, just like all biomaterials used in traditional TE approaches, bioinks must comply with the general requirements of the biological or clinical application: these substances must demonstrate adequate biocompatibility, tolerance and biodegradability properties [93]. For instance, in clinical applications, clinical-grade bioinks must be formulated to present degradation profiles that are suitable with the time needed for tissue regeneration without releasing any toxic degradation products [54]. Figure 3 summarizes the critical features of bioinks which determine their suitability for a given 3D bioprinting application.

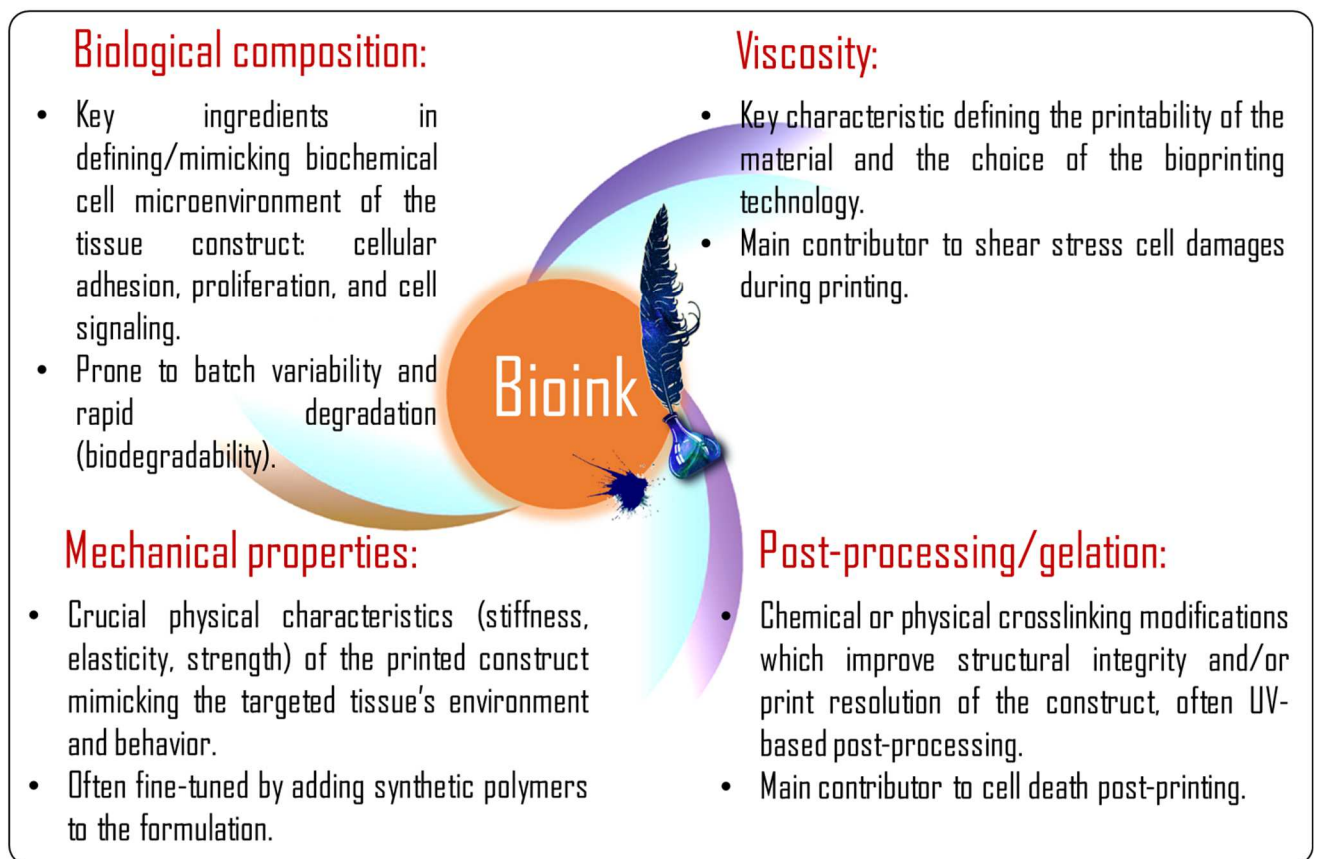


Figure 3: Key properties that determine if a bioink is an appropriate candidate for the 3D bioprinting.

Different types of bioinks are used to bioprint tissue constructs. For instance, bioinks based solely on cells have been used in scaffold-free approaches, mimicking the embryonic development [99][100]. In this approach, cells in the form of tissue spheroids, cell pellets, or tissue strands, can be set to a particular pattern where they undergo tissue fusion and maturation for the production of larger-scale functional tissues [100]. In the absence of a polymer carrier, the issues of cytocompatibility, biodegradability, degradation products and other drawbacks related to the polymer features are circumvented. The 3D arrangement of cells in the scaffold-free system, comparable with native tissues, also provides good communication between cells and facilitates both cell survival and cell function. However, these constructs require the preparation of a massive amount of cells to create the tissue, and exhibit weak fidelity and structure.

Alternately, hydrogels are currently the first choice and the most appealing bioink candidates used for cell encapsulation and printing [101]. Hydrogels are polymers capable of absorbing and retaining large volumes of water or biochemical solutions, enabling living cells to reside and sustain their function [102][103]. The natural properties of hydrogel-based bioinks (high water content, cytocompatibility, biodegradability, high porosity, softness, and ability to serve as a growth medium) make these materials the ideal candidates to mimic the ECM of human tissues in cell printing [99]. Many studies have investigated the use of hydrogels for bioprinting applications [104][105][106][107], in particular their printability which often

requires some form of compromise between all the different bioink and bioprinting requirements, e.g. rheological and crosslinking properties [105][108].

Hydrogels can either be of natural or synthetic origin [109]. Natural hydrogels are materials derived from natural polymers and display biocompatibility, biodegradability, and inherent signaling molecules for cell adhesion, proliferation, and differentiation [110][111]. These features make natural hydrogels particularly appealing for TE applications mainly due to their biochemical resemblance to the human ECM and therefore are inherently acceptable for bioprinting. Some of the widely reported natural hydrogel-based bioinks include alginate [112][113][114], chitosan [115][116][117], gelatin [118][119], collagen [120][121][122][123], silk [124][125][126], hyaluronic acid (HA) [127][128][129][130], dextran [127][131][132], and fibrin [133][134][135]. However, natural hydrogels may exhibit uncontrolled swelling, weak fidelity, and non-tunable degradation profiles [111].

Unlike natural hydrogels, synthetic hydrogels can be fabricated with defined control over their structure and function [110]. Some of the reported synthetic hydrogel-based bioinks include polyethylene glycol (PEG) [136][137][80][138], pluronic [139][140], and polylactic acid (PLA) [141][142], etc. Synthetic hydrogels can be fabricated with an extended half-life, tailored functionalities (such as release of growth factors [143][144][145]), tuned degradation profiles, adequate water absorption, and appropriate mechanical strength [146][147]. For example, huge progress has been made to fabricate synthetic hydrogels with tailored biodegradation profiles [103][148]. For instance, protease-sensitive PEG-based hydrogels can be fabricated by the inclusion of protease-targeted cleavable domains onto the PEG backbones [149][150][151]. This strategy to fine-tune hydrogel degradability in response to a cell-induced enzymatic remodeling can aid in the enhancement of cell infiltration, migration, and tissue vascularization. Some drawbacks of synthetic hydrogels include their strong propensity to stimulate inflammatory and immunological responses and to release toxic metabolites during degradation [111][147]. Thus, semi-synthetic hydrogels have attracted increasing interest in the bioengineering sector because they exert common structural and functional features of both the natural and the synthetic hydrogels. For example, methylcellulose [152][153][154][155], and gelatin methacrylate (GelMA) [156][157], are fabricated from natural polymers by grafting them with different synthetic substituents [158][159][160].

In summary, there is no ideal type of hydrogel that can fill all the critical requirements needed for bioprinting. There is rather a myriad of possibilities to choose from, making this research area equally exciting and challenging. Figure 4 provides representative cases of diverse natural and synthetic hydrogels used in 3D bioprinting. For a more detailed and comprehensive overview of hydrogels and their properties for applications in the TE and bioprinting area, we refer the readers elsewhere [103][161][162].

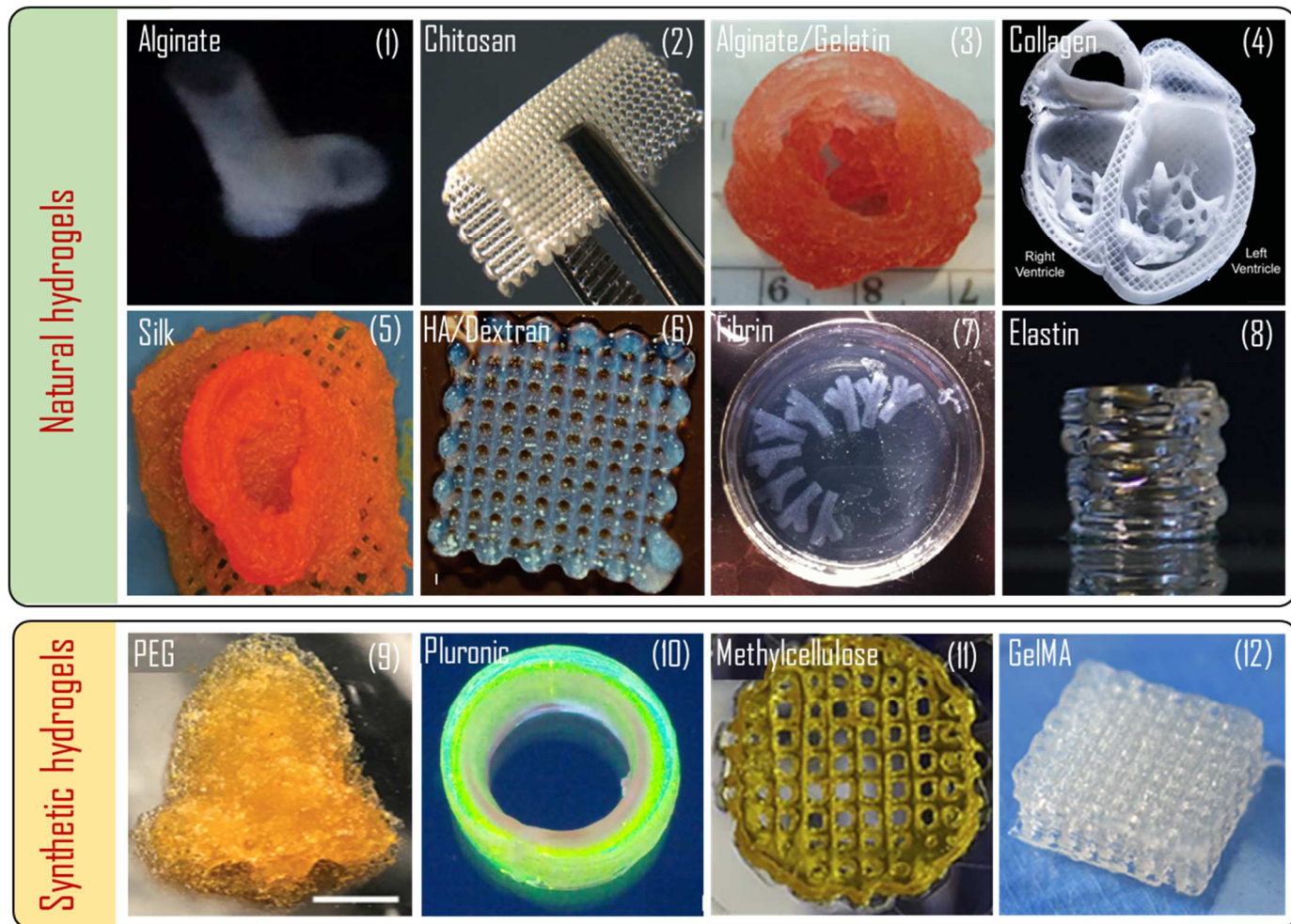


Figure 4: 3D bioprinting examples of different natural and synthetic hydrogels. (1) 3D printed bifurcated alginate tube. Adapted with permission from [114], copyright 2015, International Society of Biofabrication. (2) microstructured and stretchable chitosan hydrogel fabricated with a 100  $\mu\text{m}$  micronozzle and folded using a tweezer. Adapted with permission from [117], copyright 2017, Wiley-VCH. (3) bioprinted aortic valve conduit using an alginate–gelatin hydrogel mix. Adapted with permission from [119], copyright 2013, Society for Biomaterials. (4) A cross-sectional view of the collagen heart. Adapted with permission from [121], copyright 2019, American Association for the Advancement of Science. (5) 3D bioprinting of self-standing ear-shaped construct using silk-based bioink. Adapted with permission from [125], copyright 2018, Wiley-VCH. (6) Hyaluronic acid and dextran hydrogels fabricated by extrusion bioprinting. Adapted with permission from [127], copyright 2011, American Chemical Society. (7) 3D bioprinted Y-shaped fibrin tubes. Adapted with permission from [135], copyright 2015, American Association for the Advancement of Science. (8) genetically engineered elastin-like recombinamers with sequence-based molecular stabilization used as bioink for 3D bioprinting. Adapted with permission from [163], copyright 2020, Elsevier. (9) 3D printed nose from a PEG microgel. Adapted with permission from [138], copyright 2019, Royal Society of Chemistry. (10) Bioprinted Pluronic F-127 fluorescent tube illuminated with UV light. Adapted with permission from [140], copyright 2015, International Society of Biofabrication. (11) 3D printed methylcellulose-based scaffold. Adapted with permission from [152], copyright 2020, Elsevier. (12) 3D printed GelMA. Adapted with permission from [157], copyright 2018, American Chemical Society.

Nevertheless, an important drawback with the use of hydrogels, either natural or synthetic, is their inherent fragility. The main issue encountered when bioprinting hydrogels is the difficulty to shape them into complex geometries and to keep their structural integrity post-printing. Hence, sustaining and enhancing the fidelity of hydrogel scaffolds has become a

crucial matter in 3D bioprinting. For example, some approaches of toughened hydrogels (e.g. double network hydrogels [164], nano-composite hydrogels [165], and ionic covalent entanglement hydrogels [166]) have been investigated. Unfortunately, the fabrication of toughened hydrogels involves complex and in many cases multi-step chemical synthesis, which complicates their use in bioprinting. Recent strategies known as hybrid hydrogel bioinks have been explored to address these issues and improve hydrogel-based bioink formulation. Hybrid hydrogel bioinks are composed of chemically, functionally, and morphologically distinct building blocks, proteins, peptides, polysaccharides on one hand, and thermoplastic polymers on the other, joined by physical or chemical means. The hydrogel compartment provides an ECM-like environment to facilitate cell response and the thermoplastic polymer adds support to the scaffold and incorporates functional domains for tunable biodegradation [167]. Recently, many advances in hybrid constructs have been explored in the bioprinting field to fabricate a large range of tissue constructs including liver [168], cartilage and bone [169], as well as blood vessels [170], etc. For a comprehensive overview of the synthesis of hybrid hydrogel bioinks and applications in the bioprinting area, we refer the readers elsewhere [167].

Finally, bioprinting using exclusively curable polymers rather than hydrogels and/or cells could be an appealing approach to guarantee optimal fidelity and durability to the designed scaffold after fabrication [171]. However, these materials require a harsh processing environment (organic solvents, high temperature, toxic curing agents, UV, etc.) that is not compatible with living cells [41]. Cells can therefore only be seeded onto these materials post-printing to prevent the induced cytotoxicity [172][173], which means that cells will require much more time to invade, colonize and regenerate the targeted tissue. We are therefore convinced that a hybrid construct integrating the foremost features of hydrogels, cells, and thermoplastic polymers would be the most promising approach to bioprinting an esophageal substitute.

### 3. Tissue engineering for esophageal repair

#### 3.1. The esophagus: physiology, pathology and remediation

The esophagus is an active muscular tube which transports food from the pharynx to the stomach. In humans, it is a hollow organ of 18 to 26 cm long that stretches between the upper and lower esophageal sphincters [174]. The sphincters are muscle rings whose main role is to avoid food moving backwards [175]. During swallowing, the esophageal lumen can distend to about 2 cm in the anteroposterior direction and 3 cm laterally to accommodate the food bolus [176]. The esophageal wall is composed of four distinct layers: mucosa, submucosa, muscularis propria, and adventitia (Figure 5) and three main cell types: epithelial cells, fibroblasts, and smooth muscle cells [176]. The mucosa is the thick innermost layer, exposed to the esophageal lumen and its inner surface is covered by squamous epithelium. The

submucosa is rich in elastin and collagen and comprises a thin film of connective tissue, lymphocytes, nerve fibers, a vascular network, and salivary glands linked via ducts to the esophageal lumen to lubricate and facilitate the transport of food. The collagen gives structural support and elastin is organized circumferentially and longitudinally, which promotes the stretching of the esophagus during the passage of the food bolus and supports the propagation of a peristaltic motion towards the stomach [177], which itself occurs as the result of the progressive wavelike contraction of the muscularis propria, a layer composed of striated and smooth muscle cells [178]. The adventitia is the outermost layer which covers and links the esophagus to the adjacent tissues. It is made of loose connective tissue and comprises small vessels, lymphatic channels, and neurons [176]. Various arteries among which the inferior thyroid artery, the left gastric artery, the left phrenic artery, and the aortic esophageal arteries provide blood supply to the entire esophagus [179]. Finally, the esophagus is an innervated organ with both parasympathetic and sympathetic innervation to control glandular secretion, blood vessel capacity, and the motion of striated and smooth muscles [180].

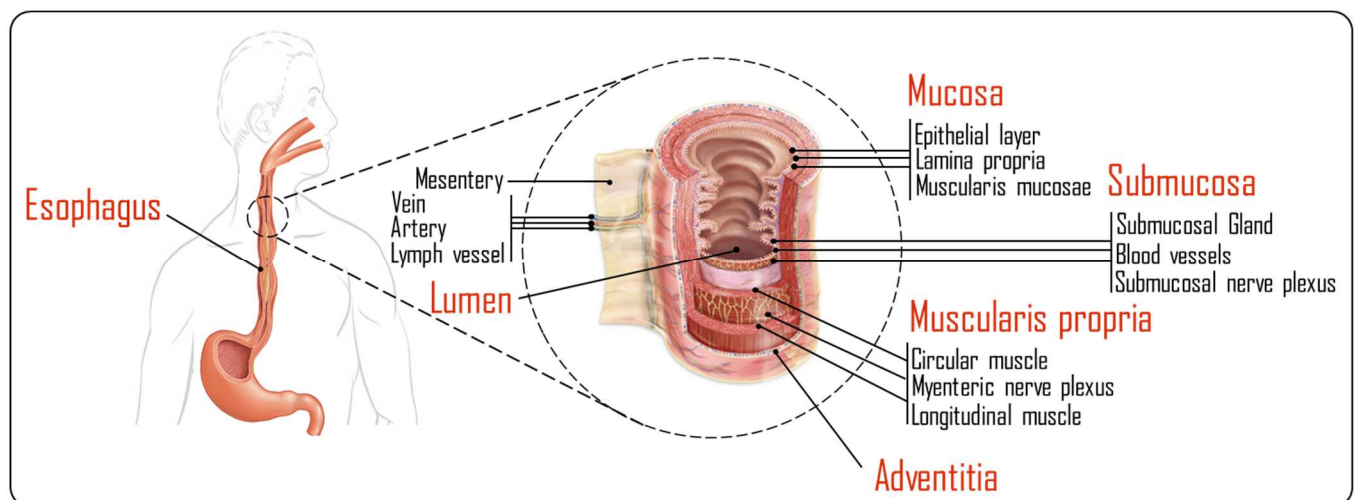


Figure 5: The anatomy of the esophageal tissue showing its four concentric layers and sublayers.

The esophagus can be the target of a myriad of congenital or acquired diseases which are associated with high morbidity and mortality rates worldwide [181][182][183]. For instance, Barrett's esophagus, a condition of metaplasia affecting the lining of the esophagus, is associated with an increased risk of developing esophageal adenocarcinoma [184], estimated to occur in about 10% of cases [185] with a 5-10% five-year survival rate in patients that do not receive treatment at the earliest stages [186]. Esophageal cancer is one of the deadliest cancers and is considered as the sixth leading cause of death from cancer worldwide [187][188]. Other esophageal anomalies include tracheoesophageal fistula and atresia, congenital esophageal stenosis, congenital esophageal duplication, and esophageal stricture, all of which are also associated with a substantial risk of morbidity and mortality [25][189][190]. The human esophagus has a poor ability to regenerate and the most common response after injury is inflammation, resulting in stricture, loss of function, and cancer development [191]. Regenerative capacity of a given organ is linked to the existence of reservoirs of stem and progenitor cells, which are scarce in esophageal tissue [192], except



for progenitors of epithelial lineage present in the basal side of the mucosal layer, and whose role are to continuously replace the epithelium of the mucosal lining [193].

Current techniques of esophageal replacement after esophagectomy using parts of the gastrointestinal tract (e.g. stomach, colon, or jejunum) or other autologous tissues (e.g. skin) are associated with high morbidity (e.g. poor functional results) and mortality [194][195]. Long-term quality of life is frequently impaired by reflux, delayed conduit emptying, and dumping syndrome [196][197][198]. The option of resorting to donor esophageal transplantation is not considered clinically relevant as vascularization of the esophagus has numerous origins, in contrast to the liver or the kidney, which have a single vascular pedicle to anastomose [199]. ETE is therefore considered as an alternative therapeutic approach that aims to substitute or reestablish the structural and physiological activity of the damaged or injured [200].

### 3.2. 3D Bioprinting in Esophageal Tissue Engineering

To replace a damaged esophagus, an ideal substitute ought to be implantable without eliciting immunological rejection, provide support for cell adhesion and proliferation, exhibit optimum porosity with suitable pore size for cell invasion, replicate the esophageal layers structure and function, in particular be soft and elastic to allow the passage of food and propagate peristalsis, have a lubricating capacity, be able to endure the acidic reflux of the gastric fluid, be suturable, and maintain the luminal topography without stenosis post-operation [201][202]. Moreover, controlled scaffold biodegradability *in vivo* post-implantation is considered a major advantage in advanced TE and regenerative medicine. Ideally, biodegradable esophageal substitutes would act as temporary templates for the regeneration of a biologically functional esophagus, and would be slowly replaced by *de novo* esophageal tissue, thus avoiding subsequent surgeries, in particular in pediatrics during a young patient's development and growth. The artificial esophagus would thus need to be biodegradable with a controlled degradation profile to compensate the regeneration rate of the tissue [203] while also decomposing through hydrolytic/enzymatic processes into harmless byproducts.

ETE studies have explored promising strategies for esophageal regeneration and several materials have been identified as efficient artificial supports to regenerate the esophagus *in vitro* and *in vivo*. Esophageal reconstruction was reported using resorbable materials [204], acellular matrices [205], decellularized patches [206], and implants of natural or synthetic polymers [207]. Prior to the introduction of the TE concept by Langers and Vacanti in 1993 [208], diverse efforts were done to restore the function of the diseased esophagus. For instance as early as 1887, Charles Symonds was the first to report the use of an endoluminal esophageal tube made of ivory and silver for the treatment of a malignant esophageal cancer [209]. Attempts to replace a portion of the esophageal tissue using synthetic polymers were reported *in vivo* since 1945 [210] and 1952 [211]. In his work, Edgar Berman explored the use of polyethylene tubes in a dog model to reestablish the continuity of the esophagus after resection [211]. Even though the outcome was unsuccessful, this study opened doors for further investigations in the domain of ETE and allowed to identify the main challenges such as anastomotic leakage, tissue stenosis, biocompatibility, and controlled degradation.

Today, TE for esophageal replacement is a rapidly growing research field as reflected by the increasing number of citations in this discipline (Figure 6). Part of this growth stems from the need to reproduce the structural and functional integrity of the esophagus by using cytocompatible and biodegradable scaffolds to abolish the need of autografts or allografts, which diminish the number and complexity of surgeries associated with classical treatment methods, and perhaps reduce the surgery-related complications. The most recent developments in ETE include a variety of tissue decellularization strategies including decellularized esophagus [39][212], decellularized small intestine [213], acellular dermal grafts [207][214], as well as other tissues or organs [215]. Tissue decellularization is a procedure that enables the suppression of cellular and immunogenic substances while maintaining the ECM topography and biomechanical features of the native tissue intact [216]. Decellularized matrices can avoid immunogenicity and allow cell adhesion, migration, proliferation and differentiation. However, severe donor shortage, deficiency of properly sized cadaveric donors, poor mechanical strength, and fast degradation profiles could ultimately limit the use of these matrices in ETE [38]. In parallel, polymeric (natural or synthetic) based scaffolds have been extensively explored to construct esophageal substitutes using a wide variety of fabrication techniques [217][218][219][220][221]. These methods (including electrospinning [222], cell-sheet technology [223], and mold-casting [224]) typically use supporting substrates (e.g. rotating rod, sacrificial mold) to build a tube-like esophageal structure and involve complex and multiple fabrication processes. Due to technical constraints, these methods have little freedom in terms of shape and size and lack fine control of mechanical strength [225]. Furthermore, classical scaffolds with a random distribution of cells, polymers, bioactive cues, and other biological components do not reflect the complexity of native esophagi. In general, all these approaches continue to face several obstacles and post-operative issues including anastomotic leakage, poor reepithelization, lack of muscle regeneration, infection and immunogenic responses, strictures in long-term implantation [214]. Thus, as will be discussed in the context of this report, ETE approaches exploiting the promising features of 3D bioprinting may provide personalized therapeutic alternatives to regenerate the injured or damaged esophagus.

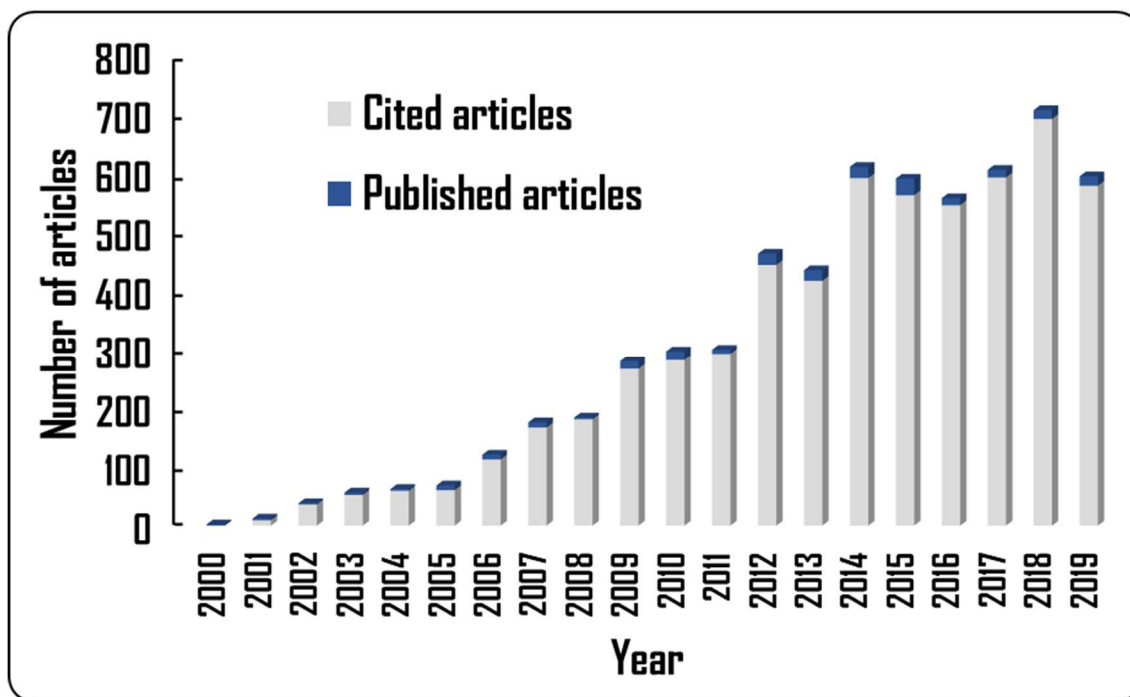


Figure 6. The number of published and cited articles related to tissue engineering for esophageal repair from 2000 to 2019 (Data were extracted from Web of Science with the search terms esophageal tissue engineering, collected in 2020).

As described earlier, the esophageal tissue has a complex structural anatomy and histology, with four distinct layers. Therefore, a perfect substitute for functional and structural repair must be designed as a composite multilayered material with cylindrical architecture and cell-accommodating microstructure. It is reported that the structural and physicochemical features (topography, pore size and thickness) of the artificial esophagus play a critical role in supporting nutrient diffusion, cell adhesion, invasion, tissue vascularization, tissue regeneration and orientation, as well as in limiting fibrosis [226][227][228][229][230]. For ETE applications, fibrosis must be as low as possible in order to avoid stricture and preserve the peristaltic movement and mechanical activity of the esophagus [226]. Thus, according to these studies, the scaffold should have interconnected pores with a pore size of about 150 to 250  $\mu\text{m}$  to facilitate cell seeding, transport of cells nutrients and waste, enhance cell proliferation and stratification of the epithelium [231][232]. In addition, others have reported that the outer layer of the esophageal substitute should bear relatively larger pore sizes, above 400  $\mu\text{m}$ , to facilitate the invasion of blood vessels and tissue vascularization [233][227].

Furthermore, the flexible and elastomeric features of the native esophagus must be reproduced in the artificial scaffold to support peristaltic propagation. The esophageal substitute should be distensible, but not flaccid, in order to accommodate the frequent cyclic stretching by the food bolus while swallowing [234]. It is reported that the human esophagus has an average maximal strength of 2.12 and 1.4 MPa for the longitudinal and radial directions, respectively. Its ultimate strain was estimated to be about 70 and 82.5 % in the longitudinal and circumferential directions, respectively [235]. Another study revealed that maximal longitudinal stress and strain for the human esophagus were about 1.2 MPa and 140 %,

respectively [236]. Finally, the esophageal substitute should demonstrate good handling and suturability properties in order to facilitate surgical implantation and prevent leakage [237].

3D bioprinting is a very recent approach in ETE, and Park *et al.* published one of the earliest noteworthy attempts in 2016 to fabricate an artificial scaffold to reconstruct partial esophageal defects [238]. In this study, an extrusion-based bioprinter was used to build a grid structure of polycaprolactone (PCL), a FDA approved biocompatible and bioresorbable polymer with rheological parameters compatible with printing, forming 5 mm diameter/10 mm high acellular cylindrical scaffolds with a wall thickness of 2 mm. A subset of these scaffolds were seeded with rabbit mesenchymal stem cells (rMSCs) in fibrin. The grafts were then implanted into the esophageal defect site in rabbits and evaluated for their performance. Three weeks after implantation the grafts did not show evidence of leakage, stenosis, infection, or graft rejection. Unlike the acellular scaffolds, the cell-seeded grafts were totally covered by regenerating mucosal epithelium and smooth muscle cells [238]. This investigation shows great promise for PCL to fabricate a 3D printed esophageal substitute and for the use of allogenic MSCs to enhance host cell migration and colonization. However, their study was limited to the repair of small and partial esophageal defects whereas circumferential reconstruction would be more clinically relevant while requiring longer constructs.

Simultaneously, in 2016, Tan *et al.* proposed a melt-drawing approach to fabricate tube-like objects with enhanced features for applications in ETE [239][240][241]. Melt-drawing is a bioprinting tool that uses a rotating mandrel with a predetermined diameter and rotational speed to continuously collect molten fibers. In one of their studies [240], they deposited poly(L-lactide-co- $\epsilon$ -caprolactone) (PLC) polymer melt onto a rotating mandrel to fabricate a hollow tube of 3 cm internal diameter. The thickness of the tube could be adjusted by changing the number of layers of the melt-drawn structure [241]. The PLC was melted at 150 °C and the mandrel rotation speed was varied between 150 and 2400 rpm. Increasing the rotation speed led to a reduction in the PLC fiber diameter (from 66  $\mu$ m to 14  $\mu$ m) and this modified the microarchitecture of the fabricated constructs as well. Analysis of the tubes' mechanical behavior showed that their tensile properties were comparable to those of native esophagi. Fibroblast cells were seeded onto the scaffolds and cell proliferation assays revealed a high ability of the seeded cells to adhere and proliferate on the substrate. A live/dead cell viability assay showed more than 90% cell viability 6 days post-seeding. In this study, the fabricated objects showed promising features for ETE. However, the architectural design of these constructs are limited by the dimensions of the rotating mandrels, and impose a smooth internal surface in order to remove it off the mandrel. Overall, this means less freedom in designing personalized esophageal constructs, in particular if more complex designs than a cylinder are required.

In 2018, Chung *et al.* reported a more sophisticated design using a similar 3D melt-extrusion method [242]. In this study, PCL was deposited as a filament onto a rotating mandrel to first form several 1mm wide rings slightly spaced apart (Figure 7A). PCL nanofibers were then electrospun over the fabricated rings on the rotating mandrel, leading to a tubular-like scaffold of about 5 mm in length and 1.6 mm in diameter. Structural analysis showed that the average pore size of the scaffolds was about 5.1  $\mu$ m and its ultimate tensile stress and yield stress were higher than that of the native rat esophagus, while the elastic modulus appeared to be similar.

The PCL-based scaffold was also able to sustain the survival and proliferation of fibroblast cells *in vitro*. The tubes were then implanted into the omentum of rats for two weeks to induce cellularization and vascularization of the materials. The scaffold was then orthotopically implanted into surgically generated circumferential esophageal defects in the same animal. All rats died prior to- or were sacrificed on the 15th day post-surgery. Histological analysis via H&E staining indicated the neoformation of blood vessels on its exterior and revealed that the host cells were able to infiltrate the construct and spread throughout its inner and outer membranes, confirming that such a construct supports cell migration and survival as well as tissue vascularization. After implantation at the defect site, the esophageal substitute retrieved full healing by the second week and the repaired esophagus showed no evidence of fistula, perforation, or necrosis. Furthermore, loosely arranged vascularized tissue attached to the interior and exterior of the implant was visible. However, very few cells were able to infiltrate the nanofibrous scaffold, most likely due to the relatively small pore size. Epithelialization at the proximal and distal anastomoses was visible after 15 days, but its progression was stopped by the internal PCL rings [242]. This study illustrates again, as many groups already have shown in TE approaches, that implantation in the omentum is an efficient step to stimulate vascularization before moving the implant in the defected site. This increases cell colonization of the graft in the early phases via the diffusion of nutrients and oxygen and limits graft rejection [243].

In another study, Tan *et al.* [244][245] used 3D bioprinting by extrusion and developed hybrid hydrogel/cell/polymer bioinks to print cell-laden cylindrical constructs for ETE. Poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres were seeded with cells (Cell Laden Microspheres or CLMs) and encapsulated in an agarose-collagen hydrogel blend (AC hydrogel) (Figure 7B). This bioink was deposited by an extrusion bioprinter to fabricate imbricated ring-like structures of 1.5 cm diameter and 0.55 cm height. Various cell types could be cultivated as CLMs, including fibroblasts, epithelial cells, myoblasts, smooth muscle cells, and human mesenchymal stem cells (hMSCs). Results indicated that the microspheres have a great capability to stimulate the cells to attach and proliferate in a pre-printing step, and supported excellent cell viability 14 days after printing. The AC blend hydrogel facilitated printability of CLMs, by reducing friction, and allowed rapid gelation of the bioink post-printing. Furthermore, the AC blend hydrogel provided a good microenvironment resembling the ECM of the epithelium. Tight packing of the CLMs within the AC blend hydrogel increased the compressive strength of the fabricated bioink to about a 100 times that of pure AC hydrogel, illustrating the point we made in the section about hybrid hydrogel bioinks (section 2.2) where the addition of supporting materials to the formulation brings extra strength to the overall construct. The hMSCs kept their stemness-associated features post-printing, and this can be exploited as an alternative to epithelial cell encapsulation in order to encourage epithelium regeneration by the host cells once the fabricated scaffold is implanted *in vivo*. While this is a pioneering study for ETE using cell-laden bioinks, the authors reported the fabrication of very short constructs, in the range of 0.55 cm high, raising the issue of the construct collapsing if longer segments were to be printed. This is in fact a recurring issue when 3D printing complex hollow architectures with voids, which can lead to collapse of the

structure if appropriate steps are not taken with sacrificial structures or supporting environments such as a slurry suspension bath [21][246][247][248][249].

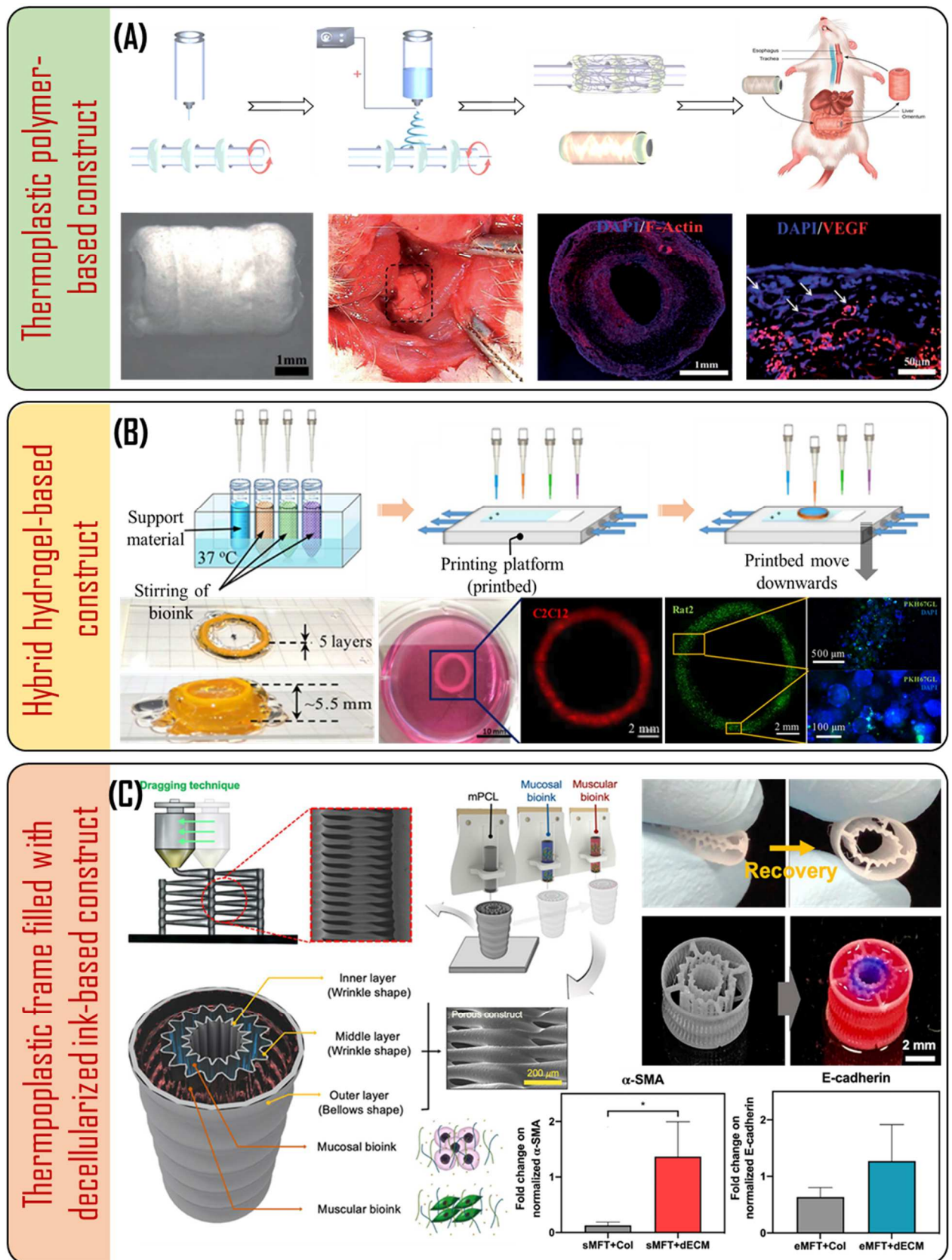


Figure 7. Additive manufacturing in ETE. (A) Design of an esophageal substitute reinforced by 3D-printed rings, from top-left to bottom-right: The fabrication process of the polymer-based esophageal scaffold using melt-drawing technology and its implantation into a rat model after maturation in the omentum; Results of this study showed the fine structure of the designed material before implantation, the orthotopic-cultured esophageal substitute implantation into the defected esophageal site, the cross-section image of DAPI/F-Actin staining and revealed that the host cells infiltrated the scaffold along the inner/outer walls, and an image at higher magnitude of the DAPI/VEGF staining which revealed that neovascularization had occurred in the implanted scaffold. Adapted with permission from [242], copyright 2018, Taylor & Francis Group. (B) 3D bioprinted multicellular scaffold with a hybrid bioink, from top-left to bottom-right: Illustration of the automated bioprinting system, images of the 3D bioprinted tubular constructs, fluorescence image of ring built with red-labelled C2C12 CLMs, fluorescent image of ring built with green-labelled Rat2 CLMs after 3 days of culture. Fluorescent signals proved that the cells were still viable. Blue fluorescence images show DAPI staining of the sample. Adapted with permission from [244], copyright 2016, Nature Publishing Group. (C) Multi-layered Free-form tubular scaffold with decellularized inner and outer esophageal tissue-derived bioinks, from top-left to bottom-right: Illustration of the bioprinting process via the fabrication of a multilayered free-form 3D tubular construct using a dragging technique, then filled with decellularized inner and outer esophageal tissue-derived bioinks, flexibility of the frame printed construct showing recovery to its original structure after an induced stress, images of the frame with and without decellularized bioink filling (mucosal bioink stained by blue and muscular bioink stained by red for the distinction), expression of alpha-smooth muscle actin ( $\alpha$ -SMA) and E-cadherin, respectively. Adapted with permission from [250], copyright 2020, Nature Publishing Group.

More recently, in 2019, Kim *et al.* investigated the use of 3D bioprinting to repair a circumferential esophageal defect in a rat model [251]. In this study, a structural and functional substitute mimicking the native esophagus was obtained by building a two-layered tubular scaffold to mimic the mucosa and the muscle of the esophagus. The inner membrane of the tubular scaffold was fabricated by electrospinning polyurethane nanofibers on rotating mandrels. PCL was then deposited on the polyurethane layer by melt extrusion to form the outer membrane of the scaffold. The internal layer of electrospun nanofibers efficiently mimicked the mucosal layer and facilitated cell infiltration and proliferation, while the outer layer had micron-size fibers, which gave the material the desired fidelity and flexibility; thus mimicking the functionality of the muscle in the native esophagus. Human MSCs were seeded on the inner membrane of the scaffold with the aim to improve epithelialization and stimulate mucosal regeneration either by prior incubation in a bioreactor, or by maturation in the omentum, or directly implanting the seeded substitute [252]. Furthermore, substitutes were covered with a thyroid gland flap to stabilize the graft and provide a vascular supply to the implanted site. This resulted in increased survival compared to the group where implantation was done without a thyroid gland flap, and where fistulas at the anastomosis site were observed. The omentum and bioreactor groups showed 80% regeneration of the mucosal layer and partial regeneration of the muscle layer compared to the MSC-seeded substitute without prior maturation. This study demonstrated a particularly efficient solution for the repair of circumferential esophageal defects by mimicking the structure of the natural esophagus and promoting regeneration of the mucosal and muscle layers. They demonstrate in particular the efficiency of a prior maturation step that can be carried out in an external bioreactor, which proves to be just as efficient as the usual maturation in the omentum,

without the clinical disadvantages. However, we believe that further attention to the biodegradation of the scaffold should have been considered. In fact, polyurethane, the inner layer of the scaffold described in this study, is barely biodegradable. Lin *et al.* reported in a biodegradation test, that after 16 weeks of incubation in either neutral PBS or acid medium, the total weight loss of the polyurethane esophageal stent was only about 1.5% [253]. Thus, the non-biodegradable feature of the designed two-layered artificial esophagus will limit its use in ETE applications. In order to overcome this complication, other biocompatible and biodegradable alternatives to polyurethane comprising natural and synthetic polymers deserve to be explored in this approach.

Very recently, in 2020, Nam *et al.* reported a multi-layered esophageal substitute fabricated by extrusion-based 3D bioprinting (Figure 7C) [250]. In this investigation, a PCL-based hollow Multi-layered Free-form porous Tubular (MFT) construct was printed using a method called ‘the dragging technique’. This technique uses the stretching phenomenon of viscoelastic polymers and prints filaments of material with decreasing widths and pore sizes in a single extrusion step. The authors clarify that the key advantage of the dragging technique is its ability to design MFT constructs with an adjustable pore structure. The different layers of the fabricated MFT construct were then filled with decellularized bioinks derived from mucosal (between the inner and middle membranes of the MFT) and muscular (between the middle and outer membranes of the MFT) layers of the native esophagus. Results indicated that the MFT is a porous 3-layer construct with significantly smaller pores on the outer layer (about 90  $\mu\text{m}$  in size) in comparison with pores of the middle (about 340  $\mu\text{m}$  in size) and the inner layers (about 250  $\mu\text{m}$  in size). Here, the pores of the inner and middle layers were designed to enhance cell migration and to provide an effective nutrient supply; in contrast, the small pores of the outer layer were designed to allow a steady influx of nutrients and to reduce the capability of soft tissue growth on the outside of the construct. The mechanical features of the fabricated construct were comparable with the native esophagus, suggesting its ability to withstand the peristaltic movement once implanted *in vivo*. Moreover, the use of specific bioinks derived from different decellularized esophageal layers is very relevant to induce tissue-specific cells phenotypes. In this approach, the decellularized mucosal bioink enhanced epithelization, and the decellularized muscular bioink induced the proliferation of muscle cells. This study proves to be a very effective approach to replicate the architecture and performance of the esophagus. However, additional analyses are needed in order to evaluate the translation of such a system into clinical practice, for example by testing the suturability to the remaining parts of the native esophagus.

In another recent study, Takeoka *et al.* used a less conventional bioprinting technique known as the kenzan method to construct a multicellular esophageal substitute (Figure 8A) [254]. The kenzan method is a scaffold-free bioprinting technique described by Koichi Nakayama which involves placing cell spheroids one by one on an array of fine needles according to a predefined plan, and to let them fuse together to form a tissue [255]. In the work of Takeoka *et al.*, a number of multicellular spheroid groups were produced from distinct cell types by mixing different ratios of normal human dermal fibroblasts,



human esophageal smooth muscle cells, human bone marrow-derived mesenchymal stem cells, and human umbilical vein endothelial cells, and these were used to build an artificial esophagus by kenzan bioprinting. The tube-like structures were cultured in a bioreactor for 7 days during which the spheroids fused together, the structure was then removed from the array of needles and incubated in a second bioreactor for an additional three weeks. [254]. Mechanical testing showed that the constructs with the highest ratio of MSCs had superior mechanical properties and showed enhanced expression of  $\alpha$ -smooth muscle actin and vascular endothelial growth factor in comparison with the other groups. The constructs from this group were implanted in rats as a secondary route between the lower part of the esophagus and stomach using a silicone stent and left *in vivo* for 30 days. After histological analysis, these constructs showed complete epithelization of the luminal surface and were capable of transporting food. The epithelialization of the inner surface is of great importance and is considered critical for sustaining the functional integrity of the esophagus. Despite the successful outcome of this pioneering method in fabricating an epithelized esophageal substitute, some limitations have yet to be addressed. For instance, the authors reported a lower mechanical strength in comparison with the native esophagus (0.3 N in tensile strength, instead of 0.5 N for the native esophagus), which will therefore limit the durability of these structures. Furthermore, this approach requires a non-biodegradable stent for transplantation, which as discussed earlier, will hinder its use in medical trials, mainly in pediatrics, with the risk of stent migration already reported in existing studies [256][257][258]. Moreover, a huge number of cells are required to create a human-sized esophageal construct and this is not very feasible in practice, primarily due to sourcing the cells (ideally from the patient), the prolonged time to obtain the needed number of cells and restrictions in the number of passages when manipulating primary cells. To solve these issues, a hybrid scaffold of cells and biocompatible and biodegradable polymers may serve as an ideal compromise to recapitulate the esophagus.

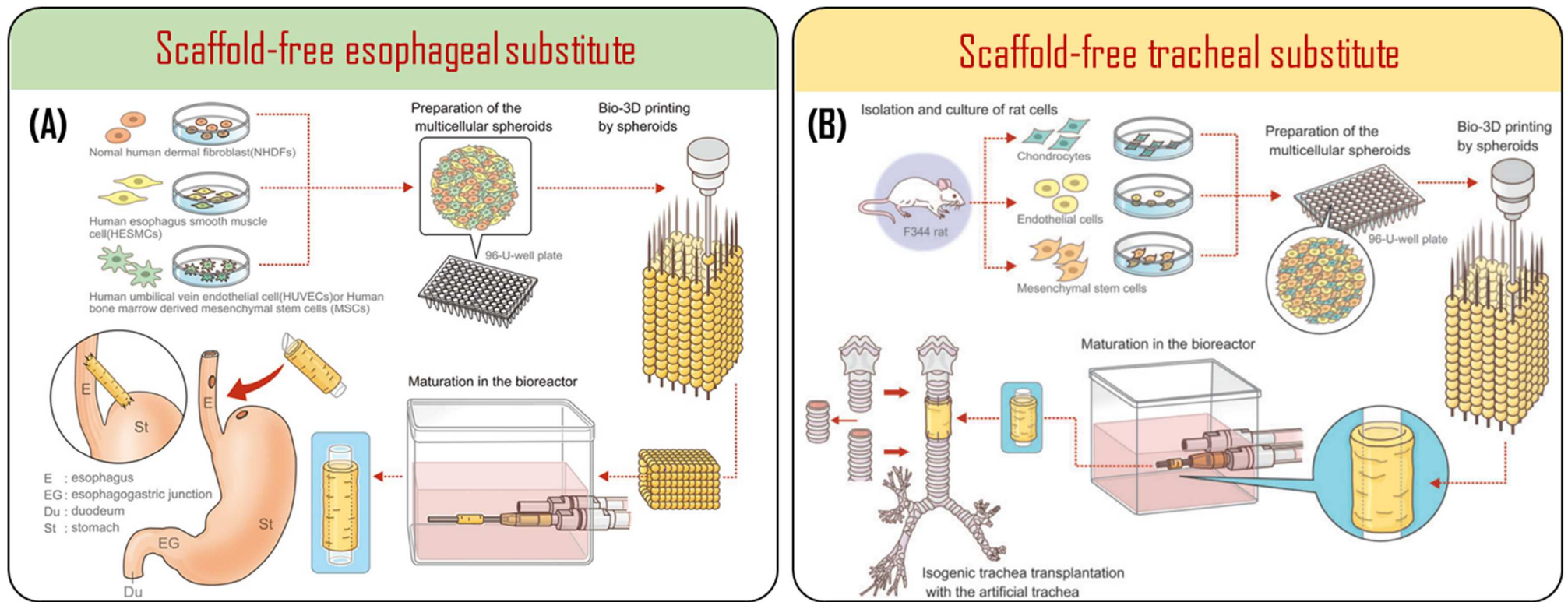


Figure 8. Kenzan 3D bioprinting method for the biofabrication of scaffold-free tubular constructs. A) Illustration of the fabrication process of the esophagus substitute and its grafting between the native esophagus and the stomach. Cells: Fibroblasts, MSCs, smooth muscle cells, or endothelial cells Adapted with permission from [254], copyright 2019, Public Library of Science. (B) Schematic illustration of the process for artificial trachea generation and its grafting at the site of interest to replace a portion of the native trachea. Cells: chondrocytes, MSCs, and endothelial cells. Adapted with permission from [259], copyright 2018, European Association for Cardio-Thoracic Surgery

In summary, 3D bioprinting of esophageal constructs is yet an emerging sector in ETE and the current state of the art is still far from the ultimate goal to replicate the sophisticated structural and functional features of the esophagus. Table 2 provides a summary of the different methodologies used to fabricate an esophagus substitute by 3D bioprinting. The majority of the reported work in the literature has used extrusion-based technologies (e.g. melt-drawing or extrusion 3D printing) to print tube-like structures, mainly from thermoplastic polymers, with mechanical properties comparable to those of the native esophagus. The fabricated constructs were often seeded *in vitro* with cells and subjected to a period of maturation *in vivo* in the omentum or *in vitro* in bioreactors. This step has shown promising results in terms of stimulating the vascularization and re-epithelialization of the artificial substitute. Substantial attention was put on the overall mechanical performance and the *in vitro* biocompatibility of the designed tubes. Different types of cells including MSCs, epithelial cells, muscle cells, and fibroblasts were used in the different investigations and examined for their viability and ability to adhere and proliferate once in contact with the fabricated scaffolds. However, the analysis of the *in vivo* toxicity and degradability of the fabricated esophagus was not studied in the majority of the reviewed reports. In conclusion, we suggest that hybrid materials encompassing hydrogels (mimicking the ECM), thermoplastic polymers (to provide mechanical support), and cell spheroids (the living component) could be the best candidate bioinks to generate scaffolds mimicking the key features of the native esophagus, although a combination of bioprinting approaches may be necessary to reach the complex structure of an esophagus and make the construct implantable and suitable for clinical practice.

Table 2. Summary of the different methodologies used to reconstruct an esophagus substitute by 3D bioprinting.

Bioprinting method	Material (polymer\hydrogel)	Cell type	Outcomes	Limitations	Refs
Extrusion-based (melt-drawing)	Polycaprolactone, extruded at 130 °C	Primary cells : rabbit MSCs	Repair of the rabbit cervical esophagus, regeneration of mucosal epithelium and recolonization by smooth muscle cells 3 weeks after transplantation	Overall design limited by the rotating mandrel. Repairs partial esophageal defects but doesn't allow circumferential reconstruction.	[238]
Extrusion-based (melt-drawing)	Poly(L-lactide-co-ε-caprolactone), extruded at 150 °C	A10 cells (myoblasts), L929 cells (fibroblasts), and primary cells: hMSCs	Tubular scaffold with comparable tensile properties to the native esophagus. Myoblasts and fibroblasts cultured on the scaffolds showed good cell viability, attachment, and proliferation. hMSCs were able to differentiate to smooth muscle lineage after seeding them on the fabricated construct in the	Overall design limited by the rotating mandrel.	[239][240][241]

			absence of soluble induction factors.		
Micropipette-based extrusion bioprinter	Hybrid cell-laden poly(D,L-lactic-co-glycolic acid) microspheres encapsulated in an agarose-collagen hydrogel blend	L929 and Rat2 cells (fibroblasts), A10 (smooth muscle) and C2C12 cells (myoblasts), TR146 cells (epithelial cells), and primary cells: hMSCs	Very good biocompatibility <i>in vitro</i> . hMSCs were capable of maintaining their stem properties after printing. Enhanced mechanical properties of the hydrogel ink: more than a 100 fold increase in the mechanical strength of the hybrid bioink in comparison with the hydrogel alone.	High risk of collapse of a longer construct	[244][245]
Dual fabrication method using extrusion-based (melt-drawing) and electrospinning dispensing systems, respectively.	Polycaprolactone, to form reinforcement rings by melt drawing then PCL (in DMF and THF) deposited by electrospinning on the rings to form a porous tube	NIH 3T3 cells (fibroblasts)	Complete healing of the surgically induced circumferential esophageal defects after 2 weeks of implantation in rat model. Fistula, perforation, or necrosis were not visible. No cell toxicity observed. Loosely arranged vascularized tissue attached to the interior and exterior of the implant was visible. Epithelization was observed after approximately 2 weeks post-implantation. Comparable mechanical characteristics with native esophagus.	Overall design limited by the rotating mandrel. Use of solvents requires extra purification and drying steps to reduce solvent toxicity.	[242]
Dual fabrication method using electrospinning and extrusion-based (melt-drawing) dispensing systems, respectively.	Polyurethane solution (in DMF) was electrospun on a rotating mandrel to form inner layer then polycaprolactone was deposited as an outer layer by melt-drawing.	Primary cells: hMSCs	Esophageal substitutes seeded with hMSCs and cultured either in a bioreactor or in the omentum showed regeneration of the mucosal (epithelization) and the muscle layers of esophagus after implantation. A thyroid gland flap was used to increase vascularization in the area leading to better outcomes.	Overall design limited by the rotating mandrel. Use of solvents requires extra purification and drying steps to reduce solvent toxicity. Poor biodegradability of polyurethane would constrain the use of such scaffolds in pediatrics	[251]
Kenzan method	N.A.	Cell spheroids derived from a combination of primary cells:	A higher proportion of hMSCs with respect to the other cell types induces better mechanical strength.	Poor mechanical properties. Requires a stent	[254]

		human dermal fibroblasts, human umbilical vein endothelial cells, bone marrow derived hMSCs, and human esophagus smooth muscle cells	Epithelization of the luminal surface and food bolus transport across the cell-based graft were observed post-implantation. Lower mechanical strength of the 3D printed esophageal construct in comparison with the native esophagus		
Pneumatic pressure, extrusion-based bioprinter	Polycaprolactone, extruded at 85 °C and 600 kPa to fabricate the complex multilayered substitute, Decellularized mucosal and muscular esophageal hydrogel-based bioinks were printed sequentially in the inner space of the substitute's layers.	Primary cells: human esophageal epithelial cells and human esophageal smooth muscle cells	The fabricated esophageal scaffold had a porous multilayered structure that promoted high cell viability and proliferation. Mechanical behavior comparable to the native esophagus.	No <i>in vivo</i> results. Suturability to the native esophagus needs to be addressed	[250]

#### 4. 3D bioprinting of other tubular organs: strategies that could be applied to ETE

Tubular organs such as those found in the cardiovascular (e.g. arteries and veins, etc.) and respiratory (e.g. trachea) systems share with the esophagus some common features. For instance, the trachea, arteries, and veins are tubular organs with a smooth muscle layer allowing them to perform transport functions. While the esophagus transports food to the stomach, the trachea transports air to the lungs and the arteries and veins transport blood to and from the tissues. In order to perform their distinct transport functions, evolution has provided each of the aforementioned organs with their own distinct set of properties with regards to mechanical performance, layer structure, size, and glandular secretions. However all these organs have a common tubular structure and it appears quite relevant in the context of this review to analyze the studies already published based on 3D bioprinting approaches of these organs. Here, we compare and evaluate the feasibility of integrating these approaches in the effective fabrication of an esophageal substitute.

##### 4.1. 3D printing of large blood vessels

Today, 3D bioprinting of parts of the blood vasculature has become a reality with potential therapeutic applications in regenerative medicine. Current clinical studies in 3D printing of vascular networks focuses mainly on creating 3D models of diseases affecting the great vessels, major arteries and veins for assisting diagnosis, choice of treatment, pre-surgery

training, as well as on designing personalized stents or prostheses [260][261][262]. For instance, Selective Laser Sintering, a laser-assisted 3D printing technology based on selectively fusing powders with a scanning laser, was used to fabricate strong and flexible thoracic aorta models out of nylon powder, replicating the anatomical features of aortic aneurysm and aortic dissection [261]. The 3D printed vessels were reproduced from a contrast-enhanced computed tomography (CT) scan of the patients' aortas. While the use of a cell-free scaffold using plastic materials alone to reproduce the mechanical and dynamic properties of tubular organs could be an appealing approach to mimic the structure of a pathologic esophagus and could be useful for *in vitro* investigations to assist in diagnosis and treatment, most of these materials are not applicable for *in vivo* implants.

In one study, Hockaday *et al.* [263] used extrusion-based 3D bioprinting with controlled photocrosslinking to fabricate aortic valve scaffolds (Figure 9A). Both natural and axisymmetric aortic valve geometries were produced from polyethylene glycol-diacrylate\alginate hydrogels. The material showed good fidelity and cell compatibility. In another report, a DLP technology was used to develop a biodegradable vascular graft from poly(propylene fumarate) with 1 mm inner diameter and 150  $\mu\text{m}$  wall thickness that was maintained *in vivo* up to 6 months [264]. This construct was proven to have good suturability and mechanical properties. In a study by Fukunishi *et al.* [265] a 3D printed mandrel template made by SLA was used to develop a patient matched tissue-engineered vascular graft by electrospinning biodegradable polymers (poly(L-lactide-co- $\epsilon$ -caprolactone)). The tissue-engineered vascular graft was subsequently tested in a large animal model and demonstrated an organized smooth muscle cell layer, extracellular matrix deposition, and endothelialization. Furthermore, Huang *et al.* [266] fabricated a triple-layer PCL based vascular graft by combining E-jet 3D printing and electrospinning techniques. The designed construct was implanted *in vivo* and showed adequate features (improved the proliferation and migration of endothelial cells towards the vascular substitute, and maintained good mechanical strength) mimicking the structure and function of native blood vessels. The aforementioned strategies to 3D bioprint vascular grafts have found similar fabrication designs in ETE and they share a comparable set of benefits and limitations, which has been discussed earlier in the context of this review.

More noteworthy and a source of additional innovations and inspiration in this field is the use of smart biinks for the fabrication of intelligent vessel constructs. For example, Haghashtiani *et al.* [267] described in 2020 a smart patient-specific aortic root construct with internal electronic sensor arrays to help model, test and select the best bioprosthetic valve for treatment and mitigate the risk of post-surgery complications (Figure 9B). In light of these new developments, it is tempting to investigate if such sensors can be added to an implantable substitute. In this emerging research field, the fabrication of smart implantable vessels is beautifully illustrated by the recent work of Li *et al.* [268]. They fabricated an artificial artery with a real-time battery-free detection system capable of sensing small variations of pressure within the human blood pressure range, thus allowing the early detection of vascular occlusion (Figure 9C). In this approach, electric field-assisted 3D printing was used to print at high temperature ( $>250^\circ\text{C}$ ) a ferroelectric composite composed of biocompatible piezoceramic particles embedded within a soft thermoplastic matrix which are polarized

during printing to give them self-powered piezoelectric behavior. The material showed excellent linearity between output voltage and pressure, as well as high sensitivity in the range of human blood pressure. The printed film was rolled into a cylinder, grafted with electrodes and embedded in a silicon elastomer to form a tube with tensile and flexibility properties comparable to the original tissue. The artificial artery was integrated with a computer-controlled syringe pump to mimic heartbeats. In response to the internal pressure change, a longitudinal strain could be induced in the ferroelectric wall, and thus piezoelectric potential was generated between the two electrodes. When thrombosis was simulated by partial occlusion of the tube, the profile of the voltage output changed significantly, demonstrating that this pioneering approach could provide early detection of thrombosis and prevent the failure of vascular implants.

We thus foresee that smart materials could also find their way into esophageal tissue engineering, for example by using a piezoelectric component to induce the self-propagation of a contraction\relaxing wave along the tube in response to stimuli (e.g. food sensation).

## 4.2. 3D printing in trachea tissue engineering

Tracheal stenosis and narrowing are rare but are nevertheless life-threatening disorders that require immediate treatment [269][270]. A number of tracheal repair and replacement strategies have been reported in the literature (such as artificial tracheal prosthesis [271], decellularized grafts [272][273], and tissue-engineered trachea using standard approaches in TE [274][275]). Recently the 3D bioprinting of artificial tracheal constructs [259][276][277][278] or tracheal stents [279][280] have appeared as game changing approaches in tracheal tissue engineering. For example, in a similar process described for the esophagus substitute, Nagayasu's research team used again the Kenzan method to 3D bioprint tracheal constructs using a combination of chondrocytes, fibroblasts, endothelial cells, and MSCs (Figure 8B) [259][276]. The printed constructs formed trachea-like tubes that could be transplanted into rat models with the support of catheters (silicone stents) to prevent their collapse. Epithelium and vascular networks formed over the grafts. As discussed earlier for the esophageal constructs, despite its advantageous features (no synthetic scaffolds, enhanced cell-cell contacts, and no toxicity), the use of cells alone to fabricate artificial organs such as the trachea or esophagus is currently not an applicable approach for clinical practice due to a number of restrictions in particular: a huge number of cells are needed for human-sized substitutes, sourcing and amplification of patient cells are limited and time-consuming, constructs exhibit very weak mechanical strengths and require stents with their associated drawbacks.

In another research project, Goa *et al.* [277] 3D bioprinted a porous PCL construct with circumferential rings mimicking the dimensions of a rabbit's native trachea (Figure 9D). In this study, the printed constructs were cultured for about 4 weeks with chondrocytes and the porous structure of the fabricated trachea proved to enhance cell adhesion and proliferation. The constructs were then incubated in the dorsal subcutaneous spaces of nude mice to stimulate their epithelization, neovascularization, and for further maturation of the cartilaginous tissue. After 6 weeks, the artificial trachea were retrieved from the mice and

implanted into induced defected tracheal sites in rabbits. The maximum survival time was about 10 weeks post-transplantation. Compared to what has already been reported in ETE and discussed in this review, this study follows the exact same approach of 3D printing of cell-free scaffolds, fabricated from thermoplastic polymers such as PCL, and “primed” biologically by incubating them in artificial or natural bioreactors (e.g. subcutaneous spaces\omentum) prior to their implantation at the site of interest. Similarly, an extrusion printer was used in another study to develop a multi-layered tracheal construct from PCL and alginate-based hydrogel encapsulating epithelial cells and chondrocytes in the inner and outer layers, respectively [281]. The tracheal scaffolds were implanted in a partial resection model and results indicated that they were able to be maintained up to 12 months without specific respiratory symptoms, in which regeneration of respiratory epithelium but not cartilage regeneration was observed.

In this research field, Park and other colleagues [278] also used DLP printing to 3D print a sacrificial frame from an alkali-soluble photopolymer to form a hollow tube with a bellow structure, bearing pores of about 300  $\mu\text{m}$  to promote effective invasion of tracheal tissue. A polymer blend of poly-(L-lactide-co- $\epsilon$ -caprolactone) (PLCL) and gelatin was then injected into the sacrificial mold, crosslinked in glutaraldehyde and the mold was dissolved in alkaline medium (Figure 9E). The scaffold showed a porous microstructure inside the wall as a result of a phase inversion between PLCL and gelatin. Heparin-functionalized gelatin sponges carrying releasable TGF  $\beta$ 1 were manually placed in between the external grooves of the bellow structure and seeded with chondrocytes. The constructs were then placed in the dorsal subcutaneous space of nude mice for 4 to 8 weeks. The fabricated constructs showed good fidelity and flexibility comparable to natural trachea along with remarkable regeneration of tracheal cartilage *in vivo*. In a similar approach, a 3D printed sacrificial frame made by SLA was used to develop a tissue-engineered tracheal graft from PCL bellows scaffold together with tracheal mucosa decellularized extracellular matrix and turbinate mesenchymal stromal cell sheets for circumferential tracheal reconstruction [282]. Here, the fabricated scaffold was consequently examined in an animal model and showed no post-operational complications such as severe stenosis, anastomotic dehiscence, dislocation, nor collapse. In a similar strategy, the use of a mold to fabricate an esophageal construct was reported in 2015 without the use of a 3D printing technology [221] and the 3D bioprinting of sacrificial mold is yet to be explored in ETE. As discussed earlier in this review, the concept of using a 3D bioprinted frame to recapitulate the mechanical nature of the esophagus was reported very recently by Nam *et al.* [250]. We suggest that using the MFT frame reported by Nam but with sacrificial polymers instead of PCL may also be an interesting approach to make the substitute more easily suturable by removing some of the inner support walls after maturation of the living tissue.

Finally, the study of a drug-releasing tracheal stent was reported recently in 2020, by Feuerbach *et al.* [280]. Here, tracheal PGLA stents encapsulating a model drug (triamterene) were fabricated by 3D printing. The stents showed a sustained capability to release the drug over several months *in vitro*. From our perspective, the design of an esophageal construct locally releasing drugs or bioactive ingredients (growth factors, cytokines, chemokines, and other signaling proteins) could help limit the diverse physiological and immunological responses post-implantation due to foreign materials, and improve cell colonization and tissue



regeneration. By allowing to print these bioactive ingredients in specific areas of the construct, 3D bioprinting would be a step forward towards patient-specific implants with tailored drug administration.

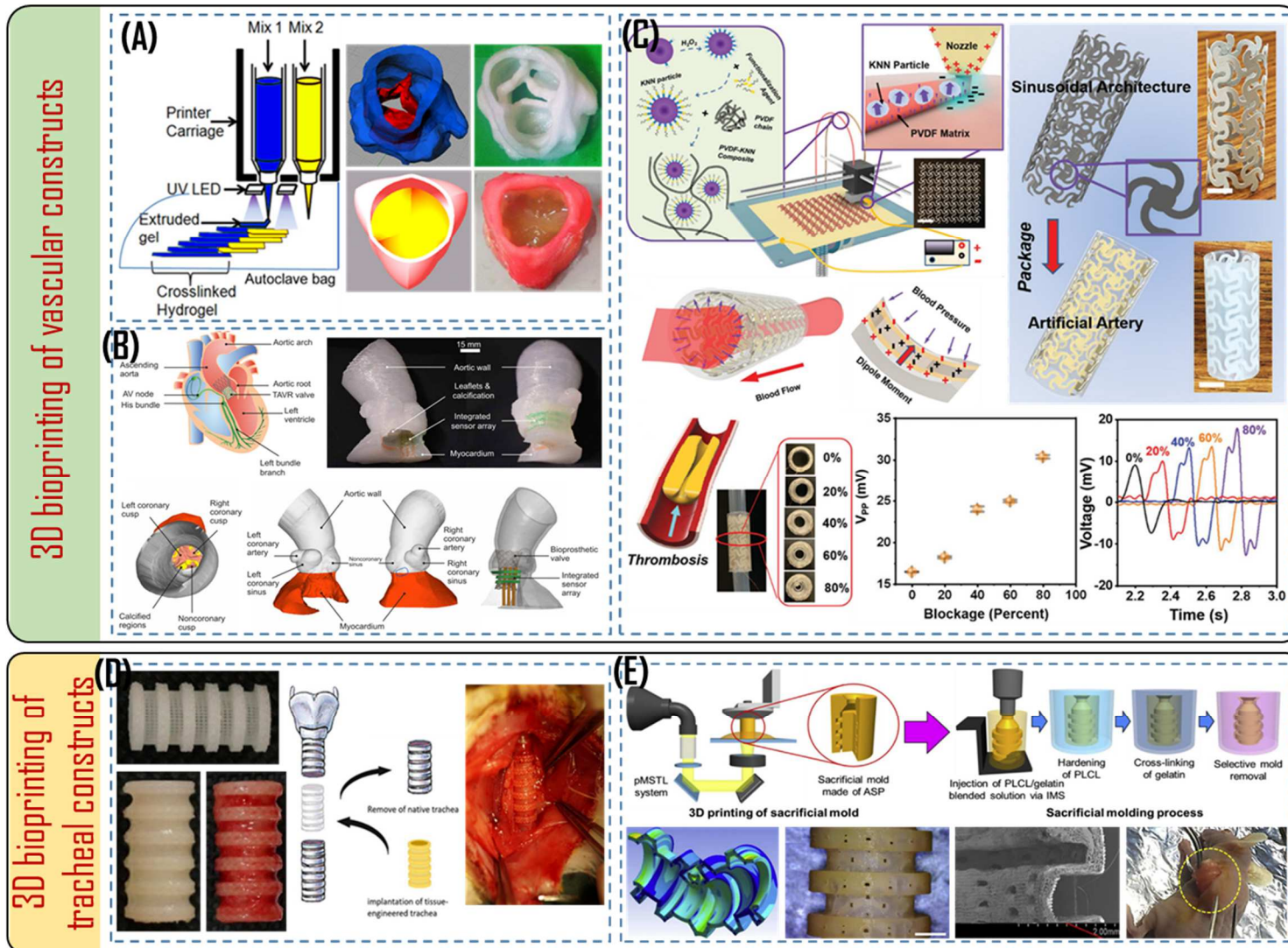


Figure 9. Additive manufacturing in tube-shaped organ TE. (A) 3D printing of mechanically heterogeneous aortic valve scaffolds, from top-left to bottom-right: Dual printheads were loaded with two distinct hydrogels and UV-LED crosslinked the deposited hydrogels during printing, porcine aortic valve model and its representative

printed construct, axisymmetric valve mode and its representative printed construct. Adapted with permission from [263], copyright 2012, International Society of Biofabrication. (B) 3D printing of aortic root models with internal sensors as models for presurgery testing, from top-left to bottom-right: illustration of the heart grafted with transcatheter aortic valve replacement prosthesis in the aortic root region, 3D bioprinted aortic root integrated sensor array, and illustrations of the constituent of the aortic root model. Adapted with permission from [267], copyright 2020, American Association for the Advancement of Science. (C) 3D printing of smart artery with built-in ferroelectricity for real-time blood flow detection and occlusion monitoring, from top-left to bottom-right: Electric field-assisted printing system in which the ferroelectric filament made up of surface functionalized KNN nanoparticles and PVDF polymer matrix and a digital image of 3D printed sinusoidal film, printed piezoelectric tube with sinusoidal lattice and PDMS package, piezoelectric effect on the designed vessel due to blood pressure, partial closure (0–80%) of the vessel by thrombosis. voltage change as a function of artery occlusion, and single voltage envelope under different levels of occlusion. Adapted with permission from [268], copyright 2020, Wiley-VCH. (D) 3D printed tracheal construct to improve whole-segment tracheal repair, from top-left to bottom-right: printed PCL scaffold, image after 4 weeks of culture with chondrocytes, image of the retrieved construct after implantation in nude mice, and surgical procedure for the replacement of the native trachea with the fabricated scaffold. Adapted with permission from [277], copyright 2017, Nature Publishing Group. (E) Fabrication of a hollow bellows scaffold as a mold for tracheal tissue engineering, from top-left to bottom-right: Design of PLCL/gelatin bellows scaffold by indirect 3D printing including 3D printing of sacrificial frame and sacrificial molding process, simulated model of the frame, image of the printed mold, SEM image of cross-sectional surface, and implantation of the fabricated trachea in the dorsal subcutaneous spaces of nude mice. Adapted with permission from [278], copyright 2015, Elsevier.

## 5. Summary and future directions

In this review, we have highlighted the current advances in ETE to provide functional esophageal substitutes with a focus on the applications of 3D bioprinting which shows great potential to overcome the severe shortage of organ donors, the need to prepare decellularized matrices, as well as some limitations of current ETE approaches. Indeed, by offering accurate arrangement and spatial control of its components (cellular and acellular constituents), 3D bioprinting can help build a substitute which better recapitulates the complex architecture of the esophageal tissue. By exploiting a wide range of tunable bioink formulations, 3D bioprinting can be used to improve biocompatibility and suturability, offer better control over size, shape and degradability, while favoring cell invasion for a more efficient reepithelialization and revascularization.

In principle, bioprinting technologies can be adapted to a clinical setting by operating them in cleanrooms or under sterile laminar hoods, and by formulating bioinks using only clinically-approved or GMP-certified ingredients or cells. It can be envisioned that, after an intensive optimization to all of its parameters, the process of bioprinting could take place *in situ* directly onto the human body during surgery, which would provide the patients with their own personalized organs for transplantation in a short timeframe. To achieve this future perspective of bioprinting, ideal processing conditions (material printability, gelation, cell encapsulation, and viability, etc.) have yet to be addressed precisely.

However, 3D bioprinting in ETE is yet an emerging research area and so far, a very limited number of studies have been published. Furthermore, the investigations in this discipline have used 3D bioprinting as an indirect procedure to produce artificial esophagus. In fact, the majority of these reports describe the fabrication of an acellular tubular-like structure followed by cells seeding or implantation into the omentum for epithelization and vascularization. The small amount of studies correlates with the challenges of printing a long hollow organ and keeping it intact throughout the printing process. A solution to circumvent this difficulty is the use of a sacrificial layer to provide support to the membrane during the printing process which would be removed post-printing. Furthermore, the artificial esophagus may encounter severe challenges post-implantation as it will be subjected to harsh environments, such as direct contact with the food bolus, contraction, expansion, and acidic reflux from the stomach, all of which would diminish the formation of the epithelial layer. Finally, the use of smart materials to fabricate artificial organs is a hot topic in the TE field which has witnessed the emergence of a new research discipline called '*4D bioprinting*', thus referring to the 3D printed objects that have the ability to modify their shape or function upon stimuli [283][284][285]. Thus, in the context of our review, as electrical signals are transmitted from the brain to stimulate the peristaltic movement of esophagus to move the food bolus towards the stomach, one could imagine the 4D bioprinting of an esophageal substitute that can undergo self-contraction\relaxing in response to stimuli (e.g. food sensation) using a piezoelectric component. This could represent a highly innovative research program aiming at integrating dynamic features in the artificial esophagus.

## Funding and acknowledgments

This work received the financial support of MSDAvenir-APHP Foundation as well as of the iLite RHU program (grant ANR ANR-16-RHUS-0005). We thank Dr. Briac Thierry (M.D.) for insightful discussions in the future of tracheal tissue engineering.

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