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Naturally-Derived Biomaterials for Tissue Engineering Applications

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Abstract

Naturally-derived biomaterials have been used for decades in multiple regenerative medicine applications. From the simplest cell microcarriers made of collagen or alginate, to highly complex decellularized whole-organ scaffolds, these biomaterials represent a class of substances that is usually first in choice at the time of electing a functional and useful biomaterial. Hence, in this chapter we describe the several naturally-derived biomaterials used in tissue engineering applications and their classification, based on composition. We will also describe some of the present uses of the generated tissues like drug discovery, developmental biology, bioprinting and transplantation.

Keywords

Naturally-derived materials; Tissue decellularization; Tissue engineering; Protein-based biomaterials; Polysaccharide-based biomaterials; Glycosaminoglycans; Extracellular matrix-derived biomaterials; Solid organ bioengineering; Regulatory landscape for naturally-derived biomaterials

23.1 Introduction

The use of new advanced experimental strategies, such as bioengineering techniques, will transform the practice of medicine in the coming years. A clear example of this is the quick advancement in the field of tissue engineering, an interdisciplinary field of research that involves the principles of materials science, engineering, life sciences, and medical research. Tissue engineering aims to replace an entire organ or provide restoration of the specific cellular functions [1, 2]. For this purpose, tissue engineering usually works with three essential tools: scaffolds, cells, and growth factors [3].

In recent years, the search and generation of new and suitable scaffolds for tissue engineering has been greatly accelerated. This is especially true in the study of natural biomaterials as they have been found to mimic the biological and mechanical function of the native ECM found *in vivo* in any tissue of the body. Natural biomaterials can be categorized into the following subtypes: protein-based biomaterials (collagen, gelatin, silk) [4], polysaccharide-based biomaterial (cellulose, chitin/chitosan, glucose) [5], glycosaminoglycan-derived biomaterials and tissue/organ-derived biomaterials (decellularized heart valves, blood vessels, livers) [6]. Depending on the final use, they usually share several prominent features: biocompatibility, biodegradability, and non-toxicity, amongst others [7]. However, when the final goal of tissue engineering is the generation of solid organs with bioengineering techniques, the use of protein-based and polysaccharide- based biomaterials presents some disadvantages: a) Mechanical strength is limited, avoiding the generation of larger constructs and restricting their applications at load bearing regions; b) manufacturing variability; c) Potential impurities from the proteins or

polysaccharides before implantation, which can be a source of immunogenicity [8, 9]. Despite these disadvantages, almost every tissue and organ in the body has been bioengineered *in vitro* with success. Within the past 20 years, most of the major achievements in tissue engineering were focused on tissues constructed using thin sheets of cells for tissue replacement, such as skin, small intestine, esophagus, bladder, bone, carotid arteries, amongst others [10–14].

Construction of thicker tissues has been slow due to the limited diffusion of nutrients and oxygen within the engineered tissue mass [15]. Nonetheless, the tissue engineering sector has grown exponentially with breakthroughs reached in this area in the last years, and right now the ultimate goal of the field is the creation of whole- organs using bioengineering techniques for human transplantation. End-stage organ diseases affect millions of people around the world, and for these patients, organ transplantation is the only definitive cure available. However, every year there is a significant gap between the number of patients on the organ waiting list, the number of donors, and the number of patients died waiting for a transplant due to the persistent organ shortage. In 2016, Europe registered 10,893 organ donors, with 59,168 patients in the waiting list for transplantation, and 3795 deceased people waiting for an organ transplant [16]. Multiple alternatives and solutions have been sought in past decades to solve this problem, and at the moment, whole-organ bioengineering seems promising [17] and it could change the actual paradigm of organ shortage in the near future.

The development of decellularization methods for the generation of whole-organ engineering provides the ideal transplantable natural bioscaffold with all the necessary microarchitecture and extracellular cues for cell attachment, differentiation, vascularization, and function [18]. Numerous attempts to generate whole- organs have been made. The most extensively, are some of the major organs: liver, heart, kidney, and lung [4, 19–22]. Although, progress so far has been quite remarkable, significant challenges still need to be overcome in whole-organ bioengineering to transfer this new technology into the clinic. These include identifying appropriate species to provide decellularized tissues, selecting ideal cell sources, localizing signals for differentiation, achieving robust vascularization, optimizing bioreactor perfusion technology along with scalability, and preventing graft immunological rejection [23–25].

23.2 Naturally-Derived Biomaterials

The current research into naturally-derived biomaterials should be considered a renaissance as its original interest started with the beginning of recorded human history.

Some of the earliest biomaterial applications have been dated as far back as 3000 BC to the ancient Egyptians who employed coconut shells to repair injured skulls, or wood and ivory as false teeth. In modern times, more sophisticated applications of natural biomaterials emerged with the first replacement surgery using ivory being reported in Germany,1891 [26].

By the 1950s and 60s, there were records of clinical trials with blood vessel replacement and the first mechanical human cardiac valve implantation [27].

In the biomedical field, natural biomaterials can be classified into several categories according to their origin. These groups can be distinguished as those derived from proteins (for example, collagen, gelatin, silk, and fibrin); polysaccharides (cellulose, chitin/chitosan, alginate and agarose), or glycosaminoglycans (hyaluronic acid, chondroitin sulfate, dermatan sulfate, heparan sulfate and keratan sulfate). In recent years, more complex biomaterials have emerged as in the case of cell/organs-derived matrices.

Despite their nature, they all shared some unique features (such as biocompatibility, biodegradability and remodeling) which have increased the scientific interest in the development of medical/tissue engineering technologies around these biomaterials. Hence, in this section, we provide a brief summary and applications of the most important naturally-derived biomaterials.

23.3 Protein-Based Biomaterials

23.3.1 Collagen

Collagen is the main structural protein of most tissues in the animal kingdom and plays an important role in maintaining the biological and structural integrity of the extracellular matrix (ECM) while also providing physical support to cells and tissues. At a cellular level, collagen is secreted mainly by fibroblasts and plays key roles in regulating cellular morphology, adhesion, migration and differentiation.

In the human body, collagen comprises approximately 25–35% of the whole-body protein content where it can be found mostly in fibrous tissues (skin, tendons and ligaments) and every other tissue that require strength and flexibility, such as bones, cartilage, blood vessels, corneas, gut, intervertebral discs and dentin (in teeth) [28].

Currently, there are 29 known isoforms of collagen that have been described. Collagen's important biological role has driven this biomaterial to the center of tissue engineering research. In fact, collagen is easily isolated and can be purified on a large scale. Moreover, it has well- documented structural, physical, chemical and immunological properties. Additionally, collagen is biodegradable, biocompatible and has non-cytotoxic proprieties.

Collagen can also be processed into a variety of forms including cross-linked films, steps, beads, meshes, fibers, sponges and others [29] expanding its potential applications. Many researchers have illustrated the use of collagen as scaffolds for cartilage and bone [30–32] as well as in bioprosthetic heart valves, vascular grafts, drug delivery systems, ocular surfaces, and nerve regeneration [33]. Additionally, microcapsules containing collagen matrices have been designed in 3-D scaffolds for soft tissue engineering [34]. Regarding liver tissue bioengineering, collagen-coated silicone scaffolds represent an important tool for the development of viable 3D hepatocyte cultures [35]. More recently, *Wang Y et al.* were able to generate crypt-villus architecture of human small intestinal epithelium using micro engineered collagen scaffolds [36]. These are just a few examples of the great potential of using collagen as a biomaterial.

23.3.2 Gelatin

Gelatin is a biocompatible, biodegradable and fully absorbable biopolymer derived from the hydrolysis of collagen. Due to its biological nature, great solubility in aqueous systems and its high commercial availability at low cost, gelatin has been commonly used and showed several advantages compared to its parent protein.

Among its many formulations (mainly derived from porcine, fish, and bovine tissue) the most used ones in biological fields include nanoparticles, microparticles, 3D scaffolds, electrospun nanofibers and *in situ* gels [37].

Another important fact regarding this polymer is that it can be crosslinked and chemically modified which expands even more its applications.

As an example of gelatin versatility, Tayebi L and co-workers, have recently characterized a biocompatible and bio-resorbable 3D-printed structured gelatin/elastin/sodium hyaluronate membrane with great biostability, mechanical strength and surgical handling characteristics which hold great potential for engineered procedures [38].

Furthermore, several other authors have described other applications of gelatin-derived biomaterials from cardiovascular [39], bone [40], skeletal muscle [41] and hepatic tissue engineering [42] to wound healing and injectable fillers [37].

Another very interesting finding was attributed to Kilic Bektas C., and Hasirci V., who recently developed a “corneal stroma system” using keratocyte-loaded photopolymerizable methacrylated gelatin hydrogels which could serve as an important alternative to the current products used to treat corneal blindness [43].

Gelatin as a biomaterial shows a wide range of applications. In stem cell research, modifications in gelatin formulations have been shown to influence stem cell fate in injectable cell-based therapies [44].

Gelatin-based delivery systems have also found to be successful in gene and siRNA delivery, by inducing the expression of therapeutic proteins or trigger gene silencing, respectively.

Overall, with the significant progress that has already been made, along with others that will be achieved in a near future, the safe and effective clinical implementation of gelatin-based products is expected to accelerate and expand shortly.

23.3.3 Silk

Silks are biopolymers formed by different fibrous proteins (fibroin and sericin) that are segregated by the glandular epithelium of many insects including silkworms, scorpions, spiders, mites and flies.

Silk fibers, in the form of sutures, have been used for centuries, and new research into different formulations (gels, sponges and films) have been encouraging [45].

In the orthopedics medical field and cartilage tissue engineering, many studies have been pointed out the enormous potential of this biomaterial.

Thus, *Sawatjui N et al....*, found very recently that the microenvironment provided by the porous scaffolds based on silk fibroin (SF) and SF with gelatin/chondroitin sulfate/ hyaluronate scaffolds enhanced chondrocyte biosynthesis and matrix accumulation [46].

In this line, another silk derived scaffold (curcumin/silk scaffold) was also described as a good candidate for cartilage repair [47] and for meniscal replacement [48].

Another recent investigation conducted by *Hu Y et al.,* lead to a silk scaffold with increased stiffness and SDF-1 controlled release capacity for ligament repair [49].

On the other hand, *Bryan S. Sack et al*, described that silk fibroin derived scaffolds showed promising repair of urologic defects in pre-clinical trials [50].

Although silk fibers also showed broad applications, the more popular ones seems to be related to cartilage engineering.

23.3.4 Fibrin

Fibrin is a non-globular protein, involved in blood coagulation, formed from the thrombin-mediated cleavage of fibrinogen.

Numerous studies have exploited fibrin's function as hemostatic plug and wound healing, which suggests fibrin has potential applications in both the medical field and tissue engineering [6].

Among its applications, three-dimensional fibrin gels have been used as scaffolds for cell proliferation and migration. According to *Ye Q., et al..,* fibrin gels can serve as a useful scaffold for cardiac tissue engineering with controlled degradation, excellent cell seeding effects and good tissue development [51].

More recently, *Seyedi F et al.*, have reported that 3D fibrin scaffolds effectively induced the differentiation of human umbilical cord matrix- derived stem cells into insulin producing cells [52].

Other studies reported that hybrid fibrin/PLGA scaffolds may promote proliferation of chondrocytes as well as cartilaginous tissue restoration and may eventually serve as a potential cell delivery vehicle for articular cartilage tissue- engineering [53].

Additionally, a new biomaterial called fibrin glue or fibrin sealant, has been formulated by combining fibrinogen and thrombin at very high amounts along with calcium and Factor XIII. This material is currently used as an adjunct to hemo-stasis in patients undergoing different types of surgeries. More specifically, *Azizollah Khodakaram-Tafti, et al*, suggested that autologous fibrin glue appears to be promising scaffold in regenerative maxillofacial surgery [54], just to name one example.

23.4 Polysaccharides-Based Biomaterials

23.4.1 Cellulose

Cellulose is the most abundant natural polymer on Earth; present in the cell walls of green plants, some forms of algae, and can also be produced by bacteria.

Although cellulose is very abundant and has several readily available renewable natural sources, major difficulties in its refinement make it a poor option for a naturally-derived biomaterial, such is the reason why there are no physiological or pharmaceutical applications [55]. Research is currently focused on the simplification of the normally intensive methods for the depolymerization of cellulose and the manufacture of its derivatives so that it may be used as a biomaterial.

Recently, chemists were able to generate useful cellulose derivatives such as carboxymethylcellulose, cellulose nitrate, cellulose acetate, and cellulose xanthate which are all gaining some interest in the medical and tissue engineering fields [6]. Cellulose acetate for instance has been used to produce cardiac scaffolds [56], while other forms were used for cartilage tissue engineering [57].

One of the lastest and curious application of this biomaterial is to use heparinized bacterial cellulose based scaffold for improving angiogenesis in tissue regeneration [58].

23.4.2 Chitin/Chitosan

Chitin is the second most abundant natural polysaccharide next to cellulose. It is mostly found in the exoskeletons of arthropods and many insects. Its derivatives which includes chitosan, carboxymethyl chitin, and glyco-chitin have all generated attractive interests in various fields such as biomedical, pharmaceutical, food and environmental industries [59].

In recent years, considerable attention has been given to chitosan (CS)-based materials and their applications in the field of orthopedic tissue engineering. It has garnered this interest because of its intrinsic anti-bacterial nature, porosity, and the ability to be molded in various geometries which are suitable for cell growth and osteocon-duction [60]. Chitosan/alginate hybrid scaffolds have also been developed in this field [61]. Moreover, chitosan was also reported to promote angiogenesis and accelerate wound healing response by promoting migration of inflammatory cells to the wound site and collagen matrix deposition in open skin wounds [62–64]. Thus, chitosan hydrogels have been developed in medical therapeutics for third-degree burns [65].

23.4.3 Alginate

Alginate is a naturally occurring anionic polymer typically obtained from brown seaweed. Among its excellent biological proprieties (biocompatibility, low toxicity, and relative low cost), alginate is a readily processable into three-dimensional scaffolding materials such as microspheres, microcapsules, sponges, foams, fibers and hydrogels.

Alginate hydrogels are one of its most popular formulations. In fact, alginate hydrogels can be prepared by various cross-linking methods and their structural similarities to ECM of

living tissues that allows a wide range of applications from wound healing management [66] to more complex drug delivery vehicles [67].

Wang Y et al. recently described a three-dimensional (3D) printing technology to fabricate the shape memory hydrogels with internal structure (SMHs) by combining sodium alginate (alginate) and pluronic F127 diacrylate macromer (F127DA), which showed a huge prospect for application in drug carriers and tissue engineering scaffold [68].

Another common application of alginate is for the immobilization of cells [69] allowing for largescale cellular expansion in different bioreactors. This immobilization application was exhibited by *Anneh Mohammad Gharravi et al.*, which have fabricated a bioreactor system containing alginate scaffolds for cartilage tissue engineering [70]. *Beigi MH et al*, also described very recently that 3D alginate scaffolds with co- administration of PRP and/or chondrogenic supplements had a significant effect on the differentiation of ADSCs into mature cartilage [71].

Besides that, encapsulated cells have been proven useful for cell therapies. According to Coward SM et al., alginate encapsulated HepG2 cells, circulating in the plasma of patients suffering with acute liver disease, maintained their hepatic metabolism, synthetic and detoxification activities, indicating that the system can be scaled-up to form the biological component of a bioartificial liver [72].

In another interesting approach has just been published, *Yajima Y., et al* performed perfusion cultivation of liver cells by assembling cell-laden hydrogel microfibers and packed HepG2 into the core of sandwich-type anisotropic microfibers, which were produced using microfluidic devices to structurally mimic the hepatic lobule *in vivo* [73].

Pipeleers D., et al., also reported that human embryonic stem cell (hESC)-derived beta cells encapsulated in alginate microcapsules were protected from the immune system and corrected insulin deficiencies of type-1 diabetic mice for at least 6 months [74]. These are just few examples of the potential of polysaccharides-based biomaterials.

23.4.4 Agarose

Agarose, the main constituent of agar, is another polysaccharide naturally found in red algae and seaweed.

Most of the beneficial features shown by alginate are shared by agarose. In fact, agarose and alginate were two of the first materials used as hydrogels for cartilage tissue, showing natural pro-chondrogenic properties and are easy to prepare [75].

On the other hand, recent findings pointed agarose as an excellent candidate for applications involving neural tissue engineering [76–78].

In this line, *Han S et al..,* reported that an agarose scaffold loaded with matrigel could promote the regeneration of axons and guide the reconnection of functional axons after spinal cord injury in rats [79].

In cardiac bioengineering and stem cell biology, agarose was shown to promote cardiac differentiation of human and murine pluripotent stem cells [80].

In other study, high quality valvular interstitial cell aggregates were generated, in agarose micro-wells, which were able to produce their own ECM, resembling the native valve composition [81].

23.5 Glycosaminoglycans

Glycosaminoglycans (GAGs) represent a group of long unbranched polysaccharides consisting of repeating disaccharide units. There are several types of GAGs components including hyaluronic acid (HA), chondroitin sulfate (CS), dermatan sulfate, heparan sulfate, and keratan sulfate.

Among these GAGs, HA and CS are the two most studied in regenerative medicine and tissue engineering field. Some of its applications are described briefly.

23.5.1 Hyaluronic Acid

Hyaluronic acid (HA) is a molecule comprised of repeating disaccharide units of N-acetyl-d-glucosamine and d-glucuronic acid being widely distributed in the most connectives tissues and some body fluids (such as synovial fluid and the vitreous humor of the eye). Its chemical properties (solubility and the availability of its reactive functional groups) make this molecule an excellent candidate for chemical modifications and a very biocompatible material for use in medicine and tissue regeneration.

Research has found that intra-articular injections of MSCs and HA in rabbits showed statistically significant improvements in osteochondral defect healing [82].

In current orthopedics medical practice, HA has a prominent place. In fact, HA injections have been shown to ameliorate osteoarthritis symptoms and shown the ability to delay prosthetic surgeries [83]. Moreover, HA has also become popular as dermatological fillers for treatment of face aging [84].

In tissue engineering, cartilage biodegradable scaffolds made of HA were engineered [85] and HA-collagen hybrid scaffolds were proven robust and offered freely permeable 3-D matrices that enhance mammary stromal tissue development *in vitro* [86]. In other research field, *Kushchayev SV et al*, described that hydrogel had a neuroprotective effect on the spinal cord of rats by decreasing the magnitude of secondary injury after a lacerating spinal cord injury [87].

To conclude, one of the most attractive advantages of HA is that it can be easily and controllably produced in large scale through microbial fermentation avoiding the potential risks of animal- derived biomaterials [88].

23.5.2 Chondroitin Sulfate

Chondroitin sulfate (CS) consists of repeating disaccharide units of d-glucuronic acid and N-acetyl galatosamine, sulfated at either 4- or 6-positions [89] and represents the second most

used GAG as biomaterial. CS could be obtained from bovine, porcine, chicken, shark and skate cartilage after various extraction and purification processes.

In biomedical applications, CS has been associated with bone and cartilage metabolism and regulation, presenting both anti-inflammatory effects and accelerated bone mineralization capabilities [90].

In animal studies, CS combined with HA and other GAGs administered after arthroscopy were described as beneficial to equine cartilage health by increasing the number of repair cells and decreasing the number of apoptotic cells [91].

In this line research, other biomaterials combinations have been hypothesized. According to Liang WH, et al., CS-Collagen scaffolds could be used to cartilage and skin applications [92].

Recently, Zhou F et al., designed a silk-CS scaffold and proved that this scaffold exhibited good anti-inflammatory effects both in vitro and in vivo, promoted the repair of articular cartilage defect in animal model [93]. Thus, CS also constitutes one of the FDA approved skin substitute component [94].

23.6 Extracellular Matrix-Derived Biomaterials

23.6.1 Cell-Derived Matrices

In recent years, the development of decellularized ECM has made the fields of cell biology, regenerative medicine and tissue engineering advance beyond the use of simple natural derived biomaterials.

Cell-derived matrices (CDMs) consist of an acellular complex of different natural fibrillar proteins, matrix macromolecules and associated growth factors that often recapitulate, at least to some extent, the composition and organization of native ECM microenvironments. As an ECM derived material, CDMs provide mechanical and biological support allowing cellular attachment, migration and proliferation; paracrine factor production and differentiation in a tissue-specific manner [34].

The unique ability to produce CDMs *de novo* based on cell source and culture methods makes them an elegant alternative to conventional allogeneic and xenogeneic tissue-derived matrices that are currently harvested from cadaveric sources, suffer from inherent heterogeneity, and have limited ability for customization [95].

The production of CDMs have undergone several evolutions. There are several ways these matrices can be produced through the use of different decellularization strategies that includes chemical, enzymatic, physical, mechanical, or a combination of methods.

Regarding chemical decellularization protocols, the surfactant sodium dodecyl sulfate (SDS) is the most used reagent, which promotes cellular lysis through phospholipid membrane disruption [56]. However, many authors prefer using acids (peracetic acid) or bases (sodium

hydroxide) agents to decellularize the ECMs. To supplement this treatment some enzymes like DNase I can be added to prevent the agglutination of DNA.

Due to the toxicity of this approach, some physical and mechanical methods have therefore been developed. These methodologies include the use of temperature (for example, freeze-thaw cycles) or pressure treatments (such as high hydrostatic pressure or supercritical CO2 content).

Among all CDMs that have been tested, small intestine submucosa (SIS), bladder submucosa, acellular dermis and engineered heart valves represent the most popular ones and will be described briefly.

23.6.2 Small Intestinal Submucosa (SIS) and Bladder Submucosa (BS)

One such CDM being extensively used is the Small Intestinal Submucosa (SIS) or the Bladder Submucosa (BS). These are naturally occurring ECM, derived from the thin and translucent tunica submucosa layer of the porcine small intestine or urinary bladder, which remains intact after removing the mucosal and muscular layers. SIS and BS have been shown to be biologically active and its unique combination of intrinsic growth factors, cytokines, GAGs and structural proteins (mainly collagen fibers, fibronectin, vitronectin, etc) provide strength, structural support, stability and biological signals which allow overall cell ingrowth [96–99].

Once the SIS or BS biomaterial is harvested, it is carefully processed to remove all living cells, disinfected and sterilized, and then able to be used as scaffold or for long-term storage in their lyophilized form [100, 101].

Over the years, many applications have emerged. The first application of SIS was associated to Lantz and colleagues, who first used SIS in animal studies as a vascular patch, reported a great tissue-specific regeneration in both arteries and veins [102, 103].

Within the urology field, SIS and BS are also very popular biomaterials. Thus, Kropp and colleagues subsequently demonstrated that SIS, used as an unseeded graft, could promote bladder regeneration, in both small and large animal models, and it was accompanied by serosal, muscle and mucosal layers regeneration, tissue contractibility and biological functionality [104, 105]. Gabouev *et al.* have used BS to bioengineer porcine urinary bladder tissue that they have seeded with smooth muscle and urothelial cells. The resulting tissue displayed the generation of 2 tissue layers with the putative markers of muscle and urothelial cells [106].

In the management of anterior urethral stricture disease, SIS showed promising results. In one study, more than 50 patients were managed with an SIS graft placed in an inlay fashion and according to a follow-up of 31 months the success rate was around 80% [107]. A second study reported 85% success when SIS was used as either an inlay or on lay patch graft with a mean follow-up of 21 months. However, when used ventrally, it failed in 6 of 10 adult patients [108].

In different clinical areas, SIS has been described as a functional bioscaffold for the intestinal tract. According to Hoeppner et al., SIS application showed a great potential in colon walls regeneration in domestic pigs [109].

In neurological and orthopedic fields, porcine SIS was used in rat peripheral nerve regeneration [110] and chest reconstruction after tumor removal respectively [111]. Based on scientific findings, many companies have been interested in this CDM with many SIS and BS derivatives being currently commercialized. SIS and BS derived devices are now available for hernia and hiatal hernia grafts; dural graft; ENT repair graft; enter cutaneous Fistula Plug and as nerve connector/protector, etc. [112].

23.6.3 Acellular Dermis

The acellular dermis (AD) is another example of a CDM, which consists of a soft tissue substitute derived from donated human skin tissue. As the others CDMs, AD undergoes a multi-step process which includes epidermis removal, disinfection and sterilization minimizing the risk for an anti-genic or rejection response.

AD was initially used as a type of graft material for the management of burn wounds [113]. However, in the recent years, AD has been used for various applications in reconstructive surgery including head and neck reconstruction as well as chest and abdominal wall reconstruction [114, 115].

Currently, AD has become an integral part of implant breast reconstruction being one of its most important applications in regenerative medicine field [97].

23.6.4 Heart Valves

The origin of decellularized cardiac valves matrices may come from xenotransplants or allotransplants showing different outcomes. Rieder, et, al reported immunological differences depending on whether the valves came from human or porcine, showing that decellularized porcine pulmonary valve does not represent a completely non-immunogenic heart valve scaffold [116].

In other studies, the aim was to develop and optimize decellularization protocols to obtain viable scaffolds for this type of applications. For example, mitral valves can be decellularized to obtain structures that generally preserved their microarchitecture, biochemistry, and biomechanics [117]. These CDMs are currently widely studied and tested being now available many commercial options on the market such as Acellular porcine heart valve leaflets from *Epic™ and SJM Biocor®*; porcine acellular valve from *Prima™ Plus* or porcine acellular heart valve tissue from *Hancock® II, Mosaic®, and Freestyle®* [118].

Another important aspect is to know what type of cells may be a functional option for valve replacement. Fang et, al, reported that human umbilical cord blood-derived endothelial progenitor cells (EPCs) may be a promising option to form a functional endothelial layer on decellularized heart valve scaffolds [119].

23.7 Bioengineering of Solid Organs

The disciplines of tissue engineering (TE) and regenerative medicine (RM) endeavor to eliminate the need for patient to patient tissue and organ transplantation by constructing analogs *in vitro* that will normalize or improve physiological functions of their respective system. The need for further research is paramount, as currently there are 115,000 patients waiting for organ transplants, however there were only 34,000 transplants performed in 2017 in the USA. Programs to reduce the wait list by increasing donors have been met with limited success. The difference in waitlisted patients and transplant recipients has grown in size over the last 14 years. TE and RM would initially eliminate the gap between the waitlisted and recipients culminating by rendering large donor pools obsolete.

23.7.1 Liver

Whole-liver engineering has made incremental advances in the recent past. Researchers have been able to successfully decellularize the liver ECM in a variety of different ways [19, 120–124]. Decellularization is achieved by effectively pumping detergents through the vasculature with the use of peristaltic pumps through the portal vein. There have been several successful attempts at reintroducing cells into the decellularized scaffold, namely Baptista and colleagues [19, 124]. Current roadblocks to generate liver tissue capable of completely assuming the full spectrum of native tissue functions are several-fold: The construction of a fully patent vascular network and generation of required number of cells to acquire a basal level of functionality. Vascular network patency is required for normal blood flow as acellular ECM will induce the formation of blood clots as it is highly thrombogenic. Recent advances in revascularization have greatly improved the efficacy of endothelial cell seeding resulting increased vascular patency. In 2015 Ko et al. were able to greatly improve the reendothelialization of the vasculature by the introduction of anti-CD31 antibodies which were injected into the decellularized arteries and veins of the scaffold after being treated with 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide esters (NHS) thus effectively conjugating the antibody to the acellular liver scaffold [125]. After conjugation, mouse endothelial cells (MS1) were statically seeded and perfused through the liver construct. This resulted in a vascular network evenly seeded with endothelial cells and was successfully transplanted into a pig [125]. This method allowed the liver construct to maintain biological blood flow and reduced platelet aggregation for a 24-h period. In 2017 Mao et al. were able to obtain a patent vascular network by using porcine umbilical vein endothelial cells (pUVEC) and its human analog (hUVEC). The use UVEC's also allowed for complete coverage of the vasculature and additionally allowed for vascular patency over a period of 72 h in a porcine model [126]. Other methods such as the refinement of the decellularization technique have also yielded positive results [127]. An average male liver is made up of ~2×10^{11} cells [128]. Even achieving the 30% fraction required for complete organ function still is proving a challenge. The preferred source of cells would be autologous liver stem cells, but alternative cell sources are also being studied such as iPSCs, Mesenchymal SCs, and humanized hepatocytes [129–132]. There have been several studies on the transplant of iPSCs into mice suffering CCl4 induced liver failure. iPSCs show much promise in that they are autologous and are a potentially limitless source for the material required to repopulate a sufficiently

sized scaffold. Currently, research has been oriented into making more functional cells similar to their native counter parts [133–136].

23.7.2 Heart

As with the liver, similar complication arises in the bioengineering of the heart in terms of generation of the adequate number of cells, and of appropriate scaffold sources. Decellularized ECM has also been successfully used as a scaffold for the seeding of cardiac cells and been transplanted into animal models with some limited success. Specifically, Ott *et al.* was able to successfully transplant the recellularized scaffold where it was able to pump in blood throughout the vasculature for a short period of time [20]. For clinical applications, porcine ECM tissue has been at the forefront of research [137–140]. Decellularized porcine heart valves have been used clinically, however this has not been plausible for the entire heart itself [141]. As with any xenotransplant the risk of immunogenicity is of paramount importance. In porcine tissues the alpha [1, 3]-galactose epitope is the general cause for rejection in the human host [142]. There have been attempts to remove the epitope through the use of galactosidase during the decellularization process and additionally to genetically engineered pigs to abolish the possibility of synthesis of this particular epitope [142]. Cardiomyocytes and endothelial cells are the two major cell types that comprise the heart. The generation of several billion cells directly from adult stem cells remains elusive in the cardiac field of research as well. Again here we see that iPSCs and human embryonic stem cells have come to the forefront of the field as a possible solution to the problem of culturing the amount of cells required for recellularization [143].

23.7.3 Kidney

The bioengineering of the kidney has proven challenging and the cellular makeup of the organ is highly complex consisting of approximately 26 different cell types that perform a litany of functions for the body including the regulation of blood pressure, blood filtration, and ion exchange [144–146]. There are more than a dozen cell based therapies in current clinical trials for the treatment of renal deficiencies [147]. However, this does not help with patients suffering from end-stage renal disease and are in need of total transplant. Recellularization of whole acellular ECM constructs have been attempted by several research institutes which demonstrated the ability to form some native like structure [148–150]. For most of the native functions that have been mimicked have been in terms of the filtration of plasma into a urine-like substance, but have not been able to control overall vascular blood pressure as a normally functioning kidney would do [151]. This has been through the growth of nephron like structures, which were found after the recellularization using pluripotent stem cells [151]. The nephron stem cells were generated by researchers Tagasaki et al. and Taguchi et al. [152] using a variation of treatments where the directed differentiation of the iPSCs was achieved through the use of Activin A or CHIR90021, a GSK3β inhibitor.

The field of tissue engineering has not yet provided clinicians with the ability to replace defective organs with wholly engineered ones. Yet, highly important steps have been made to replace some function of the organs themselves, but not all. There have been great strides made in the area of decellularization of a multitude of different types of organs.

Vascularization of the decellularized organs has also made great advances allowing for a fully patent vasculature that does not undergo thrombosis under blood flow. Finally, the use of autologous iPSCs have garnered a great deal of interest and could serve as the ultimate source of cells for the future of recellularized organ constructs. Satisfying these three needs could greatly benefit the world of transplantation needs throughout the world.

23.8 Current and Future Applications

23.8.1 Drug Development and Toxicology

The development and launching of a new drug into the market continues to be challenging [153]. The average cost is currently 3–5 billion USD and takes approximately 12–15 years of exhaustive research. After a preliminary screening, the potential drugs (or pharmaceutical candidates) are characterized *in vitro* and *in vivo* for their ADME-Tox (absorption, distribution, metabolism, excretion and toxicity) properties before continuing on to clinical trials. However, about 90% of the candidates fail during the final stages of the clinical trials, where 43% are due to a lack of efficacy, and 33% due to the presence of negative adverse effects [154], predominantly in the liver, phenomenon known as DILI (drug-induced liver injury) [155] or in the heart (cardiotoxicity). The principal problem resides in the use of conventional drug screening models (cell lines or animal cell monolayers), which possess a lack of predictive value of the human tissue response to the drug candidate. Therefore, it is necessary more physiological systems in order to relieve the burden of high failure rates.

The **liver** is the responsible organ for the metabolism, conversion, and elimination of a variety of substances. The majority of the drugs are transformed, there, into metabolites/active substances which could result in toxicity to the liver and to the rest of the body. The principal cause of failure at the clinical trials in humans is the use of unsuitable and inaccurate *in vitro* and *in vivo* hepatic models. In addition, the liver is target of many prevailing diseases, such as infectious HBV, HCV [156], malaria [157], overnutrition-induced (type 2 diabetes, NAFLD, fibrosis, cirrhosis) [158–160] or tumoral diseases (hepatocellular carcinoma represents the 6th most common cancer worldwide) [161]. Therefore, the improvement of hepatic models would be essential for the development of specific drugs for liver diseases.

To reproduce a **hepatic** environment suitable to study the efficacy and toxicology of different drugs, it is essential to maintain the liver parenchymal function *ex vivo*. Currently, models used for drug screening are simply comprised of a monoculture system that is maintained on a collagen substrate under static conditions. However, under these conditions hepatocytes suffer de- differentiation into fibroblastic-like cells and lose their liver-specific functions due to limited amount of juxtacrine signalling with neighbouring cells, while the majority are in contact with the substratum or the medium which is unlike the native environment. For this reason, this type of drug screening platform is not an optimal model for drug development, testing and efficacy suboptimal models for drug efficacy and safety testing [162]. The development of 2D culture models, such as sandwich culture, produced an rising of the basal and induced drug-metabolizing enzyme activities [163, 164]. Nevertheless, the absence of non-parenchymal cells and hepatic dedifferentiation continued being inherent disadvantages of these models [165]. The co-culture of hepatocytes with non-

parenchymal cells, such as Kupffer cells, hepatic stellate cells, and liver epithelial or sinusoidal endothelial cells helped overcome some of the previous limitations, recovering cellular functionality/longevity, and generating higher expression of CYP and Phase II isoforms than in monotypic culture [166–169]. However, these co-cultures result only in a random mix of diverse cell types without taking into account their specific anatomical relationships. More recently, in order to recreate much better the microenvironment, 3D cultures have been developed. These 3D cultures generally consist of spheroids [170, 171], 3D scaffold systems [172] or microfluidic *in vitro* systems [172, 173]. Up to the present, many commercial 3D co-culture devices are commercialized for drug screening, such as the "Hepatopac" platform [174], the 3D InSight™ Human Liver Microtissues of Insphero, the HepaChip® in vitro microfluidic system [175] or the Hμrel® microliver platforms [176]. Although several issues have been resolved with the models mentioned above, others continue to be biologically and technically challenging [163, 177].

Heart failure is the major cause of morbidity and mortality worldwide. The statistics by the American Heart Association show a decrease in the rates of cardiovascular diseases, in part because of current available treatments and improved patient supervision, however, the burden of disease continues to be high [178]. Consequently, robust translational models that mimic the environment of the heart failure are needed to address questions during development of new therapeutics related to target validation, pharmacodynamic's research and pharmacokinetic, and biomarker discovery.

The **cardiotoxicity** is a frequent side effect of many novel drugs. A clear example of is the recent research regarding peroxisome proliferator-activated receptor-gamma modulators employed in the treatment of type 2 diabetes. While this drug presents metabolic benefits in the treatment of type 2 diabetes [179, 180] several studies show that this drug is related to a significantly higher risk of heart failure [181]. Therefore, it is essential to establish a translational model for cardiac safety assessment to increase the limited capacity of preclinical screening assays used for the detection of cardiotoxicity. Currently, *in vitro* assays measure the toxicity in two different cell lines (CHO and HEK cells), which have been genetically modified to artificially express cardiac channels. However, due to the genetic aberrations accumulated in these cells and the failure of ectopically expressed channels to accurately model the same channels found in human cardiomyocytes [182, 183], it is very easy to obtain false negatives and positives, which can lead to the commercialization of potentially lethal drugs and the discard of valuable drugs [184–187]. As mentioned above, in pharmaceutical industries, the cardiotoxicity test models are based on cell lines, animal cardiomyocytes, and small/large animal models [188, 189]. To improve the precision of toxicity screening, preclinical drug tests should be done on adult human cardiomyocytes. However, it has not been possible due to the difficulty of obtaining these cells from patients, and the inability of expanding them in culture. The discovery and development of the iPSC, and the following derivation into cardiomyocytes have done feasible circumvent these hurdles [190, 191]. Generation of iPSC from patients suffering inherited heart disease and their differentiation into cardiac cells have been predicted to serve as a model to study disease pathogenesis and to discover novel drugs [192]. The employment of patient-specific iPSC-cardiomyocytes provides an exceptional opportunity to

renovate drug toxicity screening [193]. However, the maturation of these cells has been compared to 16-week old human fetal cardiomyocytes [194], questioning the validity of these cells as compared to the classical animal models. However, the actual progress in differentiation protocols and the combination of many innovate organ-on-a-chip platforms [195], have opened new avenues for *in vitro* engineering as they recapitulate mechanics and physiological responses of tissues in the 3D manner [196, 197]. Hence, it is vital to develop appropriate models of disease, and identify new biomarkers that are more sensitive predictors of the early on-set and/or progression of heart failure to facilitate drug discovery and development.

Kidneys are the main organs of the urinary system consisting of complex organs involved in the secretion of waste substances through urine and the maintenance of osmolarity of blood plasma, homeostasis of body fluids, the balance of electrolytes and pH of the internal environment. Furthermore, they are involved in the production of different hormones like erythropoietin (contributing to erythropoiesis) and renin (contributing to hypertension regulation).

In developed countries, hypertension, obesity, diabetes, and exposure of environmental contaminants are the main detonators of renal failure. Acute kidney injury (AKI) is the rapid loss of renal function, which can develop chronic kidney disease (CKD), reduction of the glomerular filtration rate (GFR) and terminate with end-stage renal disease (ESRD) and death [198]. Furthermore, nonrenal complications can de also developed, like cardiovascular disease (CVD), which seems to be one of CKD complications [199].

Despite this imperative urgency in finding clinical tools to reduce the effects of kidney dysfunction, therapeutic advances have failed until today. The main reasons being the lack of knowledge in renal pathophysiology, its association to CVD, poor characterization of predictive biomarkers involved in essential molecular mechanisms, and a lack of precise selection of clinical validation criteria, among others [199, 200].

The complexity in achieving a successful pharmaceutical drug development for kidney disease resides in the fact that many processes can be activated and the many cell types that are involved. Serum creatinine and blood urea nitrogen have been popular biomarkers for renal disease, but resulted in poor candidates for clinical trials because of their poor diagnostic capabilities. Currently, the most relevant molecular pathways include the deeper study of RAAS (renin-angiotensin-aldosterone system), inflammation, hypoxia, phenotypical modulation processes and extracellular matrix (ECM) remodeling [201]. Furthermore, new candidates as KIM-1 (Kidney injury molecule-1), fibrinogen and small RNA species are considered as emerging biomarkers due to characteristics like their stability, sensitivity and predictive capability in animal models, early expression and less complexity [198].

Lungs are the principal organs of the respiratory system, with the critical role of extracting oxygen from the air while removing the gaseous waste from the body. Due to the fact that airway epithelial cells are located at the interface with the external environment, this tissue

not only acts as a first barrier against inhaled irritants and allergens, but it is also involved in the immune system.

There are several cell culture models for lung drug delivery [202]. For example, SOPC1 (rat tracheal globet cell line) is used as a model for mucus secreting drug absorption, allowing the evaluation of the effect of mucus layer on drug transport in the tracheal epithelium. Another example is the use of the human cell line Calu-3, utilized in the study of drug transport at the bronchial level.

More realistic models of lung tissue are those made in a 3D configuration. For example, Klein et al. developed a system where four kinds of cells (alveolar type II cell line, differentiated macrophage-like cells, masts cells and endothelial cells) where seeded on a microporous membrane to mimic the alveolar surface, making it able to study of the toxic effects of particles within the lungs [203].

In the last few years, companies like Epithelix Sàrl or CellnTec had introduced *in vitro* cell culture models to the market that mimic airways like trachea and bronchi, with the aim to provide alternative solutions for drug development and toxicity assays. Others, like Charles River, offers services related to drug development such screening assays, *in vivo* pharmacology studies, and biomarker services.

Hug et al. Developed a human-cell based, “breathing,” lung-on-a-chip microdevice, forming an alveolar-capillary barrier on a thin PDMS membrane previously coated with ECM that recreated physiological breathing movements [204]. This represents a novel strategy that closely mimics the microarchitecture of the alveolar-capillary unit, constituting an excellent screening platform for toxicity and drug development studies.

Most of the new treatments approved for respiratory diseases are improvements of existing drugs, due to the difficulty in finding new ones. Some targets of these drugs are leukotriene receptor antagonists (used in asthma control), several epithelial growth factors receptor inhibitors (used for lung cancers) and endothelin receptor antagonists, phosphodiesterase-5 inhibitors and prostanoids (used for Group 1 pulmonary hypertension treatment) [205].

23.8.2 Developmental Biology Research

The **liver** is the largest internal gland in the body. It plays a fundamental role in metabolic homeostasis due to provide many essential metabolic exocrine and endocrine functions such as the detoxification and elimination of many substances, maintenance of blood homeostasis, regulation of glucose levels, and production of numerous products such as lipids, proteins, vitamins, and carbohydrates. In addition, the liver possesses a unique regenerative capacity, being able to regenerate most of its function after losing up to three-quarters of its mass because of a partial hepatectomy or toxic injury.

In the third week of gestation, hepatic development and organization begins continuing into postnatal period. The first morphological characteristic is the formation of the hepatic diverticulum on the ventral surface of the foregut cranial to the yolk sac. The anterior portion

of the hepatic diverticulum gives rise to the liver and intrahepatic biliary tree, while the posterior portion forms the gall bladder and extrahepatic bile ducts.

The majority of *in vitro* models employ human embryonic and iPSCs [206, 207]. However, these models do not completely recapitulate the simultaneous differentiation of liver progenitors into hepatocytic and biliary fates. The formation of mature bile ducts is particularly laborious *in vitro* [208, 209] thus needs the presence of a 3D environment for effective and suitable cellular polarization [210, 211]. Wang *et al*. demonstrated that scaffolds made from liver ECM possessed that required environmental cues [212]. In 2011, it was demonstrated as the human fetal liver progenitor cells cultured inside a ferret liver ECM developed into a native liver tissue including hepatocytic and biliary structures [19] indicating a preservation of cell differentiation signals from the ECM among different species. Recently, Vyas *et al*. showed as human fetal liver progenitor cells self-assembled inside acellular liver extracellular matrix scaffolds to form 3D liver organoids, which mimicked many aspects of hepatobiliary organogenesis and resulted in concomitant formation of progressively more differentiated hepatocytes and bile duct structures [213]. In this study, after 3 weeks in culture, there were clear changes in the phenotype of the human hepatoblasts, suggesting parallel lineage specification into hepatocytes and polarized cholangiocytes. Hence, liver tissue scaffolds contains specific and necessary ECM molecules that surround the diverse hepatic zones and regulate specific cell differentiation, function, expansion, and regeneration [214]. As mentioned above, 3D scaffold systems are an excellent model, not only for human liver development, but also for drug development and toxicity screenings. On the other hand, conventional 2D differentiation from pluripotency fails to recapitulate cell connections happening during organogenesis. Recently, the team headed by Professor Barbara Treutlein have used single-cell RNA sequencing to reconstruct hepatocyte-like lineage progression from pluripotency in 2D culture hepatic cells [215]. Then, they developed 3D liver bud organoids by reconstituting hepatic, stromal, and endothelial interactions, and deconstruct diversity during liver bud development. They found that liver bud hepatoblasts diverge from the 2D lineage, and express epithelial migration signatures characteristic of organ budding. In addition, they compared 3D liver buds to fetal and adult human liver single-cell RNA sequencing data, and that their lab-grown liver buds had molecular and genetic signature profiles very similar to those found during human liver cellular development.

The **heart** is the first functional organ in human body, which begins to beat 3 weeks after gestation pumping blood throughout the body via the circulatory system, supplying oxygen and nutrients to as well as extracting wastes from the rest of the organs. The heart is an organ with a complex hierarchical molecular, electrical, and mechanical organization thus i*n vitro* models take into account.

Despite the effort in studying the anatomy and physiology of the human cardiovascular system, little is known about the normal development of human heart and dysregulation in disease at the molecular and cellular level. Until now, the most of our understanding of the cellular and molecular basis for cardiogenesis is based on studies of murine cardiovascular development. Recently, researchers at UC Berkeley and Gladstone Institutes have demonstrated that induced pluripotent stem cells can differentiate and self-organize into

cardiac microchambers when spatially confined [216]. In this study, after 2 weeks in culture the cells, which initiated in a 2D surface environment, started taking on a 3D structure as a pulsating microchamber. Furthermore, the cells had self-organized based upon whether they were located along the perimeter or in the middle of the construct. The cells positioned along the edge experienced greater mechanical tension and stress compared to the center of the cellular mass. On the other hand, the cells in the center developed into cardiac muscle cells. Such spatial establishment was perceived as soon as the differentiation began. Hence, it is the first time that it has been demonstrated the cardiac spatial differentiation *in vitro*.

It is crucial to have an in-depth understanding of **kidney** development and regulatory pathways in order to achieve successful tissue engineering.

Kidney formation consists of two processes: nephrogenesis (where glomerulus and tubules are formed), followed by branching morphogenesis (which involves the formation of collection tubes, calyces, pelvis and ureter) [217]. Kidney development starts with the formation of the metanephric kidney, derived from the metanephric mesenchyme (the source of the epithelial cells constituting the mature nephron) and the ureteric bud (originating the epithelial tissue present in the caudal portion of the Wolffian duct). The fully developed kidney is preceded by transient kidney- like structures which do not contribute to the fully functional organ such as the pro-nephros (which degenerates in mammals) and the mesonephros (which originates male reproductive organs).

The ureteric bud formation starts at week five in human fetal gestation, induced by signals produced by the metanephric mesenchyme. Afterwards, the metanephric mesenchyme is invaded by the ureteric bud and ureteric bud branching follows. Simultaneously, cells that are in contact with the invading ureteric bud differentiate from mesenchymal to epithelial cells, which become new nephrons. This process continues up to 20–22 weeks of gestation, when ureteric branch is completed and peripheral branch segments give rise to the collecting duct development.

From weeks 22 to 44 of gestation, the cortical and medullary areas of the kidney are well defined and become morphologically different. Finally, after birth, many medullary stromal cells suffer apoptosis and are replaced by developing loops of Henle. Additionally, stromal cells from nephron tissue differentiate into fibroblasts, lymphocyte-like cells and pericytes.

Kidney ECM is composed of heparin sulfate proteoglycans, hyaluronic acid, collagens, fibronectins and laminins and ECM binds to growth factors like FGF (fibroblast growth factor), VEGF (vascular endothelial growth factor) and HGF (hepatic growth factor) to regulate their activity to support cell growth and differentiation [218, 219].

Despite the fact that there are several well defined processes of kidney development, there is still much to be understood. These investigations are crucial for having a whole knowledge for kidney origin in order to apply it in scientific studies. For example, Kaminski et al. demonstrate the transformation of human and mouse fibroblasts into induced renal tubular epithelial cells (iRECs) using four transcriptional factors (Emx2, Hnf1b, Hnf4α and Pax8) [220]. The resultant cells have many morphological, transcriptional and functional characteristics of fully differentiated kidney epithelial cells making them available for

nephrotoxic agent screening and for the study of hereditary tubular diseases and drug toxicity.

Abolbashari *et al.* used primary adult renal cells isolated from kidney cortical tissue, with the *in vitro* expression of aquaporines 1, 2, 4, ezrin and podocin, showing the presence of cells from different renal segments. Most of them expressed aquaporin 1 and ezrin, indicative of proximal tubular cells, and other expressed aquaporin 2 and 4, indicative of collecting duct cells. These cells were then seeded in kidney scaffolds, showing promising results regarding to electrolyte and protein absorption, hydrolase activity and erythropoietin production [221].

Embryonic stem (ES) cells have also been used in kidney recellularization with promising results. Song *et al.*, infused these cells via renal artery and ureter, showing their distribution into tubular and vascular structures, with cell multiplication [222]. Despite the fact that ES cells offer promising results in organ recellularization, their use is limited due to ethical questions and their teratogenic potential. For these reasons, other source of cells is employed, such as bone marrow mesenchymal stem cells (BM-MSCs), adipose tissue or amniotic fluid stem cells.

Taguchi *et al.* used human and mouse pluripotent stem cells (PSCs) to derive metanephric mesenchyme. They demonstrate that these progenitors are able to generate 3D nephric tubules and glomeruli with podocytes [223].

Lungs are tree-like structures divided into two anatomical zones, the conducting airways and alveoli.

The conducting airways begin with the trachea, which split into two main bronchi, which further branch into smaller airways, the bronchioles. These conducting airways are covered by three different epithelial cells (ciliated, globet and basal cells) surrounded by fibroblasts, smooth muscle, cartilage, vasculature and neurons. The alveolar epithelium is composed of two epithelial types I (AETI) and II (AETII) [224].

Lungs and trachea arise from the anterior foregut endoderm, the gut tube which will further originate the gastrointestinal tract and organs like lungs, thyroid, liver and pancreas. Lung development starts arounds week 3 during human gestation, with two forming buds that undergo a highly and regulated branching process to form the typical tree-like network of airways of the organ [225, 226], with an established proximal-distal axis. This is followed by the canalicular and saccular stages, where alveoli are formed, in preparation for respiration at birth. Finally, around the third trimester, full maturation of the alveolus follows, which persists for up to 3 years postnatally. During the stages of endodermal development, lung mesoderm interacts with lung endoderm, promoting branching and cell differentiation into lung lineages [226].

Despite advances in the understanding of lung development and cellular components of its epithelium the diversity and function of all mesenchymal cell types is still poorly understood. Additionally, researchers recently realize how important the vascular and neuronal networks are during lung development [227].

Most of the current models for lung development and homeostasis are based on rodent models.

ALI (Air-Liquid-Interface) method developed in the 80's supposed a great advancement in the field. This system consists on a cell monolayer of epithelial tissue grown on a porous filter that physically separates lung epithelial tissue from the underlying media, resulting in the achieving of proper apical-basal polarization. These systems are combined today with ROCK inhibitors, allowing long term cell culture.

3D environments are more recently used for primary adult human lung tissue culture. One example is the "bronchospheres", where basal stem cells from human or mouse origin are embedded in a gel, allowing the formation of spherical colonies [228]. With these structures, the self-renewal capacity of stem cells and its capability for giving rise to proximal secretory and ciliated cells is known, and they are also used as screening platforms for the study of epithelial responses against different stimuli.

Ghaedi *et al.* reported an efficient differentiation method to obtain definitive endoderm, anterior foregut endoderm and a homogenous population of alveolar epithelial type I and II cells from human iPSCs, which were then seeded into rat or human lung scaffolds. They demonstrated that iPSCs-derived AETII were able to proliferate and give rise to lung cell types [229].

Similarly, Dye *et al.* seeded hPSC-derived lung spheroids in mice lung scaffolds, obtaining promising results like the formation of airway structures, the creation of many mesenchymal cell types and ciliated and secretory functional cells [230].

Recently, proximal (airway) and distal (alveolar) models can be studied in vitro. However, full integration of both parts in a unique system is still challenging, due to intrinsic differences in each cell type such as the physical environment or ECM composition where different cell types develop [227].

23.8.3 Bioprinting

3D Bioprinting consists in the manufacture of artificial constructs. This process uses small amounts of biomaterials and cells that are precisely placed to the most miniscule detail of the organ [231]. This novel technology can be classified according to the final use in tissue and organ fabrication, or pharmaceutical investigation. Nowadays, several medicinal and therapeutic applications include the creation of personalized implants and prosthetics, drug discovery, drug delivery or dosage forms [232], as well as disease models and regenerative medicine.

The principal issue in organ failure is the large number of people waiting for a transplant, which results in long waiting lists due to the few number of available donors. 3D Bioprinting could resolve this problem using cells from the patient's own organ to create a tissue substitute, decreasing the risk of rejection and eliminating the necessity of taking immunosuppressants for live [233]. Although, the main objective of tissue engineering and regenerative medicine is to alleviate the organ donor shortage, 3D Bioprinting possesses some advantages. For example, bioprinting presents a highly precise cell placing,

concentration, and diameter of printed cells [234]. At present, 3D Bioprinting is capable of producing complex organs with a high degree of cell density [235]. However, to achieve a correct vascularization is a big challenge. Currently, none of cardiovascular tissue have been bioprinted with entire functions similar to native tissue.

In the field of the **liver research**, Organovo™ has performed 3D vascularized liver constructs containing stellate, endothelial, and hepatocyte cells with high cell viability and a solid zonation, mimicking the native hepatic lobules. Organovo™ currently offers testing services thanks to ExVive™ 3D Bioprinted Human Liver. This tissue can be used in the assessment of drug exposure for acute and chronic toxicity and metabolism studies for more than 28 days. At the present, Organovo is working on bioprinted organs for therapeutic use in humans with a therapeutic tissue program. In this program, the company is emphasizing on developing clinical solutions for pediatric inborn errors of metabolism, and for acute on-chronic liver failure and it plans to develop and conclude its liver tissue design over the next 18 months. The preclinical studies into diseased animal models have shown good engraftment, vascularization, and functionality 60 days after implantation. The company expects to file an Investigation New Drug application in 2020.

Currently, there are two options for **heart** valve replacement surgery: using a mechanical heart valve or using a biological heart valve [236]. However, using the first option requires the patient to take an anti-coagulant for life and on the other hand, biological heart valve has a shorter lifespan which may require replacement [237]. Thus, the capacity to produce bioprinted native heart valves has a direct clinical impact. Sodian *et al.* have employed them for many surgical procedures to correct everything from congenital heart defect [238] to aortic valve replacement operation [239–242] or patients with rare cardiac tumors [243]. Though the surgical models were non-living, it was a step towards bioprinting heart valves. In fact, Sodian and his colleagues were the first in using 3D printing to manufacture engineered heart valves [244, 245]. Recently, Jonathon Butcher's lab have been using this novel technology to fabricate living alginate/gelatin hydrogel valve conduits [246, 247]. When heart tissue suffers damage, the heart pumps blood inefficiently due to the loss of contractile muscle and the formation of stiff scar tissue [248] which can lead to ischemia [249]. One approach to repair the heart is to transplant cells at the site of the damaged tissue [250], however, one of the main limitations to survival of the implanted cells is the immediate availability of oxygen [251]. To solve that, Yeong *et al.* used 3D bioprinting to produce porous structures, facilitating and ensuring efficient mass transport trough the construction [252]. Others used this technology to create a construct containing human cardiac-derived cardiomyocyte progenitor cells and RGD- modified sodium alginate as the ECM [253]. Another approach is the use of bioinks generated from decellularized ECM. Cho's group encapsulated rat myoblasts cells into the heart-derived bioink and observed an increase in many cardiac-specific genes compared to collagen constructs [254]. Thus, 3D printing is a promising field, however, despite the major limitation is still the source of human cardiac cells.

The structure of a whole heart includes multiple cell types, ECM, and multi-scale structures for pumping blood. Thus, a replication using this new technology involves a great effort. BioLife4D is working in to be the next great medical achievement within heart transplants. It

is currently developing bioprinted hearts in combination with unspecialized adult induced pluripotent stem cells, which will convert into cardiac cells.

In the field of **kidney research**, Homan *et al.* reported a bioprinting method that creates 3D human renal proximal tubules [255]. The process starts with the printing of a renal proximal tubule with a a thermos degradable ink, which models the convoluted pathway of the proximal tubule. Afterwards, a layer of ECM is deposited on top of the printed structure. Then, the ink is removed, resulting in a proximal tubule mold, with a perfusable inlet and outlet. Finally, live human kidney cells are pumped into the mold and adhere, forming a confluent epithelium. This system is placed on a chip and it is able to persist more than 2 months *in vitro*. They describe a method that combines bioprinting, 3D culture and organ-on-a-chip concept, showing an epithelial morphology and functionality comparable to those observed in the same cells cultured in 2D conditions.

Once more, Organovo had performed a 3D bioprinted kidney tissue (ExVive ™ Human Kidney Tissue), which is a complete human bioprinted tissue consisting on an apical layer of polarized primary renal proximal tubule epithelial cells sustained by a collagen IV-rich interface of renal fibroblasts and endothelial cells. This architecture provides an extraordinary system for phenotypic and nephrotoxicity studies.

In contrast, very few works have been published regarding **lung** 3D bioprinting. Horváth *et al.* reported the biofabrication of a human air- blood tissue barrier analogue to lung tissue. It consists on a two cell-layer model of endothelial cells printed in a matrigel ECM bioprinted layer. They achieved the creation of an automated and reproducible way to obtain thinner and homogenous cell layers, resembling to the naturally occurred environment of the native tissue, where the epithelial cell layer is separated by a thin basement membrane [256].

23.9 Regulatory Landscape for Naturally-Derived Biomaterials

23.9.1 Regulatory Landscape

Development of a tissue-engineered product for clinical use can be challenging. Because of the novelty, complexity and technical specificity, it is essential to understand the regulations that guarantee the quality and safety of these novel products. For this goal, this section of the chapter will be focused on two regulatory agencies with similar objectives, but different systems of operation: The Food and Drug Administration (F. D. A.) and the European Medicines Agency (EMA).

23.9.1.1 Food and Drug Administration—In the US, the FDA's Center for Biologics Evaluation and Research is responsible for ensuring the safety, purity, potency, and effectiveness of many biologically derived products. The term "tissue engineered medical products," (TEMP) has been defined in a standard document of the American Society for Testing and Materials, and this terminology has been included in the FDA-recognized consensus standards database [257].

TEMP can consist of a variety of different constituents (cells, scaffolds, device...) or any combination of these and the FDA classifies these products as combination products.

Congress recognized the existence of combination products when it enacted the Safe Medical Device Act of 1990, and it was defined in the 21 Code of Federal Regulation 1270/1271 Part C 210/211/820 [258, 259].

A combination product's primary mode of action (PMOA) establishes its regulatory and product development framework and determine which center will be responsible for a particular combination product [260]. The PMOA is such an important concept that the FDA published a docket in August 2005 entitled Definition of Primary Mode of Action of a Combination Product. The PMOA is defined as "the single mode of action of a combination product that provides the most important therapeutic effect of the combination product.

23.9.1.2 European Medicines Agency—In the European Union (EU), an Advanced Therapy Medicinal Products (ATMP) is defined as being a Somatic Cell Therapy Medicinal Product (SCTMP), a Tissue Engineered Product (TEP), a Gene Therapy Medicinal Product (GTMP) or a combined ATPM [261].

The Committee for Advanced Therapies (CAT) is a multidisciplinary committee and it was established by EMA to offer high-level expertise to assess the quality, safety and efficacy of ATMPs, so this committee is the responsible for reviewing applications for marketing authorization for Advanced Therapy Medicinal Products [262]. In 2007, the European Parliament and Council of the European Union (EU) issued an amendment to Directive 2001/83/EC and Regulation No. 776/2004 to include regulatory provisions for ATMPs defined in Regulation EC No 1394/2007.

According to this regulation, when a product contains viable cells or tissues, the pharmacological, immunological or metabolic action of those cells or tissues shall be considered as the principal mode of action of the product. Therefore, a natural-derived biomaterial could be not the only actor in these fields. However, the biomaterial biocompatibility is still an essential requisite, and the new products should be subjected to the same regulatory rules as the others biomedical devices (Regulation EC No 1394/2007) [263].

In addition to the requirements laid down in Article 6 of Regulation No 726/2004 [264], the application for the authorization of an ATMP containing medical devices, biomaterials, scaffolds or matrices shall include a description of the physical characteristics and performance of the product. It should also include the description of the product design method, by the Annex 1 to Directive 2001/83/EC [265].

23.9.2 GMP Production

Control of clinical products manufacturing in both EU and the USA is exerted by the use of Good Manufacturing Practice (GMP) regulations and guidelines, to protect the patient from receiving poor quality, unsafe or products that vary from their specifications. Each regulatory body has the responsibility to apply the GMP requirements. The regulatory bodies are:

- The Food and Drug Administration in the USA

- The European Medicines Agency (EMEA) for centrally authorized products in Europe
- The National Regulatory Authorities within the various EU member states

GMP regulation includes Good Practice for Tissue and Cells and Good Engineering Practice (GEP) [266]. GMP facilities follow GMP guidelines promulged by each regulatory agency, with specialized facility designs and highly trained personnel to produce the first clinical prototype faithfully in a controlled and reproducible fashion.

The EU regulates by the publication of GMP directives and GMP Guidelines which are prepared and published in one volume by the European Commission under the auspices of Directorate General Enterprise. The US control procedures are comparable to EU's practices, whereby the GMP Regulations are published in the Code of Federal Regulations by various executive departments and agencies of Federal Government [267]. In the last years, FDA and EMA are making significant progress toward mutually recognizing each other's GMP inspections. The result of this attempt is the creation of a joint pilot program, allowing more sites to be monitored and reducing unnecessary duplication through the implementation of the International Council for Harmonization (ICH) and relevant regulatory requirements [268].

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