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**Chapter**

# Tissue-Specific Bioink from Xenogeneic Sources for 3D Bioprinting of Tissue Constructs

*Sriya Yeleswarapu, Shibu Chameettachal, Ashis Kumar Bera and Falguni Pati*

## Abstract

3D bioprinting brings new aspirations to the tissue engineering and regenerative medicine research community. However, despite its huge potential, its growth towards translation is severely impeded due to lack of suitable materials, technological barrier, and appropriate validation models. Recently, the use of decellularized extracellular matrices (dECM) from animal sources is gaining attention as printable bioink as it can provide a microenvironment close to the native tissue. Hence, it is worth exploring the use of xenogeneic dECM and its translation potential for human application. However, extensive studies on immunogenicity, safety-related issues, and animal welfare-related ethics are yet to be streamlined. In addition, the regulatory concerns need to be addressed with utmost priority in order to expedite the use of xenogeneic dECM bioink for 3D bioprinted implantable tissues for human welfare.

**Keywords:** 3D bioprinting, xenogeneic tissues and organs, xenogeneic decellularized extracellular matrix (dECM), dECM bioink

## 1. Introduction

The field of tissue engineering centers on development of tissues that are capable to regenerate and has a capacity to restore the damaged organs both structurally and functionally [1, 2]. Scaffolds that are developed to serve this purpose should be able to provide cell attachment sites and allow cell proliferation and migration while maintaining its structural and mechanical integrity [2]. Along with this, the placement and uniform distribution of cells in the scaffold play a major role to determine its functional efficiency [3]. This precise positioning of multiple cell types in an organized manner can be achieved with 3D bioprinting [4]. Plenty of natural materials, such as gelatin [5, 6], alginate [7–9], collagen [10, 11], and synthetic materials like polycaprolactone (PCL) [12–16] and polyethylene glycol (PEG) [17–22], come in handy while printing a structure. Although the above-mentioned natural materials are biocompatible, disadvantages such as mechanical instability, limited degradability, restricted cell proliferation, and differentiation challenged researchers to investigate more on natural materials [23–25]. As a result, human organ/tissue specific extracellular matrix (ECM) emerged as a best source to develop a functional tissue in laboratory conditions [23, 26, 27]. Yet, the major

limitation for this best material is its availability [28–30]. The next alternative source of ECM is to use from other species that are anatomically, physiologically, and metabolically similar to the recipient such as nonhuman primates (like apes, monkeys, and porcine) [31–33]. However, due to the risk of infections from nonhuman primates to human patients and organs from apes, baboons are abandoned, and hence pig became a suitable candidate as an organ donor for humans [33]. There is growing interest of xenogeneic ECM material as printable bioink (biomaterial formulation used for bioprinting) in the field of bioprinting due to easy access and the availability in required quantity. A process termed decellularization allows maximum removal of cellular content while retaining the ECM components from the native animal tissue to reduce the chance of immune rejection when implanted in the patient [29]. The first ever reported *in vivo* study of decellularized tissue was reported in 1991 by Krejci et al. [34], where human decellularized skin was used in mouse model. In 1995, Badylak's group used decellularized xenogeneic small intestinal submucosa for Achilles tendon repair [35]. Later, a number of decellularized ECM (dECM)-based devices are introduced, e.g., human dermis, porcine urinary bladder, porcine small intestine submucosa, and porcine heart valves [36] (**Figure 1**; for details refer to **Table 1**). In the recent past, there are several preliminary reports demonstrating the use of animal-derived dECM in the form of bioinks for developing functional tissues [27, 37]. Not only high cellular viability, these dECM-based constructs also showed enhanced differentiation and proliferation of cells into specific cell types when embedded in tissue-specific ECM [23, 27, 38]. Apart from the need to develop a fully functional construct, the foremost reason for not implanting these structures into human beings is due to high risk of xenotoxicity. Other species, being the source of material for the scaffold that has to be transplanted into human, have to undergo several stringent laws and clear all the clinical trials and ethical concerns. In this book chapter, discussion on the status of xeno-sourced dECM-based bioprinting, including the few reported preclinical studies, is included. The processing steps for dECM preparation and associated
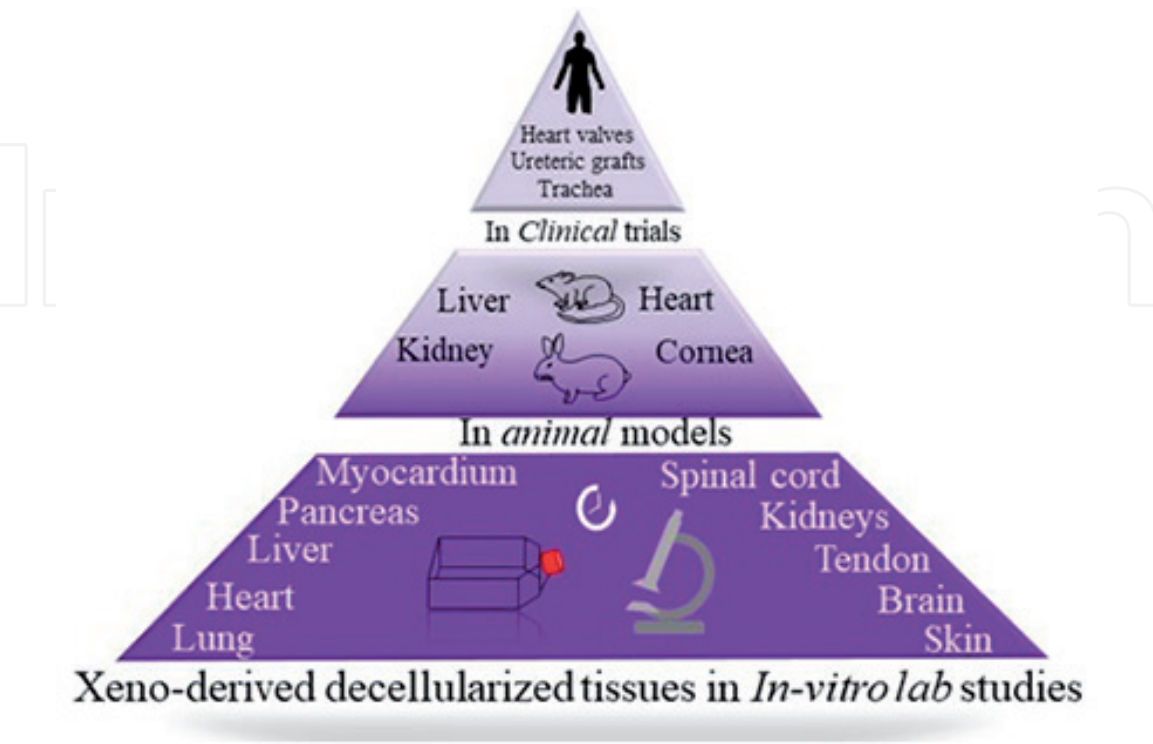


**Figure 1.**
*An upright triangle representing number of decellularied xeno-transplants that are being tested at various stages viz in vitro lab experiments, animal and human trials.*

| Source | Tissue | Cell types | Recipient | Result | Reference |
|---|---|---|---|---|---|
| Porcine | Pericardium | Human sheath synoviocyte, human adipose derived stem cells | *In vitro* culture | • Production od synovial fluid with hyaluronic acid | [126] |
| Porcine | Myocardium | Porcine adipose derived stem cells, rat adipose derived stem cells | Rat myocardial infarction model | • Stem cells expressed endothelial marker<br>• Increased vascular formation in the myocardial tissue | [127] |
| Porcine | Myocardium | Human embryonic stem cells | *In vitro* culture | • Myocardial maturation | [128] |
| Porcine | Liver | Rat endothelial cells | *In vitro*, culture and *in vivo* porcine model | • Clinically relevant vascularized bioengineered liver | [129] |
| Balb/c Mice | Liver | Balb/c Mice derived mesenchymal stem cells | *In vitro* culture | • Maturation of hepatic like tissue | [130] |
| Rat | Liver | Adult rat hepatocyte | *In vitro* culture | • *In vitro* maturation of liver with albumin secretion, urea synthesis and cytochrome P450 expression | [131] |
| Porcine | Liver | Second trimester human fetal liver cells-hepatocytes, stellate cells | *In-vitro*, culture and *in vivo* porcine model | • *In vitro* maturation of liver with albumin secretion, normal metabolic parameter | [132] |
| Rat | Heart | Rat neonatal cardiocytes, rat aortic endothelial cells | *In vitro* culture | • Increasing of left and right ventricular pressure<br>• Contraction after 8 days of *In vitro* culture | [133] |
| Mice | Heart | Human induced pluripotent stem cell-derived multipotential cardiovascular progenitor cells | *In vitro* culture | • Engineered heart tissues exhibited spontaneous contractions, generated mechanical forces<br>• Drug responsive | [134] |
| Mice | Lungs | Mesenchymal Stromal cells derived from bone marrow of adult male mice | *In vitro* culture | • Matrix from decellularized fibrotic lungs support prolonged growth of cells<br>• Decellularized lungs that are diseased can significantly affect the cell growth and differentiation | [135] |
| Porcine | Kidney | Immortalized murine hematopoietic support endothelial cell line | *In vitro*, culture and *in vivo* Yorkshire porcine | • Unseeded implanted scaffolds sustained blood pressure, renal ultrastructure maintained | [136] |

| Source | Tissue | Cell types | Recipient | Result | Reference |
|---|---|---|---|---|---|
| Porcine | Kidney | *Mice* embryonic stem cells | *In vitro* culture | • Reseeded scaffold showed HGF and VEGF levels similar to native kidney | [137] |
| Porcine | Pancreas | Human amniotic fluid derived stem cells | *In vitro* culture | • Acellular pancreas supported stem cell and pancreatic islets growth<br>• Could serve as a platform for bioengineering pancreas to treat diabetes mellitus | [138] |
| Female ICR mice | Pancreas | Acinar AR42J and beta MIN-6β cell lines | *Mice* model | • Strong up-regulation of insulin gene | [139] |
| Rats | Spinal cord | Acellular scaffolds for in-vivo, NIH3T3 cells for in-vitro studies | *In-vitro* culture and *in vivo* Sprague-dawley Rats | • Induce the regeneration of injured nerves (*in vivo*)<br>• Enhanced adhesion and proliferation of cells (*in vitro*) | [140] |
| Porcine | Brain | iPSC derived neural progenitor cells (NPCs) | *In-vitro*, culture and *in vivo* mice model | • NPC expressed neural markers in brain matrix gel (*in vitro*),<br>• Formation and assembling of larger microscale fibril like structure in gel (*in-vivo*) | [141] |
| Porcine | Skin | Human dermal fibroblasts | *In-vitro* culture | • Gene ontology showed skin morphogenesis, epidermis development | [142] |
| Porcine | Cornea | Acellular Cornea | *In-vitro* rabbits | • In-vivo good biocompatibility,<br>• Translucent cornea within 8 weeks<br>• Implants integrated into rabbit cornea without rejection signs | [143] |
| Porcine | Cornea | Rabbit corneal keratocytes, epithelial, endothelial | *In vitro* culture | • Epithelial cells showed high expressions of CK3, spindle shape keratocytes displayed vimentin | [144] |
| Porcine | Cornea and limbus | Acellular scaffolds | *In vivo* rabbit model | • Corneal transparency and epithelial integrity with no graft rejection<br>• Basal epithelial cell matured to limbal epithelial cells | [145] |

| Source | Tissue | Cell types | Recipient | Result | Reference |
|---|---|---|---|---|---|
| Porcine | Myocardium Slice | Acellular patch | Acute myocardial infarction rat model | • Firm attachment and integration with the infarcted region<br>• Neovascularization within 1 week, contraction of left ventricle wall and cardiac functional parameters improved significantly | [146] |
| Porcine | Liver | Hepatoblastoma (HepG$_2$) | *In-vivo* rodent model | • Intact liver capsule with porous acellular lattice structure with cell supportive behaviour<br>• No immunogenicity observed | [147] |
| Porcine | Heart valves (Synergraft™) | Acellular scaffolds | Human study 4 male children | • 3 Children died of graft rupture<br>• Severe inflammation<br>• Significant calcific deposits<br>• No cell repopulation of porcine matrix | [56] |
| Bovine | Ureter graft | Acellular scaffolds | Human study, 9 patients | • Acute and chronic transmural inflammation<br>• Graft failure with aneurysmal dilation and thrombosis in complex arteriovenous conduits | [148] |
| Human (allograft) | Trachea | Patient epithelial and MSC derived chondrocyte | Human study | • Immediate functional airway<br>• No immunogenic reaction | [149] |

**Table 1.**
*Various decellularized xeno derived organs that are used in in vitro, animal and human studies.*

benefits in terms of immuno-compatibility, possible immunological reactions during xenotransplantation, importance of xenografts, ethical concerns, and regulatory restrictions are also discussed.

## 2. Immunogenicity against dECM

Xenotransplantation may be the best way to alleviate the burden of allograft organ shortage from the last decade. The most enormous barrier to xenotransplantation is the immunological rejection which de-emphasizes this technique. The profound immunological rejection happens by both antibody-mediated immune response as well as cell-mediated innate or adaptive immune response. Several carbohydrate antigens have been identified that could act as targets for human natural

antibodies to inhibit immune rejection; these include Galα1-3Galβ1-4GlcNAc (referred to as α1,3Gal), Hanganuziu-Deicher (H-D) antigen, Tn, Forssman antigen, Sda antigen, etc. [39, 40]. Two antibody-mediated processes are hyperacute rejection (HAR) and acute humoral xenograft rejection (AHXR), which attack mainly the vascular system of graft tissue. HAR is mediated by natural antibodies against α-1,3Gal epitope, present in vascular endothelium of mammals except for humans, or their most recent ancestors, the Old World monkeys [31, 41]. α-1,3Gal epitope is expressed in other organisms, because of increased human interaction with these animals; anti α1,3Gal is being developed in human sera. When it binds to its antigen determinant site of anti α1, 3Gal, it activates the complement system and coagulation system to reject the graft within minutes to hours. HAR is histologically characterized by the presence of interstitial hemorrhage edema and thrombosis in small blood vessels. The depletion of α1,3Gal antibody or complement inhibition may be the best strategies to prevent HAR. But early attempts to reduce antibody by injecting a competitive antagonist of α1,3Gal antigen were unsuccessful [42] because AHXR can reject graft with a very low concentration of α1,3Gal antibody after several days or weeks. On the other hand, non-alpha Gal antigens Hanganuziu-Deicher (H-D) antigen and Sda antigen are present in vascular endothelium and on the surface of erythrocyte of all mammals except humans. The antibody against these H-D and Sda antigens is responsible for HAR and AHXR reaction via activation of complement (classical pathway) and coagulation system in α1,3Gal transferase gene knockout (GalT-KO) pigs [40, 43, 44]. The complement can also be activated via alternative pathway by islets transplantation and cause instant blood-mediated inflammatory reaction (IBMIR), resulting in an early rejection of transplanted islets [45]. The most successful approach to prevent antibody-mediated xenograft rejection is (i) transgenic pigs that express human complement regulatory protein that inhibits antibody-mediated complement activation [46] and (ii) pigs with a knockout α1,3Gal transferase gene [47, 48]. The elimination of α1,3Gal epitope extended the survival of xenograft to 2–6 months [43]. On the other hand, combination of both strategies at a time has increased the graft survival. Recently significant prolongation of graft survival was documented more than 900 days in a pig-to-baboon cardiac xenograft from α1,3Gal transferase knockout, which express human complement regulatory protein CD46 and human thrombomodulin (GTKO.hCD46.hTBM) [49, 50]. The strength of cellular rejection of xenotransplantation remains uncertain, because of difficulty in avoiding HAR and AHXR.

Xenografts are more prone to rejection when compared to allografts due to the antibodies produced by T-cells dependent activated B-cells. Inclusion of T-cell suppressive treatment significantly prolonged the survival rate (>400 days) of xenograft, where natural antibody-mediated immune rejection was suppressed [49–51]. The initial immune reaction by HAR and AHXR produced pathogen-associated molecular patterns (PAMPs) which activate the innate immune system, such as NK cells, macrophages, and neutrophils. Overcoming these barriers needs severe and sustained exposure to immune-suppressive drugs, which is very much harmful to host tissue.

All biologists are focusing on cells and intracellular contents and their regulation to escape from immune reaction, but the scenario has changed after Hauschka and Konigsberg's work in 1966 [52]. It was reported that only the ECM can differentiate myoblast to myotube formation. As the ECM has inbuilt tissue-specific matrix composition and topological cues, it may be an ideal scaffold for the use in tissue engineering. Both antibody-mediated and innate immune responses trigger by the specific receptor present on their respective target cells and inflammatory molecules like TNF, IFN, and different cytokines released upon activation of specific

cells. Decellularization is the best strategies to evade immune reaction by removing cells as well as receptors present on their surface membrane. Unfortunately, the implantation of decellularized allograft into a human produced the mixed type of result of compatibility and recipient immune response. In spite of all the hurdles, some early clinical success of ECM scaffold was achieved [53, 54], but a low level of immune reaction was identified by some group. The heart and lung xenotransplantation working group in the National Heart, Lung, and Blood Institute (NHLBI) has identified xenogeneic immune response against ECM to be a major problem to use in clinical medicine [55]. Cryopreserved human allografts are extensively used in cardiac valve reconstruction; immunologic response of these allografts has been investigated by several groups to activate the anti-HLA antibody. Hawkins et al. reported that HLA class I and II antigens reduced by 99% in the decellularized human allograft, and postoperation reactive antibody levels of HLA class I or II did not increase in children up to 12 months [56]. The inhibition of the immunomodulatory effect of decellularized tissue is obtained mainly by the removal of predominantly alpha-gal epitope along with other non-gal antigen in vascular endothelium and by removal of MHC class I and II molecules during decellularization. Although the donor-derived MHC class I became undetectable at the time of decellularization, it again reached measurable value following implantation (host-derived MHC class I) and is vascularized with host tissue [57, 58]. The underlying mechanism of decellularization on host immune response remains to be determined. Due to low or zero levels of MHC class I and II, T-cell proliferative response as well as B-cell activation is inhibited, and the anti-inflammatory effect can be seen *in vitro*, which results in the reduction of IL-2 and IFN-γ as well. As there is no MHC class antigen-presenting receptor, T cell does not recognize the foreign antigen, and T-cell-mediated immune response is suppressed. But the elevation of IL-10 fails to conclude the underlying mechanism because it has the only source from activated T cell, B cell, and macrophages [58]. It is reported that M2 phenotype in the graft prevents rejection of the xenogeneic donor tissue; however, the mechanism of macrophage activation to release IL-10 remains unknown. Till now, it is not well understood which protein and in which way decellularized xenogeneic material promotes immune reaction. The decellularized tissue may expose new protein, and the decellularization protocol may also have a significant impact on the response of human mononuclear cells [59]. Rieder et al. [60] reported that decellularized vascular wall elicited more immune cell proliferation than native equivalent, and hence, it proved the above hypothesis. It also hypothesized that opsonization would be the way of inflammation response and can occur through preformed antibody or binding of unspecific plasma protein to the surface. In genetically modified organism, (pig) alpha-gal epitope is knocked out, and it does not elicit immune response in decellularized tissue, but in unmodified xenogeneic tissue, some amount of alpha-gal antigen may be retained, and that could be enough to stimulate immunogenic response. However, further study is needed to find out the mechanism of immune response with regard to decellularized matrices.

**2.1 Strategies to resolve immune reaction against xenogeneic DECM**

Xenogeneic dECM has a huge potential to be used in tissue engineering and regenerative medicine; some early enthusiastic studies in animal and clinical trials using decellularized tissues resulted in severe inflammatory reaction, fibrous overgrowth, and tissue destruction [61–64]. Despite all these immunological reactions, in recent years xenogeneic biomaterials are being used in abdominal surgery [65–67]. There have been some early studies, where glutaraldehyde cross-linking in native matrix inhibits immune response by the modification of surface area of tissues

that inhibit the interaction with peripheral blood mononuclear cell (PBMC) and in turn T-cell activation [68]. But the problem of glutaraldehyde fixation is that it can change the tissues' topology and promote their degradation by calcification [69]. The natural cross-linking product quercetin, a plant flavonoid pigment, may be more effective, which increases mechanical strength and reduces immunogenicity [70].

## 3. Importance of xenografts in dECM-based bioprinting

Organs in the human body are extremely complex structures consisting of multiple cell types arranged in defined spatial organization, with varied ECM composition. It is due to this balanced and organized compositions that organs achieve perfect functionality [71]. Any disruption to this native structure alters the functionality of the organ drastically. The demand for organ transplantation is increasing exponentially due to the rise in traumatic injuries and changes in lifestyle, while the supply of organs increased marginally over time. The demand for organ transplantation is estimated to further rise with the advancements in diagnostics leading to early detection of diseases [72]. Researchers all over the world have been striving hard to find alternative strategies to reduce this gap for many years, using a combination of many materials along with cells [73]. As a result, researchers developed comparatively simple organs using tissue engineering approaches, such as artificial skin [74], cartilage [75], and trachea [76] that display a part or nearly full functionality of the particular tissue. Xenotransplantation is another promising approach that was started in early 1920s and has a potential to serve as a temporary measure to save patient's life in the absence of allogenic organ [77]. Nevertheless, the barriers such as graft failure due to immune reaction [63] and infections from the graft to the patient prevent the acceptance of xenotransplantation as a treatment option. Consequently, an emerging technique, 3D bioprinting, revolutionized the field of tissue engineering and regenerative medicine exhibiting its potential to develop complicated organs [78]. To fabricate a scaffold, this technique uses materials that are biocompatible and cells that are tissue-specific, while the best biomaterial to develop a tissue that eventually goes to human body is the material derived from that specific tissue, viz., ECM, as it can provide reseeded cells with local tissue environment [23]. This property of tissue-derived material can anchor cells and provides sufficient biochemical and mechanical cues allowing them to proliferate and differentiate to those tissue-specific lineages which ultimately aid in complex tissue formation [79, 80]. Ideally, autologous tissues are expected not to illicit an immune response after implantation, thus reducing the chance of organ rejection. However, due to the lack of sufficient autologous tissue, allogeneic tissues are chosen for transplantation. Allogeneic tissues also suffer from rejection from the host due to antibody-mediated rejection or T-cell movement into the allograft [81]. Genetic dissimilarity between donor and recipient turns out to be the main cause to induce immune response and eventually rejection of the graft [81]. Hence, the process of decellularization when applied on allogeneic tissues reduces the amount of genetic material, thereby allowing graft survival in the host [82]. But, the final yield of material after all the processing of tissue is very low and is insufficient for printing a higher volume 3D structure. Because of which, considering patient's own tissue or tissue from the same species for development of bioink is not practical. The very next alternative that researchers explored was to obtain tissue source from other species and use its matrix as a bioink for tissue development [23]. The concept of using other species (porcine) tissue as a source of material for humans emerged due to the anatomical and physiological similarities between both the species [83, 84]. Apart from the cellular content, organs are rich in the noncellular component, i.e.,

ECM [85]. In almost all the tissues, ECM proteins are produced by the resident cells [85, 86]. Many macrolevel molecules, growth factors, and fibrillar proteins in varied quantities constitute this considerable volume of the tissue [85]. Polysaccharides and proteins such as glycosaminoglycans (GAGs), hyaluronan, collagen, fibronectin, laminin, and elastin are the major ECM components in an organ [85]. These ECM components allow cell adhesion and cell migration, provide biochemical and mechanical properties, and impart elasticity that helps cells to obtain morphological orientation and physiological functionalities. Of all the ECM components mentioned, collagen is the most abundant protein which almost covers 30% of the protein content present in multicellular organisms [85, 86]. In vertebrates, as many as 28 different types of collagen are recognized with 46 distinct polypeptide chains, and the sources of collagen are abundantly available from marine animals to animals that live on land [87]. The main role of this profoundly available protein is to provide mechanical strength, maintain cellular adhesion, and support migration and other cellular functionalities that direct mature tissue formation [85]. To develop tissues like bone [88], skin substitutes [89], small intestine tissue [90], skeletal muscle tissue [91], collagen that is extracted from xenogeneic sources has been used extensively in research works. Elastin is another ECM component that connects with collagen to provide elasticity to the tissue. It is due to this close association; elastic nature of tissue is being maintained. To develop constructs *in vitro*, along with the exposing cells to abundant proteins, enough mechanical properties are to be provided [85]. Hence, it is necessary to include elastin components into the engineered scaffold which imparts mechanical properties to the tissue. By combining the proteins, viz., different types of collagen and elastin, a reasonable amount of work has been done on blood vessel engineering, heart valve development, tissue-engineered vascular grafts, musculoskeletal tissues, cartilage, and skin engineering [92]. The other fibrous protein that contributes for organization of ECM and is responsible for cell functionality such as cellular attachment is fibronectin. Scaffolds that are functionalized with fibronectin enhanced properties such as cell adhesion [93, 94], promoting elastin deposition [95]; cellular migration responsible for tumor metastasis [96, 97] has been reported in literature. When it comes to engineering a tissue *in vitro* using 3D bioprinting, the material should be biocompatible as well as print friendly. Components of ECM such as collagen, elastin, and fibrin were explored for them to be used as bioinks either separately or in combination with one another in 3D bioprinting technology. The potential of collagen as bioink was displayed for developing human skin model with keratinocytes and fibroblasts [98], cartilage tissue engineering [99], 3D collagen-based cell blocks that exhibited osteogenic activity [100], and osteochondral mimicking structures [101] and in bone regeneration applications [102]. The use of fibrinogen as a bioink was also reported for developing cartilage [103, 104] and vascular grafts [105]. The immune response to xenogeneic collagen in human models was reported to be not adverse, and in most of the cases, the presence of antibodies for xeno-derived collagen was due to by-products during acceptance of implanted graft by host [106]. It is also reported *in vitro* experiments conducted with collagen and elastin derived from porcine and bovine did not trigger immune cells nor trigger proliferation of isolated B and T cells [107]. Nonetheless, to mimic native tissue environment for enhanced cellular functionality, a combination of all the proteins and macromolecules is required. Hence, instead of using all the macromolecules separately in varied amounts, researchers started using ECM of the tissue for tissue engineering and 3D bioprinting applications (**Figure 2**), thereby providing all the necessary cues to the reseeded cells in essential amounts. For better acceptance of the 3D printed structure with ECM, decellularization of animal tissue is done to remove the maximum cellular content prior to 3D printing process. This reduces the chances of xenogeneic
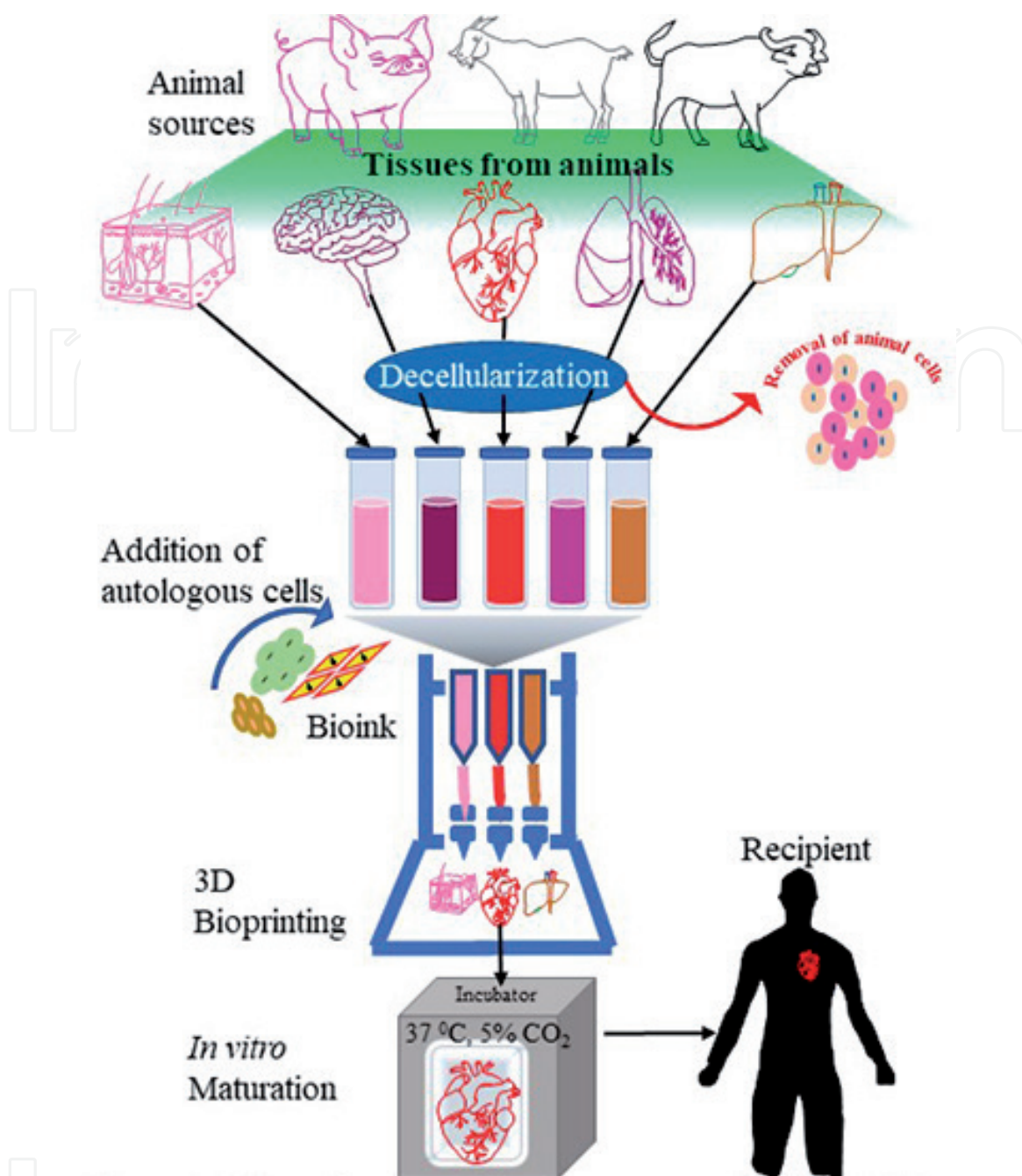
**Figure 2.**
*Schematic representing the process of 3D bioprinting, in vitro maturation and transplantation of tissues developed from animal tissue derived decellularized extracellular matrix.*

rejection in human body. In the next section, the use of dECM as a bioink for 3D printing applications is discussed.

## 4. Current status of the xenografts application in bioprinting

The process of decellularization dates to 2000s, wherein organs such as skin, vascular tissue, and bladder were decellularized. In 2014, it was first shown that after decellularization process, the ECM that is devoid of cellular material could be used as a bioink for 3D printing applications [23]. In the recent past, almost all the organs have been subjected to the process of decellularization and used for 3D bioprinting. With 3D bioprinting of decellularized organs such as the heart, liver, cartilage, adipose tissue, skeletal muscle, skin, etc., researchers have demonstrated the potential of dECM-based constructs in terms of cell compatibility, cell

attachment, migration, and proliferation. Decellularized heart matrix derived from porcine showed an enhanced expression of myosin heavy chain [23] and expression of transcription factors by cardiac progenitor cells [108]. The functionality of 3D engineered heart, developed from decellularized rat heart, was also demonstrated in one study [109]. Similarly, decellularized liver matrix from porcine exhibited consistent secretion of urea and albumin up to 14 days of culture [110] and higher levels of markers suggesting hepatocyte maturation [27]. Early adipogenic marker and lipoprotein lipase were notably observed in human-derived decellularized adipose tissue [23]. However, there is a need of further *in vitro* experiments on decellularized matrices, to completely replicate the complex geometry of the organs. With the current state of art, the *in vitro* models can be tested for immune response in animal models. For any biological material that is being implanted should contain as less as 50 ng/mg of DNA content for not eliciting the immune response in host body. To ensure this low level of nucleic acid content, the process of decellularization of xeno tissues must be stringent and harsh. Detergents such as SDS and Triton X served as chemical agents to remove the maximum DNA content from tissues in decellularization process. Using chemical treatment, acceptable level of DNA content was achieved in almost all the tissues decellularized so far. Apart from DNA nuclear material, Gal epitopes present in animals are also found to be responsible for acute implant rejection [23]. There are few reports from literature wherein 3D dECM scaffolds have been implanted in animal models to understand the host response. In one study, scaffolds that were fabricated using decellularized adipose tissue derived from porcine were implanted into mice. Due to significant reduction in DNA content and gal epitopes, the ECM grafts showed no signs of inflammation or necrosis. Also, there was formation of neo-adipose tissue with mature adipocytes supporting adipogenesis and acceptance of a xenograft [111]. Porcine-derived skin was also subjected to decellularization to show its potential in skin tissue engineering. Using chemical such as trypsin/EDTA and Triton X, the decellularized skin matrix was digested to form bioink, and a skin substitute was printed. This, when implanted into the wound of 10 mm in mice, accelerated wound healing was observed when compared to control groups. Further, immuno-fluorescence staining showed early differentiation markers for epithelial tissue and CD-31 signifying re-epithelialization and vascularization, respectively [112]. The reported results exhibit the acceptance of xeno-derived dECM-based 3D bioprinted scaffolds by the host tissue. This is made possible due to the stringent chemicals and enzymes involved in decellularization process. Nevertheless, much more studies and experiments both *in vitro* and *in vivo* must be done for using these scaffolds as replacement of deceased parts in the human.

## 5. Regulatory facets of xeno dECM-based tissue transplantation

Although the prospective benefits are unquestionable, the use of xenogeneic products in human health care raises a number of issues; hence it has to be controlled strictly by the regulatory bodies to avoid complications. The duty of regulatory bodies is to regulate the indiscriminate use of animal-sourced material intended for human health application. The challenges include (1) the potential risk of transmission of infectious agents from source animals, (2) informed consent related issues, and (3) animal welfare issues [113].

From the preclinical testing, the regulations are made strict for the human welfare before use in clinical trials. In general, enough studies have to be performed for safety characterization of therapeutic agents including the efficacy or the activity and the toxicity or undesired effects to the host system. This type of potential clinical

risks constitutes an important component of an FDA regulation. Transfer of animal microorganisms to the recipient with the graft during xenograft transplantation is another major concern for regulatory authorities [114]. There are reports that HIV, hepatitis B and C, Creutzfeldt-Jakob disease, and rabies can be transmitted between humans during transplantation. It is also proved that contact between animals and humans during animal husbandry and from pets or food products can lead to zoonotic infections. So, the use of animal cells, tissues, and organs in any forms keeps the public health at risk with known and unknown infections. Hence it is advised to go for thorough screening for all kind of possible zoonotic infections by following the standard protocol [113]. Moreover, the risk of these microorganisms or virus getting adapted to human-to-human transmission is also a major factor that has to be considered, which might be a concern for general population [115]. When it comes to cross-species whole organ transplantation, there is unavoidable transfer of endogenous retrovirus that is existing in the genome of all porcine cells into the patient receiving the organ. However, there exists no documentation regarding the transfer of these viruses in humans who are exposed to pig organs [116], probably due to the lack of long-term observation.

Preclinical studies provide valuable insight into the safety issues before being used in the human volunteers. Animal welfare is a major concern during the application of xenogeneic products in humans. Since animals' welfare is a major ethical issue, it is considered by regulatory bodies before approving any product of animal origin for clinical use.

Also, during the clinical trial stages or in long term, the volunteers or the patients and the close contacts should be educated about the chance of infectious disease risks and about how to manage those risks. Moreover, such counseling should also be continued for long term as some infection may take years to get manifested. Also, lifelong surveillance is advised by FDA irrespective of the status of the implant or graft or other xenotransplantation product.

Conversely, 3D bioprinted *in vitro* organs and tissues that are being developed using dECM are expected not to pose potential threat to recipients. This is because the cell and nucleus materials are being removed from the tissue using harsh chemicals during the process of decellularization. However, the regulatory bodies ensure that xenotransplantation is allowed only when there are evidences that show near-zero chance of recipient getting infected and informed consent, and acceptance for lifelong postoperative care from the patient was collected [116]. Nevertheless, stringent regulations will be required from regulatory bodies to monitor the pros and cons for a longer duration.

## 6. Ethical and safety concerns

There are numerous challenges and hurdles being faced for translating xenogeneic products to the clinical level. Though the potential of tissue- or organ-derived bioink for 3D bioprinting is getting proved and accepted, to reach human level it must overcome ethical concerns apart from dealing with technological and regulatory challenges. The opinions expressed on ethics behind using xeno-derived material for humans are based on the source of material and the consequence after transplants, which are already mentioned in the regulatory facets [117]. There are few groups who argue that the primary idea of using animal organ into human is unethical, while few claiming that the detrimental outcomes after the transplant are unacceptable [118]. The apprehension on the outcomes of the xenotransplantation seems valid as there are reports in the literature suggesting that patients who received the animal organs survived only for a short span [77]. The use of animal

organ in patient started in the twentieth century. Organs such as liver, heart from baboon [119], and kidney from chimpanzee [120] were transplanted to patients who survived for a very short lifespan ranging from 20 to 195 days after the implantation [77]. Immune rejection is the primary reason for failure of the graft [77]. Apart from immune response from the host, there are insufficient scientific evidences about the risk of transmission of pathogens that are passive in animal species [117]. Though it is proven that these microorganisms that are existing in animal species are not harming them, it could be fatal when they enter other species [117]. It is ethical to have an informed consent from the patient, not only regarding the transplantation but also about all the further complications that could arise due to the foreign material being placed inside [117, 121]. With xeno-organ transplantation, the risk of animal virus and microorganisms entering human body is expected to rise [121]. Apart from this, there are a lot many unknown viruses that are hosted by animal species whose effects are not at all predictable [117]. Hence, the recipient should also be informed about the risks and preventions that he/she must take posttransplantation, restricting his freedom [121, 122]. Further, to increase the success rate of transplants, recipients are constantly under the influence of immunosuppressant drugs, which would enhance his chances of other infections [117]. However, immunological reactions are not reported much after using dECM 3D bioprinted constructs. Additionally, one has to justify whether the amount that is being spent on xenotransplantation research for translation to clinical level is really worth, as it can help a relatively smaller group of people. Furthermore, for animal welfare, there are animal-related ethical issues which are considered important similar to human ethical issues [123]. Some groups believe that, the use of animals to fulfill human needs is strongly unethical, while few accept that if the benefits surpass the degree of suffering of animals, then there is no harm to use animal organs for saving human life [124]. Almost all the vertebrates suffer and perceive pain in a similar way [121]. Producing transgenic animals for organ transplantation also received criticism, as during this process, much more pain and suffering is imposed on animals due to multiple experiments in succession. In order to reduce the chance of viral transmissions, these transgenic animals are quarantined and kept in isolation [121]. Hence, the supports for animal welfare argue that the animals that undergo genetic engineering technique will be deprived of its natural habitat and are forced to live in a secluded place with pain and agony [117]. Will this suffering of an animal be the guarantee that its organ is successfully put into use remains as an unanswered question. Apart from ethics, religious feelings also come into play. A pig that is considered to have similar genetic and physiological traits similar to human [125] is considered unclean in many religions but is considered as a versatile model in biomedical research. On the other hand, if the benefits and safety of xenotransplantation is proven for human well-being, dealing with animal ethics could be vindicated. Nevertheless, how well the community approves and agrees to the use of transgenic animal organs for transplantation to serve humans is yet to be understood.

## 7. Future perspective

We believe that the severity of some disease conditions will be able to justify the use of xenogeneic therapeutic options, but the risk and benefits must be evaluated and concluded at the earliest. The most important concern, infectious disease transmission, including the chance of latent viral infections, must be studied in a larger picture including all possible disease transmissions. Though studies are limited, severe immunological reactions are not reported by using decellularized bioinks till date indicating its future potential in regenerating organs and tissue. Large

population studies are required to rule out the possibilities of rejection. A well-defined animal source is also required as species close to humans are not preferred. The animal husbandry conditions must be defined and should start dedicated farms isolated from other animals and be monitored regularly to avoid unexpected or non-listed diseases. Moreover, an unquestionable monitoring system for animal welfare conditions is also important during the raise in the use of xeno-products in human.

## 8. Conclusion

The tissue-derived decellularized extracellular matrice bioink is the latest trend in the field of 3D bioprinting. The 3D bioprinted constructs from xenogeneic dECM are yet to be studied and analyzed extensively. However, the immune response to xenogeneic collagen, the major dECM-derived bioink component, in human models is not induced by any complicated immune reactions in the host. Though studies are in progress, the 3D bioprinted constructs with xenogeneic dECM bioink are least studied for safety and efficacy despite immune reactivity studies. The animal welfare-related issue is untouched. The initial studies using xenogeneic decellularized matrices are tempting; therefore it is worth to speculate that 3D bioprinting with xenogeneic dECM can revolutionize the field of regenerative medicine.

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## Conflict of interest

The authors declare no conflict of interest.

## Author details

Sriya Yeleswarapu, Shibu Chameettachal, Ashis Kumar Bera and Falguni Pati*
Department of Biomedical Engineering, Indian Institute of Technology Hyderabad, Sangareddy, Telangana, India

*Address all correspondence to: falguni@iith.ac.in

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