Review Article

Clean Bioprinting – Fabrication of 3D Organ Models Devoid of Animal Components

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Abstract
Bioprinting is a rapidly developing technology that enables the exact positioning of living cells embedded in biomaterials in precise spatial arrangements to fabricate engineered tissues and organs. While the ultimate goal of bioprinting approaches is to produce organs for transplantation purposes, bioprinted organ models also hold great potential for research purposes to serve as alternatives to animal experiments. By using human cells, humanized organ models can be generated that may produce more relevant results for human (patho-)physiology than animal models. However, standard bioprinting procedures currently use numerous hidden animal components. Virtually all studies published in the field to date make use of cells grown in media with fetal bovine serum (FBS). In addition, Matrigel, the extracellular matrix (ECM) harvested from Engelbreth-Holm-Swarm sarcoma grown in mice, is widely employed to cultivate stem cells and 3D organ models. Finally, most bioinks currently in use contain gelatin or comparable animal components to improve cell viability and adhesion. The present review will give an introduction to the potential of bioprinting to fabricate 3D models that may be substituted for animal experiments and will go on to describe strategies to replace animal components currently included in standard procedures of bioprinting. These approaches comprise the adaptation of cells to FBS-free media, the use of bioinks composed of synthetic or plant material, and the replacement of animal ingredients by materials of human origin. We propose denoting bioprinting strategies devoid of animal components as *clean bioprinting*.

1 Introduction

The current biomedical research paradigm is based on initial studies in 2D cell culture followed by animal experiments. Conventional cell culture, however, does not reflect the three-dimensional architecture of natural organs that influences cell features, such as gene expression patterns. Animal models provide the opportunity to study (patho-)physiological phenomena in a functional biological system. Their major scientific drawbacks are species-specific differences that limit the relevance of animal studies to humans. The degree of this problem is highly controversial. A prominent example is the discussion centered around the predictivity of animal models in inflammation research. While the initial study by Seok et al. (2013) came to the conclusion that genomic responses to inflammatory stimuli in mice poorly correlate with human inflammatory diseases, a subsequent study analyzing the same dataset came to the opposite conclusion (Takao and Miyakawa, 2015). A more recent publication suggested that some mouse models can provide predictive insights, while others cannot (Weidner et al., 2016). A field in which species differences become particularly apparent is infection biology. Many pathogens have a narrow host tropism and only infect a single or a small number of species. A common strategy to cope with this problem is to adapt a human-pathogenic virus to the test animal, which, however, often results in a different course of disease in animals, as will be outlined in more detail below.

Furthermore, the high failure rate of drug candidates in clinical testing can, at least to some degree, be ascribed to differences in animal and human physiology. Although pre-clinical testing involves multiple animal models to evaluate efficacy and toxicity of a substance, approximately 90% of the candidates fail during clinical development, a number that varies substantially between different indications and is as high as 97% in oncology (Wong et al., 2019a). The main reasons for failure in clinical development are low efficacy and unexpected toxicity, which is to a certain extent due to species-specific differences in physiology between test animals and humans. Protein-based biologics, such as monoclonal antibodies and recombinant proteins, are among the most advanced therapeutics. Their immunogenicity in humans, however, is particularly difficult to predict in animal models. In

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addition to failure in clinical trials, a large number of drugs has to be withdrawn from the market after their approval. As of 2016, a database comprised 578 withdrawn drugs, almost half of which were discontinued due to adverse reactions and toxic effects (Siramshetty et al., 2016).

Due to the uncertainties involved in predicting human toxicity, our current approach to investigate properties of drug candidates in pre-clinical development with animal experiments is under debate (Van Norman, 2019). Recent progress in the development of 3D organ models may help to overcome the problems inherent in predicting the efficacy and toxicity of drug candidates (Weinhart et al., 2019). As human cells can be used for *ex vivo* experiments, these models can be expected to reflect the human (patho-)physiology better than animal models. Conventional 2D cell cultures with human cells provide some insight, but their significance is limited, as cells behave differently in natural organs with 3D cell-cell contacts and interactions with different cell types. Bioprinting is a particularly promising technology for the generation of organ models with high spatial precision (Crook, 2020). Compared to other 3D technologies, a key feature of bioprinting is its high accuracy and reproducibility. However, bioprinting is a highly sophisticated technology, and extensive training and expertise are required to fully exploit the potential of the technology. A major issue that will be discussed here in detail, is that virtually all bioprinting studies reported so far (as well as all other methods to produce 3D tissue models) include the use of animal components such as fetal bovine serum (FBS), animal extracellular matrix (ECM, e.g., Matrigel™) or gelatin.

The present review will summarize the current state of the bioprinting field to produce organ models that have the potential to replace animal experiments. We will then identify components of animal origin that are widely used in bioprinting and discuss alternatives for their replacement with synthetic or plant-derived materials. We suggest denoting approaches completely devoid of components of animal origin as *clean bioprinting*. Most importantly, we will argue that this concept will only have a chance to become widely established in the scientific community if the advantages of avoiding human-animal chimeric systems for obtaining human-relevant research results can be demonstrated, rather than just citing the singular consideration of improved animal welfare.

2 Bioprinting technologies

Three-dimensional printing refers to a process of building 3D objects by successively adding material in a layer-by-layer manner. It is often referred to by the technically more precise term *additive manufacturing* (AM). The process is normally controlled by computer-aided design (CAD) programs (Fay, 2020). Bioprinting is a specific variant of the AM process, which is characterized by the inclusion of living cells, biocompatible materials and, in many cases, biologically active factors. The procedure aims at fabricating multi-cellular tissues or organ equivalents with high spatial precision. In a broader sense, bioprinting also includes printing of biocompatible materials to produce scaffolds at high 3D resolution that can subsequently be populated with cells. The present review, however, will focus on approaches that work with cell-laden bioinks, i.e., include living cells during the printing process.

Multiple 3D printing technologies are available, but the majority of current bioprinters make use of material deposition techniques such as extrusion bioprinting, inkjet bioprinting or modern light-based techniques, including laser-assisted and stereolithography bioprinting (Fig. 1). These major technologies will only be briefly introduced here, and the reader is referred to excellent and exhaustive review articles on general approaches to bioprinting published recently that go into depth with the technology for further details (Heinrich et al., 2019; Matai et al., 2020; Mota et al., 2020; Sun et al., 2020). It should be noted that each of the bioprinting technologies has its specific advantages and disadvantages, and the appropriate method needs to be chosen for each biological application.

The most widely used bioprinting technology is extrusion bioprinting, in which a viscous bioink is extruded through a nozzle and then remains localized upon deposition (Fig. 1A). For the pneumatic systems, air pressure extrudes highly viscous bioinks as seamless filaments that are then cross-linked by light, enzymes, chemicals or temperature to form mechanically durable structures. The mechanical systems are usually controlled by a piston or a screw. The continuous extrusion of the bioink without interruptions is advantageous to maintain the integrity of the printed constructs in comparison to dropwise methods described below. The extrusion process also allows processing of highly viscous bioinks and high cell numbers; however, the printing speed is relatively low, and the resolution of the printed constructs is lower than with laser-based methods. In addition, cells may experience harmful sheer stress on extrusion, so the bioink and printing conditions must be optimized to maintain high cell viability.

The technology of inkjet bioprinting is derived from conventional 2D inkjet printers (Li et al., 2020). It is a non-contact process during which picoliter-sized droplets are deposited in a computer-controlled manner (Fig. 1B). The liquid is dispensed by temporal deformation of the internal space within the nozzle due to piezoelectric or digitized thermal actuation. Advantages of inkjet bioprinting include the simplicity of the method and its low cost as well as comparatively good resolution and high cell viability. Major disadvantages are the low cell density in the bioprinting process and the restriction to bioinks of low viscosity.

Laser-based technologies differ fundamentally from the nozzle-based bioprinting approaches described above and allow very high spatial resolution. Bioprinters based on laser-induced forward transfer (LIFT) usually consist of a pulsed laser, whose beam is absorbed by a layer below which the bioink is located in a donor ribbon (Fig. 1C). When the focused laser beam reaches a desired site of the energy-absorbing layer, the corresponding location of the supporting donor layer is vaporized, which causes ejection of a droplet of the bioink that falls onto the collector platform. Another light-based bioprinting technology is stereolithography (SLA). The basic concept of this method is to selec-

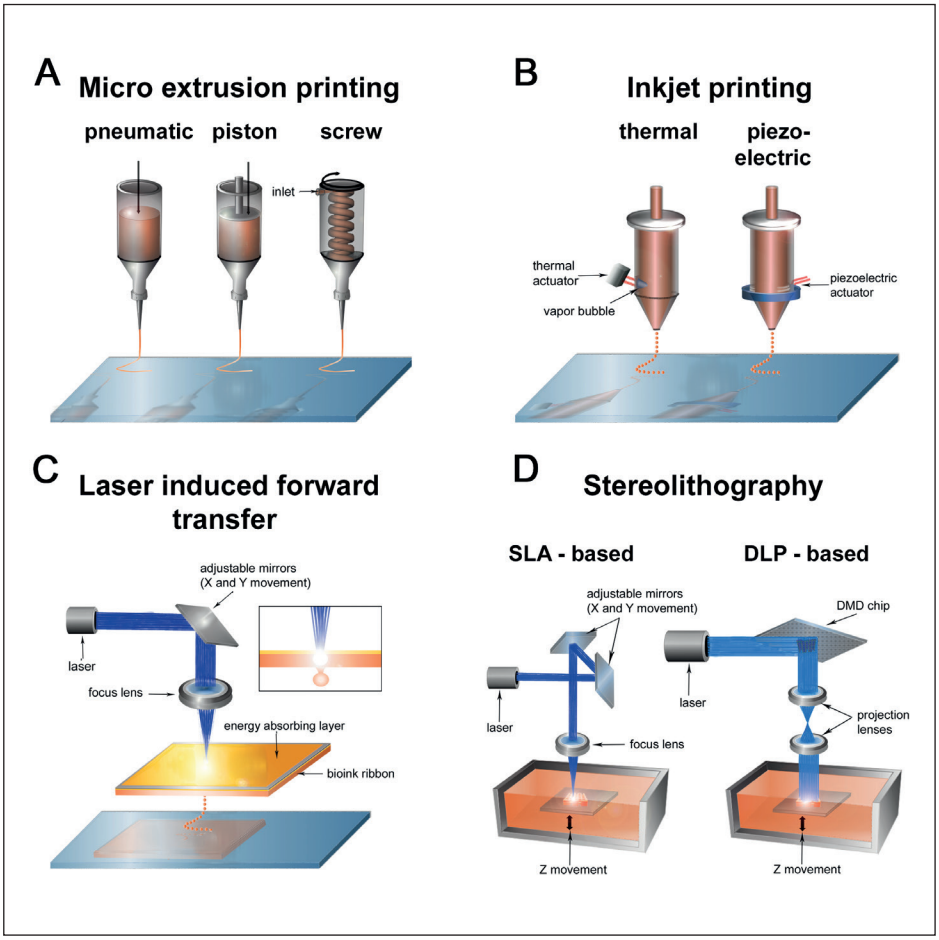

Fig. 1: Schematic representation of the most commonly used bioprinting technologies
(A) Extrusion bioprinting, (B) inkjet bioprinting, (C) laser-induced forward transfer (LIFT), and (D) stereolithography. SLA, stereolithography; DLP, digital light projection

tively cure a cell-laden bioink in a layer-by-layer process to build up materials (Fig. 1D). A laser-beam cross-links photosensitive bioinks by projecting a 2D pattern of the plane of interest onto the bioink reservoir. Digital light projection (DLP) operates similarly to SLA bioprinting. The main difference between the two is the source of light. In contrast to SLA, where a laser beam solidifies the material of each layer in a point by point-like manner, in DLP a complete layer is solidified at once using a digital micro-mirror device chip. The laser-based systems are usually fast and cell-friendly, as there is no direct contact between the dispenser and the bioink; however, the laser beam and the cross-linking photo-initiators may harm the cells or their genetic material. The instrumentation is also comparatively complex, making the costs of the technology high.

3 Bioinks

In addition to the bioprinter hardware, the material used during the bioprinting process determines the ultimate outcome of the process. It is commonly denoted as bioink and can be defined as "a formulation of cells suitable for processing by an automated biofabrication technology that may also contain biologically active components and biomaterials" (Groll et al., 2018). Bioinks need to combine multiple properties: They must initially be fluid to be printable but then rapidly transition to the solid state to form and maintain the printed structure. At the same time, they must be biocompatible to guarantee high viability of the printed cells. Common currently used bioinks are cell-laden hydrogels, i.e., cells in cross-linked polymeric substances capable of absorbing and retaining large quantities of water. Innumerable different types of bioinks have been developed, and it is of utmost importance to match the bioink, the printing technology used, and the cell types, as their interplay determines the outcome of the bioprinting approach (Fig. 2). For the most widely applied method, extrusion bioprinting, the bioink flows through the nozzle in a low viscosity state and then rapidly gels by cross-linking, either induced by chemical treatment or irradiation.

It should be noted that the description of natural and synthetic materials used for bioink development given below is far from complete. We have focused on the most commonly used materials and their origin, as well as their strengths and disadvantages. For further details on these substances as well as on bioinks with additional components such as hyaluronic acid, silk and other materials, the reader is referred to excellent and exhaustive reviews published in recent years (Gopinathan and Noh, 2018;

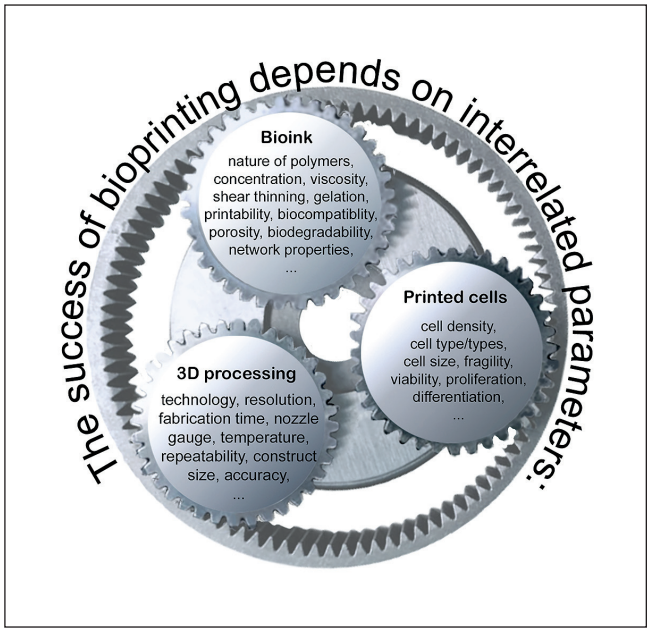

Fig. 2: Parameters determining the outcome of a bioprinting approach
A basic classification of bioinks can be made into natural materials derived from living organisms and synthetic materials. The present review aims at establishing the concept of clean bioprinting free of animal components to avoid chimeric systems for scientific reasons and for the sake of animal welfare. In this respect, it is important to sub-classify the natural bioinks further into materials of animal and non-animal origin, which is discussed in more detail in Section 6.

Gungor-Ozkerim et al., 2018; Hospodiuk et al., 2017; Sun et al., 2020). None of these articles, however, specifically investigated the use of animal materials in humanized organ models as we do below.

3.1 Natural bioinks

3.1.1 Collagen and gelatin

Collagen is the main structural protein in the ECM in various connective tissues, making it the most abundant protein in mammals. Approximately 30 types of collagen are commonly differentiated, type I collagen being the most common form by far. It belongs to the fibril-forming collagens and consists of three polypeptide chains that form a triple-helical structure. Collagen is a widely used component of hydrogels in bioprinting applications, as stated in recent reviews: "Collagen-containing hydrogels are currently the most popular cell scaffold and material for tissue engineering" (Osidak et al., 2020) and "the use of collagen-based bio-ink is prevalent in skin bioprinting" (Ng et al., 2016). Collagen for research purposes is usually of bovine or porcine origin or derived from rat tails.

Collagen has various desirable properties for its application in tissue engineering, including high biocompatibility and comparatively low immunogenicity (Hospodiuk et al., 2017). Furthermore, it facilitates adhesion of cells and enhances their growth. On the downside, the mechanical properties of collagen pose some challenges to its use in bioinks. Type I collagen molecules are mainly acid-soluble and remain in a liquid state at low temperatures. They start to gel when pH and temperature values are adjusted to near physiological conditions. Collagen's slow gelation kinetics, however, make its use in bioprinting of 3D constructs difficult. A solution to this problem that is described in more detail in Section 4.2 is the use of a support bath into which the collagen construct is printed (Lee et al., 2019). Following complete gelation of the collagen structure, the support material is removed and the stable form is set free.

Gelatin, widely used in food and pharmaceutical industries, is the denatured form of collagen and can also be used as the basis of bioink. Disruption of the typical collagen triple helical structure and its degradation result in lower immunogenicity of gelatin compared to collagen (Su and Wang, 2015). A great advantage is that gelatin still supports cell adhesion, as it retains the arginine, glycine and aspartate (RGD) motifs present in its precursor. Gelatin is water soluble, and the behavior of its solution is determined by certain factors, including concentration, temperature, pH as well as the method of preparation. Gelatin has a comparatively low gelation temperature (Wang et al., 2017b). During gelation, non-covalent cross-links are formed that are thermo-reversible, i.e., the gelatin can easily liquefy at 37°C, so the gel dissolves completely. This can be utilized by employing gelatin as a structure-maintaining hydrogel component, also known as sacrifice material, that can be flushed out during cultivation.

The thermo-sensitivity of gelatin enables a broad spectrum of applications; however, this property, at the same time, limits its structural integrity of printed models during culture. It may thus be advisable to stabilize gelatin-based hydrogels by chemical cross-linking. One of the most widely used functionalized variants is methacrylated gelatin, known as GelMA (Nichol et al., 2010), in which methacrylate groups are conjugated to side groups of the protein. In the presence of a photo-initiator, this functionalization enables covalent cross-linking of the gelatin by irradiation, thereby enabling rapid and stable polymerization of the hydrogel. GelMa provides an aqueous cell environment and supports cellular growth, adhesion as well as proliferation, and combines biological properties of the natural gelatin molecule and controllable mechanical properties due to chemical modification, resulting in higher stability at physiological temperatures.

3.1.2 Matrigel™

Matrigel is a murine ECM that has been widely used in advanced cell culture technologies (Benton et al., 2014). The gelatinous protein mixture is harvested from murine Engelbreth-Holm-Swarm (EHS) sarcoma, where it constitutes the basal membrane. It is commercialized under the name Matrigel™ by the company Corning, Inc., but is also available from other companies under other names. The thin basal membrane sheets of ECM surround most animal tissues and have an essential function, serving as a barrier to separate different tissue types. They comprise the matrix of most tumors that are of epithelial origin.

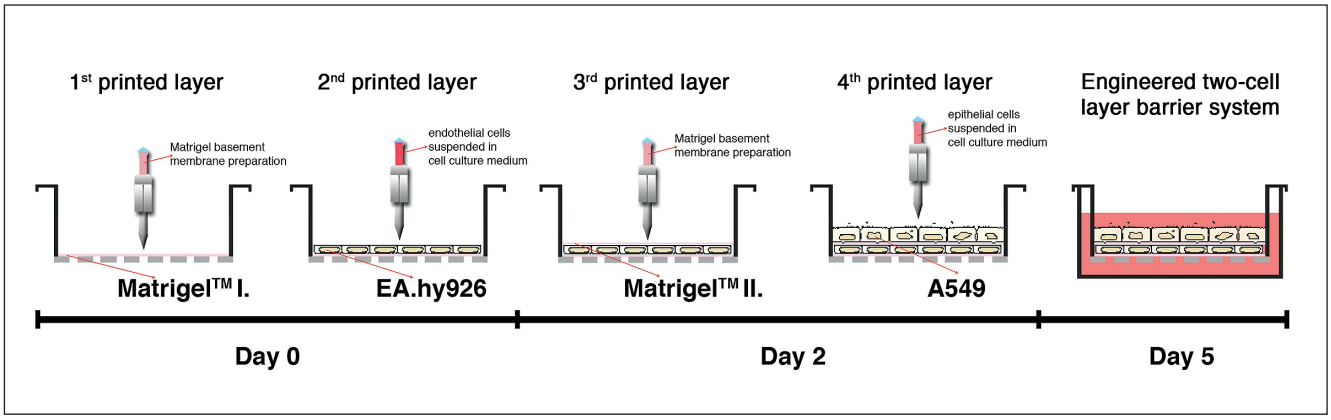

Fig. 3: Timeline for bioprinting of an air-blood barrier system
Endothelial cells are printed on top of a Matrigel layer. Another layer of Matrigel is printed to ensure adhesion of the top layer of epithelial cells. Taken from Horvath et al. (2015) in accordance with the Creative Commons Public License (https://creativecommons.org/licenses/by-nc-nd/4.0/).

The complex composition of Matrigel has been analyzed in depth by mass spectrometry (Hughes et al., 2010). Main components are laminin-111, collagen IV, entactin, and heparan sulfate proteoglycan. In addition, it includes numerous growth factors, which are largely responsible for the ability of Matrigel to enhance cell proliferation.

Matrigel is frequently utilized in cell biology *in vitro* and *in vivo* (Benton et al., 2014). It is commonly used as a basement membrane matrix for stem cells, as it retains the stem cells in an undifferentiated state. Furthermore, it is frequently included as a substrate in 3D cell culture and in suspension cultures of spheroids. A field that makes intensive use of Matrigel is cancer research, for example in angiogenesis, invasion and dormancy assays. In animal models, it is also used for angiogenesis assays and to promote growth of xenografts and patient-derived biopsies.

More recently, Matrigel has been explored as a bioink component for bioprinting approaches. The material has interesting physicochemical properties, as it is liquid at ambient temperature and reversibly solidifies at elevated temperature, forming a hydrogel at 37°C. It facilitates the creation of strong, 3D bioprinted constructs with high cell survival rates. An example of the potential of Matrigel in bioprinting is its application to generate an air-blood barrier (Horvath et al., 2015). Production of the two-cell layer barrier system starts with a layer of Matrigel printed on porous membranes, on top of which a layer of endothelial cells is printed (Fig. 3). On day two, a second Matrigel layer is printed onto the endothelial cells to ensure the adhesion of the next printed layer of epithelial cells. In another study, a 3D printable hydrogel of Matrigel and agarose was developed to support the growth of intestinal epithelial cells and cell-matrix interactions (Fan et al., 2016). Here, a particular combination of Matrigel with agarose was found to overcome disadvantages of individual hydrogels. The authors conclude, "Given that Matrigel is used extensively for 3D cell culturing, the developed 3D-printable Matrigel-agarose system will open a new way to construct Matrigel-based 3D constructs for cell culture and tissue engineering" (Fan et al., 2016). The advantageous properties of Matrigel have been confirmed in many studies (Berg et al., 2018; Schiele et al., 2010; Schmidt et al., 2019; Snyder et al., 2011; Swaminathan et al., 2019).

3.1.3 Alginate

Alginate is one of the most popular non-animal materials that is well-suited for 3D bioprinting, particularly in extrusion-based approaches (Abasalizadeh et al., 2020; Axpe and Oyen, 2016). The polyanionic linear polysaccharide is obtained from brown algae and is composed of (1-4)-linked β-D-mannuronic (M) and α-L-guluronic acids (G), which are ordered in mannuronic or guluronic blocks, separated by regions in which both acids are mixed. Alginate is a biocompatible material that does not intensively interact with cellular surfaces. Water and small molecules are trapped in its matrix, but they are still able to diffuse and can provide cells with a sufficient supply of nutrients.

For bioprinting approaches, alginate is extruded in its low viscosity state and is subsequently cross-linked by treatment with divalent cations such as Ca^{2+}. The divalent cations form ionic bridges between the G-blocks of adjacent polymer strands. Pre-cross-linking of alginate during the printing process by mixing with low calcium concentrations can be used to achieve good printing properties, followed by strengthening the printed construct with higher concentrations of the cross-linker. However, a balance must be found, as high viscosity during the printing procedure requires high pressure during the extrusion process, which can cause damage to the cells as a result of shear stress, whereas, if the viscosity is too low, slow gelation will hamper structural reproducibility and resolution of the printed model.

Alginate encapsulates the cells of the bioink, which has both advantages and disadvantages. The encapsulation substantially reduces shear stress in extrusion bioprinting and thereby increases cell viability; however, following the printing process, encapsulation prevents cell proliferation and proper formation of cell-cell contacts as desired in 3D models. A way to solve this problem

is to incubate the cross-linked alginate with sodium citrate (Wu et al., 2016). Citrate is a chelator for divalent cations and thus mediates slow and controllable degradation of alginate hydrogels.

While the weak interaction of alginate with human cells is desirable to have an inert scaffold that does not influence cellular behavior, it is at the same time disadvantageous, as cell attachment to the bioink is minimal and therefore cells tend to sediment in the printed constructs. This problem may be solved by modifying the alginate surface with RGD motifs that provide binding sites for the cells and strengthen their attachment (Daly et al., 2016a).

For many applications, it has been advisable to combine different biopolymers and make use of the desirable characteristics of each material. Blends of alginate and gelatin are frequently used for extrusion-based bioprinting to combine the thermo-sensitive properties of gelatin with the chemical cross-linking capabilities of alginate (Berg et al., 2018; Han et al., 2020; Mondal et al., 2019). In these blends, the gelatin component confers good printability to the bioink and ensures rapid, temperature-induced gelation immediately after the printing process to provide the initial stability of the printed construct. The slower Ca^{2+}-induced gelation of alginate can then occur, the gelatin dissolves over time during cultivation at 37°C, and only the alginate component remains to maintain the structural integrity. However, while the remaining alginate provides desirable biocompatibility and high mechanical stability, it has poor biomimetic properties due to the above-mentioned lack of cell adhesion motifs. In addition to the already described approach to link RGD motifs to the alginate, the bioink can also be blended with suitable protein mixtures such as Matrigel (Berg et al., 2018) or human ECM (Hiller et al., 2018).

3.2 Synthetic bioinks

3.2.1 Poly(ethylene glycol) (PEG)

One of the most widely-used synthetic bioink components is poly(ethylene glycol) (PEG), a hydrophilic polymer that is resistant to protein adsorption. While this is a desirable property in some respects, it also means that many cell types require cell adhesion components, such as RGD peptides, to strengthen the interactions between the cells and the scaffold. The advantage of a synthetic material is that its mechanical properties can be adjusted through variation of its chemistry. PEG-based hydrogels can be used with photo-cross-linking in the presence of a photo-initiator. Methacrylate can be added to increase the mechanical strength of the printed construct (Cui et al., 2012).

3.2.2 Pluronic F-127

Another synthetic polymer used in bioprinting approaches is Pluronic® F-127, which belongs to the class of poloxamers, i.e., nonionic triblock co-polymers composed of a central hydrophobic chain of polyoxypropylene, flanked by two hydrophilic chains of polyoxyethylene. Its most interesting feature is its ability to undergo reverse gelation, as it starts to cross-link with increasing temperature. This behavior can, for example, be used to produce a vascular network (Wu et al., 2011). For this approach, the Pluronic F-127 fugitive ink is deposited as a branching network in a gel reservoir that can be cross-linked by photo-polymerization after printing is completed. The fugitive ink, which is not chemically modified, can be removed by liquefaction at 4°C and modest vacuum extraction to yield the desired vascular network within the matrix.

4 Bioprinted organ models

Models for all major human organs have been produced by 3D bioprinting during the last few years. A recent review thoroughly covers publications related to bioprinting for each organ (Mota et al., 2020). Here, only some exemplary and very recent studies for selected organs (lung, heart, liver, and skin) will be discussed in more detail in order to keep the focus on the current status of bioprinting aiming at generating new tools for the replacement of animal experiments by humanized 3D organ models. Researchers use the term "organ" somewhat loosely, as it usually only contains one tissue type, which, in the best case, consists of several cell types, rather than truly representing an organ in all its natural complexity. Still, we will use the term organ or organ model for bioprinted constructs in accordance with general use.

4.1 Lung

The lungs are part of the lower respiratory tract, which begins at the trachea and branches into the bronchi and bronchioles. The respiratory bronchioles divide into alveolar ducts that give rise to the alveolar sacs, which finally contain the alveoli, where gas exchange takes place. The gold standard for *ex vivo* lung models was developed by the group of Donald E. Ingber at Harvard University's Wyss Institute in 2010 (Huh et al., 2010), without using bioprinting technologies. It consists of endothelial and epithelial cells separated by a porous membrane, which is part of a device that recreates physiological breathing movements by applying vacuum to two-sided chambers, thereby causing cyclic mechanical stretching of the membrane. Despite the unquestioned value of this model, bioprinting may help to overcome some of its inherent limitations. In addition to the use of immortalized cell lines instead of primary cells, the comparatively thick and artificial membrane does not reflect the organization of natural alveoli particularly well. This shortcoming was addressed with a valve-based bioprinting approach to create an air-blood barrier consisting of alveolar epithelial type II cells (A549) and endothelial cells (EA.hy926) that were separated by a thin layer of Matrigel (Horvath et al., 2015). This experimental strategy is described in more detail in Section 3.1.2 and illustrated in Figure 3. Compared to a manually seeded co-culture that formed overgrowing multi-layered clusters, the layer-by-layer bioprinted construct spread over the entire surface to form confluent thin monolayers (Fig. 4A).

A recently developed bioprinting method named SLATE (stereolithographic apparatus for tissue engineering) allows the production of functional intravascular topologies in biocompatible hydrogels consisting of photo-cross-linked GelMA derived from

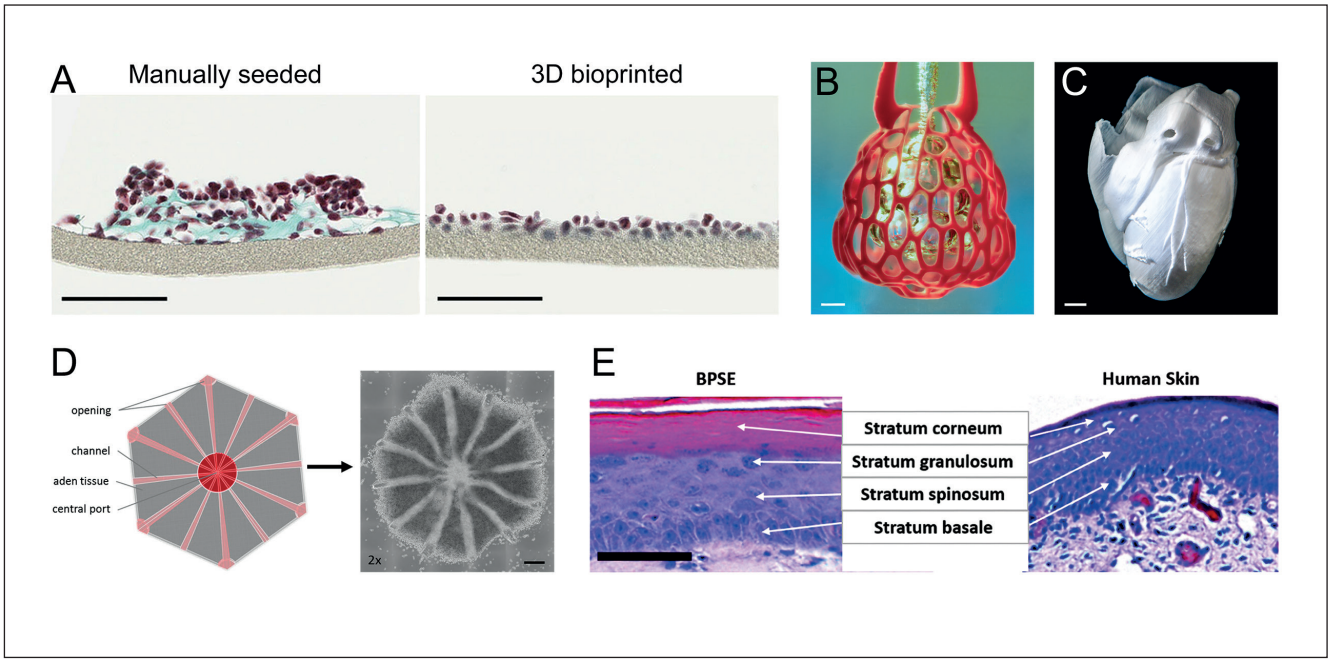

Fig. 4: Examples of bioprinted organ models

(A) Comparison of manually seeded and bioprinted air-blood barriers consisting of endothelial and epithelial cells separated by a Matrigel layer (Horvath et al., 2015). Scale bar: 100 μm. (B) Bioprinted vascular network using SLATE (stereolithographic apparatus for tissue engineering) to mimic a pulmonary alveolus (Dasgupta and Black, 2019). Scale bar: 1 mm. (C) High-resolution replica of the heart made of collagen and produced by the FRESH (freeform reversible embedding of suspended hydrogels) technology (Dasgupta and Black, 2019). Scale bar: 5 mm. (D) Liver organoid generated by stereolithographic bioprinting. The computer 3D model is translated into a construct consisting of a cell-laden multi-material hydrogel. The hexagonal structure including channels reproduces the biological topology of a liver lobule (Grix et al., 2018). Scale bar: 500 μm. (E) Histological comparison of bioprinted full skin equivalent and native human skin (Derr et al., 2019). H&E staining shows the layers of the dermis. Scale bar: 100 μm. Copyrights: (A) Taken from Horvath et al. (2015) in accordance with the Creative Commons Public License (https://creativecommons.org/licenses/by-nc-nd/4.0/). (B, C) Reproduced with permission from The American Association for the Advancement of Science. (D, E) Taken from Grix et al. (2018) and Derr et al. (2019), respectively, in accordance with the Creative Commons Public License (https://creativecommons.org/licenses/by-nc-nd/4.0/).

porcine skin tissue (Dasgupta and Black, 2019). The technology was used to generate bio-inspired alveolar air sacs (Fig. 4B) (Grigoryan et al., 2019). The complex network, composed of 185 vessel segments and 113 fluidic branch points, was then perfused with deoxygenated erythrocytes at the blood vessel inlet. Cyclic ventilation of the airways with humidified oxygen led to efficient oxygenation of the erythrocytes.

4.2 Heart

The heart is a highly compartmentalized organ that is composed of numerous cell types and has a sophisticated architecture, as well as a complex vascular system. Attempts at using bioprinting technologies for the generation of cardiac patches or whole hearts mainly aim at producing transplantable tissue rather than organ models for biomedical research and drug development. For example, human cardiac progenitor cells were printed with cardiac ECM and GelMA, and the resulting cardiac patches were implanted into rat hearts, where they were retained and showed vascularization over 14 days, thus demonstrating the potential of bioprinted patches for the repair of damaged myocardium (Bejleri et al., 2018). Another active field of personalized bioprinting approaches is the generation of heart valves that can be used to replace defective valves in patients (Duan et al., 2014).

Figure 4C shows a high-resolution replica of the complex structure of the human heart that was produced by an advanced extrusion bioprinting technology called FRESH (freeform reversible embedding of suspended hydrogels) (Lee et al., 2019). The special feature of this technology is the use of a thermally reversible, viscous gelatin support bath into which the 3D construct of interest consisting of a bovine collagen type I hydrogel is printed. The support bath maintains the structure of the construct during the printing process and is melted away by incubation at 37°C to set free the actual workpiece. Using this approach, an unprecedented resolution was achieved with an extrusion bioprinter. When cardiac ventricles were printed with human cardiomyocytes using this advanced technology, they showed synchronized beating, directional action potential propagation, and wall thickening during peak systole.

Another important step towards the use of bioprinted organs is the use of patient-specific materials. In a recent study, Noor et

al. (2019) produced personalized perfusable cardiac patches and hearts. They used primary human omental tissue to reprogram cells into induced pluripotent stem cells (iPSCs) that were then differentiated into cardiomyocytes and endothelial cells, while the ECM was processed into a personalized hydrogel. The bioink was used to produce vascularized cardiac patches according to the patient's anatomy and whole hearts with a natural architecture, which were, however, only the size of a rabbit heart and did not beat. Still, the study demonstrates that bioprinting may eventually produce personalized tissues for drug-screening and transplantable artificial organs to overcome the shortage of natural grafts from human donors.

4.3 Liver

The largest organ in the human body is the liver, which consists of multiple cell types. It radiates from the central vein and is surrounded by the portal vein, hepatic arteries, and bile ducts. The liver fulfills multiple vital functions including the production and secretion of important proteins, the metabolization of nutrients, and the bioconversion of many drugs and toxins. It does not come as a surprise, therefore, that bioprinting technology has been used to produce sophisticated liver models. The high resolution of stereolithographic bioprinting allowed reproduction of the natural hexagonal structure of the liver with high precision (Fig. 4D) (Grix et al., 2018). The bioink consisted of a multi-component hydrogel containing porcine GelMA, modified PEG, and a photo-initiator that was mixed with a co-culture of the hepatic cell line HepaRG and human stellate cells. Compared to 2D cell cultures, the bioprinted tissue expressed higher levels of liver markers and tight junction proteins. Furthermore, perfusable channels printed into the organoid reproduced the micro-vascularization of the natural organ.

The goal of producing 3D liver models that mimic the biological physiology exemplifies another general challenge of organ reproduction, i.e., the choice of adequate cells. HepaRG cells are widely used and acknowledged to exhibit a hepatocyte-like physiology; however, they still have the general limitations of a hepatoma-derived cell line, such as the expression of some typical liver markers at unphysiological levels. To reflect the natural liver physiology even better, models consisting of primary hepatocytes or hepatocyte-like cells derived from human iPSCs are desirable. The latter would even allow the generation of patient-specific liver models by using iPSCs from the respective individual. However, the limited cell numbers available from primary isolates or cells differentiated from iPSCs still prohibit their widespread application in bioprinting approaches. In addition, cell lines are usually more robust than primary cells or iPSC-derived cells, and strains during the printing process (such as shear stress, UV treatment, and the formation of initiator radicals, depending on the technology used) may have a greater impact on the viability of primary and iPSC-derived cells. Despite these challenges, some reports have already demonstrated the suitability of iPSC-derived hepatocyte-like cells for bioprinted liver models. For example, Ma et al. (2016) used DLP-based 3D bioprinting and hepatocyte-like cells derived from iPSCs in co-culture with human umbilical vein endothelial cells (HUVECs) and adipose-derived stem cells. The main components of the bioink were methacrylated porcine skin gelatin and glycidal methacrylate-hyaluronic acid. Compared to a monolayer culture or a 3D hepatocyte-only model, the 3D multi-cell type model showed improved morphological organization, higher liver-specific gene expression levels, and increased metabolic product secretion. Key drug-metabolizing enzymes were significantly induced upon treatment with rifampicin, a bactericidal antibiotic drug with potential risk of hepatotoxicity. A comparably significant increase was not observed in cells in 2D monolayer culture or in a 3D monoculture in the absence of HUVECs and adipose-derived stem cells.

4.4 Skin

A combination of consumer pressure and the ban on animal testing in the cosmetics sector in many countries, e.g., in the EU in 2013, initiated intensive efforts to develop sophisticated 3D skin models for research purposes and toxicity testing (Dellambra et al., 2019). As a result, skin models are among the most advanced 3D tissue culture systems. The skin has a multi-layer organization that is integral to its barrier function, so specific cell-cell and cell-matrix interactions and precise positioning of the cell layers are important. These requirements can be met by bioprinting technologies. Advanced procedures include a multi-step bioprinting approach to produce the different layers of the skin (Derr et al., 2019). This complex model required a sophisticated bioink consisting of rat tail collagen and porcine skin gelatin in addition to human plasma-derived fibrinogen. It was cultured under standard conditions including 10% FBS. As commonly done for 3D skin models, the constructs were initially cultured submerged and were then lifted to the air-liquid interface (ALI) to reflect their biological function. Histological analysis of the model demonstrated a morphological multi-layer organization that is similar to normal human skin (Fig. 4E). Furthermore, various markers for tight junctions and epidermal differentiation were expressed comparably to those present in natural skin.

The given examples demonstrate the potential of bioprinting technologies to reproduce biological organ structures with high precision. However, ongoing efforts aim at improving currently available models so that they better reflect normal physiology. Major challenges are the inclusion of multiple cell types and the modeling of their natural interactions, as well as the precise spatial arrangements of all components of the model organs.

5 Application fields for bioprinted organ models

Despite the need to further improve physiological features of bioprinted organ models, some studies have already shown that these biomimetic systems can be used to study processes of biomedical relevance. Some selected examples in the fields of toxicology, cancer research and infection biology are outlined in this section.

5.1 Toxicology

The high failure rate of drug candidates in clinical trials is largely due to the inability of animal models to predict human toxici-

ty, underscoring the need for test systems that better recapitulate *in vivo* human biology. Bioprinted tissues and 3D culture models, in particular, are promising tools for toxicity studies in humanized systems (Nguyen and Pentoney, 2017). The liver is the major organ that metabolizes a large fraction of endogenously produced or exogenously applied substances, including almost all drugs in clinical use, and drug-induced liver toxicity (DILT) is one of the major reasons for failure of candidate substances. Several typical liver enzymes, such as albumin or cytochrome P_{450} enzymes (CYPs), are expressed at physiological levels only in 3D arrangements, but not in conventional 2D cultures of hepatocytes. Furthermore, non-parenchymal cells, including sinusoidal endothelial cells, phagocytic Kupffer cells and hepatic stellate cells, are essential for the correct function of hepatocytes. As there are substantial species-specific differences in liver physiology, animal models produce results that are of limited relevance for humans when studying liver toxicology of exogenously applied substances.

To overcome these shortcomings, the Organovo team produced a bioprinted, humanized multi-cell type liver model and demonstrated its capacity to investigate the toxicity of pharmacological substances (Nguyen et al., 2016). The model consisted of primary human hepatocytes that were co-printed with human stellate and HUVEC cells. Unlike 2D cultures, the bioprinted tissue maintained high levels of liver-specific markers, including albumin and CYPs. The liver model was then used to assess the toxicity of two clinically approved drugs, levofloxacin and trovafloxacin. The latter had to be withdrawn from the market due to hepatotoxic side effects in humans that remained undetected with standard pre-clinical models. In the bioprinted liver model, but not in conventional 2D cell culture, dose-dependent toxicity of trovafloxacin was observed at clinically relevant doses, while it was absent in levofloxacin. The study thus demonstrated that such tissue models can predict liver toxicity in humans better than standard pre-clinical models and can distinguish between highly related substances with a differential toxicity profile. A recent study on the toxicity of aflatoxin B1 showed that liver cells in a 3D culture survived longer and were less susceptible to drug-induced toxicity than those cultured in 2D, making the bioprinted organ model suitable for long-term studies (Schmidt et al., 2020).

In addition to metabolization in the liver, absorption and first-line metabolism are crucial steps that determine the level and thus the efficacy and toxicity of a pharmaceutical substance. Oral delivery is the most common method for drug administration, and the intestine is the primary organ for absorption of a drug, in addition to being a site of off-target toxicity for certain compounds. A bioprinted intestinal tissue composed of human primary intestinal epithelial cells and myofibroblasts with an architecture reflecting the native intestine was shown to develop a physiological barrier function that was disrupted in response to the known toxicants indomethacin and tumor necrosis factor-α (TNF-α) (Madden et al., 2018). The bioprinted model has thus the potential to support safety assessment and absorption, distribution, metabolism, excretion and toxicity (ADMET) studies in drug development. As approximately 7% of new drugs fail in the clinic due to nephrotoxicity (Sanchez-Romero et al., 2016), there is substantial interest in producing a kidney model to study adverse effects of new candidates. Human renal proximal tubules were generated by 3D bioprinting, and the tubular structure could be maintained for more than two months (Homan et al., 2016). The model allowed investigating adverse drug effects as exemplified for the known nephrotoxin cyclosporine A, which disrupted the epithelial barrier in a dose-dependent manner.

By virtue of its biological function, the skin is exposed to environmental agents in general and cosmetics in particular. Due to the previously mentioned ban on animal testing for cosmetic agents, the latter can only be tested in non-animal test systems. The success of this approach can be seen in a comprehensive study, in which Wei et al. (2020) tested the toxicity and irritation potential of 451 topical-use compounds in various two-dimensional cellular and three-dimensional bioprinted skin models. The study identified toxic compounds and defined the concentration ranges in which they had irritant potential or allergic potential without causing irritation.

The predictive value of animal studies in the pre-clinical development of new drug candidates is particularly limited with respect to potential drug-induced toxicity. This is mainly due to different expression levels of factors involved in uptake, distribution and metabolization of xenobiotic substances. The examples discussed in this section demonstrate the potential of bioprinted models consisting of human cells to provide reliable predictions of adverse effects of a substance of interest.

5.2 Cancer research

While 2D cultures of tumor cells have increased our knowledge of genetic alterations that contribute to cell proliferation and the induction of tumor phenotypes, they cannot investigate the important interactions between a tumor and its microenvironment. These shortcomings may be overcome with the help of 3D bioprinting and bridge the gap between conventional 2D cultures and animal models by generating a tumor environment with human physiology (Liu et al., 2019; Oztan et al., 2020). By using patient-derived cells and materials, it may even support the aim of personalized medicine, i.e., the adaptation of a treatment to the disease of a specific individual (e.g., by adjusting the treatment to the specific mutations of a tumor) rather than the standard therapy. It is difficult to model the vascular network of tumors with conventional methods for 3D tissue engineering, though this aspect is considered very important in the development of effective treatments. Using bioprinting technologies, microchannels can be produced with a sacrificial material that is removed after the printing process, followed by populating the interior surfaces of the microchannels by endothelial cells (Liu et al., 2019). Alternatively, blood vessel formation can also occur by self-organization when endothelial cells (HUVECs) are printed together with lung fibroblasts in a bioink consisting of gelatin, alginate and fibrinogen (Han et al., 2020). The microenvironment of blood vessels and fibroblasts had a strong influence on proliferation, angiogenesis, and epithelial-mesenchymal transition when seeding glioblastoma spheroids onto the vascularized tissue. Treatment of a tumor grown on vascularized tissue with a combination of an

anti-cancer drug (temozolomide) and an angiogenesis inhibitor (sunitinib) was more efficacious than the anti-cancer substance alone. Importantly, these results were similar to those obtained in mouse models.

In another study aiming at analyzing the signaling between the tumor and surrounding cells, Langer et al. (2019) modelled tumor phenotypes by 3D bioprinting. They printed the core with cancer cells, including patient-specific tumor tissue, surrounded by several stromal cell types. Importantly, the hydrogel used as the bioink, consisting of alginate and gelatin, was removed during subsequent culture so that the cells deposited ECM and self-organized. Extrinsic signals and therapies were found to alter the tumor phenotypes, proliferation and migration. This study demonstrates that bioprinted tumor models can be used to investigate the interaction between cancer cells and their microenvironment and to study the effects of anti-cancer therapeutics on both the tumor and the stroma.

To investigate the suitability of 3D printed constructs in characterizing anticancer drugs, breast cancer cells and adipose-derived mesenchymal stem/stromal cells were co-cultured in 2D and 3D and treated with doxorubicin (Wang et al., 2018). Treatment induced less apoptosis in the 3D printed constructs. In addition, treatment with an inhibitor of lysyl oxidase helped to overcome drug resistance. Altogether, the study demonstrated that a 3D bioprinted breast cancer model reproduces the biological system better than a conventional 2D culture.

5.3 Infection biology

Although *in vivo* infection studies are usually comparatively harmful to the test animals, as they cause fever, pain, weight loss and further symptoms, approximately 10% of all animal experiments fall into this category. This is even more startling, as the transferability of results from animal experiments to human pathology is questionable. Many viruses have a narrow host tropism. For example, mice are not natural hosts of Influenza A virus (IAV) and are therefore not susceptible to infection (Radigan et al., 2015). As the majority of IAV strains replicate poorly in the murine respiratory tract, they are usually adapted to the mouse by serial passaging (Matsuoka et al., 2009). This procedure, however, leads to ambiguous results, and the course of disease differs between humans and rodents (Bouvier and Lowen, 2010), for example the induction of different pathways by pathogens in human lung tissue and mice (Berg et al., 2017; Cakarova et al., 2009).

To close the gap, we recently investigated the potential of humanized bioprinted organ models to serve as tools for infection studies. Using a bioink composed of alginate, gelatin and Matrigel for optimal growth of the alveolar basal epithelial cell line A549, we were able to demonstrate efficient replication of IAV in infected constructs and observed a clustered pattern of virus distribution that is also found in lungs, but not in 2D cell culture (Berg et al., 2018). Furthermore, infected cells in the lung model released the proinflammatory interferon IL-29. In a subsequent study using a bioprinted liver model, Matrigel was replaced by human ECM (Hiller et al., 2018) to overcome the limitations associated with the use of Matrigel discussed in Section 6.3. The bioprinted liver model promoted replication of human adenovirus, which can cause fatal liver failure in immunocompromised patients. In addition, adeno-associated virus (AAV) vectors, which are promising delivery tools in gene therapeutic interventions, efficiently transduced the liver model, indicating that the model can support the further development of gene transfer strategies.

Taken together, these studies demonstrate the potential of bioprinted organs to serve as humanized models for the study of infection with human-pathogenic viruses. The ongoing corona pandemic is demonstrating the need for readily available research tools to develop new antivirals. It can therefore be expected that bioprinted tissue models will soon be used more frequently to study infection processes. In addition, they can be used to determine the transduction efficiency of viral vectors.

6 Animal components in bioprinting and their alternatives

6.1 Fetal bovine serum

Fetal bovine serum (FBS), also known as fetal calf serum (FCS), is a commonly added supplement used in cell culture media in virtually all life science laboratories around the world. Consequently, it is also a standard component used to supply cells in bioprinted 3D models with nutrients and factors that ensure high cell viability and growth. FBS was introduced as a stimulant of cell growth in the late 1950s (Puck et al., 1958). It is harvested by means of cardiac puncture of calf fetuses discovered when slaughtering pregnant cows. It is controversial whether this procedure is distressing and painful to the animals. While distributers of FBS claim that the blood is collected from dead fetuses (Nielsen and Hawkes, 2019), critics state that the blood is obtained from living calf fetuses (van der Valk et al., 2018). Furthermore, critics point to the problem that FBS is mainly produced in countries with less restrictive rules on animal welfare than, for example, the EU, which is not a major producer of FBS. The exact volume of FCS used worldwide is unknown, but has been estimated to be in the range of 500,000 to 800,000 liters annually, which accounts for 1-2 million bovine fetuses, and global demand is steadily increasing (Gstraunthaler et al., 2013; van der Valk et al., 2018). Commercialization of FBS has a long history of scandals and abuse, including sales of volumes of FBS that are higher than the officially reported amount collected in the specified area and contamination of the product (Gstraunthaler et al., 2013).

Apart from animal welfare, the use of FBS also adds complications from a strictly scientific point of view. Blood is a complex mixture. A proteomic analysis of human plasma discovered far more than 1,000 proteins (Anderson et al., 2004), and a metabolomic study found more than 4,000 metabolites to be present in human serum (Psychogios et al., 2011). To complicate matters further, biological material is subject to substantial fluctuations. Batch-to-batch variations for FBS are a well-known phenomenon in mammalian cell culture, and it is therefore common practice to test new lots used in research projects. The outcome of a project may vary depending on the specific FBS lot used.

In fact, the reproducibility crisis, according to which more than 70% of researchers have tried and failed to reproduce another scientist's experiments (Baker, 2016), has – at least partly – been attributed to the use of different batches of FBS (van der Valk et al., 2018). In addition, safety issues have been brought up for the use of FBS. A metagenomic analysis of 26 bovine serum samples from 12 manufacturers detected viruses in all samples except one (Toohey-Kurth et al., 2017). Except for the virus-free sample, the other samples contained up to 11 different viruses.

For the sake of animal welfare, as well as for scientific reasons, there is a long tradition of efforts to at least reduce the amount of FBS required or even replace it with alternatives (Gstraunthaler, 2003). A widely used component in serum-free media is bovine serum albumin (BSA). This protein, which is the most common protein in FBS, has multiple intracellular functions and interacts with numerous ligands or bioactive factors and serves as an extracellular transport protein (Francis, 2010). These features help improve cell growth and survival. The use of BSA has primarily been driven by the need, for reasons of safety, to culture mammalian cells in serum-free media for biopharmaceutical applications, i.e., the production of recombinant proteins. However, in the context discussed here, replacement of FBS by BSA does not contribute to avoiding chimeric systems composed of human cells cultivated in hydrogels or media containing substances of animal origin. Furthermore, the substitution of FBS by albumin of bovine origin does not contribute to improving animal welfare. Recombinant human albumin offers a solution to both problems but is substantially more expensive than BSA.

A promising alternative to the use of FBS is human platelet lysate (hPL). It is commonly obtained from donated human thrombocyte units that are past their date of expiration. It is therefore clinically tested, xeno-free, i.e., free of animal components, and contains factors that support cell growth and proliferation (van der Valk et al., 2018). A comparative study confirmed that cancer cells cultured in media supplemented with FBS or outdated hPL grow very similarly and have practically identical proteomes (Pons et al., 2019). Furthermore, they responded equally to different drugs and stress conditions that were tested in the study, demonstrating that hPL can substitute for FBS in various experimental settings.

Adequate *in vitro* reproduction of biological organs requires the co-cultivation of multiple cell types, which is often complicated by the fact that requirements for media composition may differ between cell lines. An example is the co-culture of primary human macrophages and human mesenchymal stem cells (MSC). While macrophages are commonly cultured in media with human serum, media for MSCs are usually supplemented with FBS. To solve this problem, hPL was tested as a supplement and found to be the best option to co-culture both cell types, maintaining their phenotypes, expression profiles, and the phagocytosis activity of macrophages (Tylek et al., 2019).

In a recent bioprinting study, Medes et al. (2019) developed a platelet lysate-based bioink containing cellulose nanocrystals. The 3D constructs were initially printed into an alginate support bath, from which they were removed when the cross-linking reaction was completed. Human adipose tissue-derived stem cells were cultured in the printed constructs without additional animal-derived media supplementation. In contrast to cells printed in widely used alginate-based and GelMA bioinks, cells in the newly developed bioink readily spread, proliferated and produced ECM. This work can thus be regarded as a substantial step in the direction of clean bioprinting.

Although a promising alternative to the use of FBS, hPL is still connected to the general problem associated with all materials of biological origin, in that there are substantial variations between different lots. Thus, the use of fully synthetic media has been proposed to solve the FBS-associated problems described above (van der Valk and Gstraunthaler, 2017). Unlike media of biological origin, their composition is chemically well-defined and controlled so that the reproducibility of experimental results is higher and the risk of contamination with pathogens can be almost eliminated. Interestingly, a comprehensive review found that synthetic media are available for many industrially relevant cell lines to avoid the problem of batch-to-batch variations, whereas serum-containing media are still in general use in basic research (Yao and Asayama, 2017).

Currently, a major hurdle is that there is no universal chemically-defined medium, so that a synthetic medium needs to be optimized for each cell line individually. While this occasionally may be desirable, for example, to prevent primary cells from being overgrown by fibroblasts, in most cases it is a laborious and costly step that prevents wide-spread use of serum-free media. The development of FBS-free media supporting the growth of multiple cell lines would greatly facilitate its widespread adoption. For example, the commercially available medium Neuro-Pure™ has been used to maintain and differentiate various cell types, including neuronal lineages, fibroblasts and primary cancer stem cells (Usta et al., 2014). For some cell types, serum-free media are commercially available; however, they are usually substantially more expensive than standard media with FBS. Further support can be obtained from databases such as the FCS-free database[1] that collects formulations of serum-free media for individual cell lines.

For the development of a new serum-free, chemically defined cell culture medium, a basal medium of a 50:50 (v/v) mixture of Ham's F12 with Dulbecco's modified Eagle's medium (DMEM) is recommended that is supplemented with insulin, transferrin and selenium (van der Valk et al., 2018). A further common recommendation is to transfer cells cultured in FBS-containing medium to a synthetic medium supplemented with the same amount of FBS. Then, the concentration of FBS is progressively reduced, while monitoring cell growth and viability, until the cell line is completely weaned off FBS. This procedure was exemplarily demonstrated for the human monocytic cell line THP-1 (Marigliani et al., 2019). For cells that have successfully been adapted to FBS-free conditions, it is also important to adjust the freezing

[1] https://fcs-free.org

medium. Normally, dimethyl sulphoxide and FBS are added for the cryoconservation of serum-dependent cell lines. As a possible alternative, the synthetic surfactant Pluronic F68 was shown to act as an active cryoprotectant that significantly increased the fraction of viable cells after thawing and promoted cell proliferation (Gonzalez Hernandez and Fischer, 2007).

To date, only very few bioprinting studies have used chemically-defined media for cultivation of cell-laden scaffolds. One example is a co-culture of non-small cell lung cancer patient-derived xenograft cells with lung cancer associated fibroblasts (Mondal et al., 2019). In this study, DMEM/F12 and RPMI media were supplemented with B27 and recombinant growth factors (human epidermal growth factor (EGF) and human basic fibroblast growth factor (bFGF)). However, B27 contains bovine serum albumin and transferrin, which are of animal origin and can exhibit batch-to-batch variations (Chen et al., 2008). In addition, the printed hydrogels contained bovine gelatin. Thus, although aiming at replacing media containing FBS, the final system was still a chimeric composition of human cells with media and hydrogels containing components of animal origin.

Furthermore, a careful general revision of the composition of culture media for humanized organ models may be called for. Currently, media are optimized to support maximum cell growth. The success story of FBS is based on optimal proliferation of numerous cell types due to the high content of growth factors. Likewise, alternatives such as hPL were selected because they contain multiple growth factors that ensure rapid doubling times of the cells. Even chemically-defined media are commonly supplemented with factors that enhance cell proliferation. However, it should be kept in mind that, with the exception of cancer cells, adult cells rarely proliferate at high rates. Depending on the organ type, doubling times may be extremely long. For the generation of better *in vitro* organ models, it may therefore be necessary to choose media that support a steady state of high cell viability rather than solely focusing on rapid proliferation. Nevertheless, the goal of replacing FBS with human-derived components or chemically-defined media will only be successful if the scientific community can be convinced not only of its contribution to animal welfare efforts but also of the advantages for the relevance and reproducibility of experimental results in an adequate humanized setting. While this is a general issue for all types of cell culture research, it may be particularly relevant to bioprinting approaches, as cells experience severe stress during the printing process (e.g., shear stress in extrusion bioprinting or UV-irradiation in stereolithography). As FBS provides optimal conditions for cell viability, the bioprinting process may cause even more severe damage to the cells when using media without FBS, indicating the need to carefully optimize and characterize the alternatives.

6.2 Collagen and gelatin

Collagen is widely used in biomedical research. It is largely extracted from animals, mainly from bovine, porcine or rodent sources, and to some extent from marine organisms. Approximately 30 types of collagen have been identified. Depending on the type of collagen needed, it may be extracted from different parts of the animal. The most abundant type I collagen is usually bovine-derived and isolated from the Achilles tendon for industrial purposes, while rat-tail tendon is the main source of collagen type I for research purposes (Davison-Kotler et al., 2019). Type II collagen is normally isolated from bovine skin. Gelatin is the hydrolyzed form of collagen. Thus, gelatin used for research purposes or food technology is also usually of porcine or bovine origin.

Collagen, gelatin and its derivative methacrylated gelatin have become widely used components of bioinks (Ng et al., 2016; Osidak et al., 2020; Wang et al., 2017b). Numerous examples of bioprinting approaches discussed in this article rely on the use of collagen or gelatin (among others, Berg et al., 2018; Derr et al., 2019; Grigoryan et al., 2019; Grix et al., 2018; Han et al., 2020; Hiller et al., 2018; Ma et al., 2016; Mondal et al., 2019; Monferrer et al., 2020; Schiele et al., 2011; Schmidt et al., 2019). Furthermore, gelatin is not only used for the bioprinted construct itself but also has attractive properties for use as a support bath, as it dissolves under standard cultivation conditions at 37°C after the printed model has solidified. This was exemplified above for the reproduction of the human heart at high resolution by extrusion bioprinting (Lee et al., 2019). The main component of the bioink was collagen of bovine origin, while the fugitive support bath consisted of gelatin. In addition, gelatin is a frequent component of blended bioinks. It can, for example, be combined with alginate, as the gelatin component confers stiffness to the printed structure at ambient temperature, while cross-linking of alginate occurs by the addition of calcium ions. During subsequent cultivation at 37°C, gelatin dissolves, but the chemically cross-linked alginate maintains the structure (Berg et al., 2018; Han et al., 2020; Hiller et al., 2018; Mondal et al., 2019).

Despite many desirable properties for bioprinting approaches, collagen and gelatin of animal origin have several shortcomings in addition to the obvious need to use material from slaughtered animals or to kill animals for their isolation. Bioinks composed of human cells incorporated into animal collagen or gelatin produce chimeric systems. As the amino acid composition of collagen types vary between species, this may affect chemical and physical properties, thermal stability, solution viscosity, and cross-linking density (Davison-Kotler et al., 2019). Specific amino acid sequences in collagen interact with cellular integrins and facilitate cell-ECM adhesion, and recent research revealed that collagens have many regulatory and physiological functions in addition to their mechanical properties (Davison-Kotler et al., 2019). In addition, characteristics of different collagen batches may vary depending on isolation procedures, age and genetic composition of the animals, and immunogenicity of animal proteins in humanized systems may also influence the experimental outcome (Wang et al., 2017a).

An alternative to the use of collagen from animal sources is the recombinant expression of human collagen. This procedure, however, still suffers from several issues, including the difficulty to produce full-length collagen and to reproduce the native pattern of post-translational modifications (Wang et al., 2017a). Prolyl-hydroxylation is one of the main modifications required to achieve functional collagen. Prokaryotic production organ-

isms lack endogenous hydroxylase, and the artificial expression of hydroxylases in bacteria has not yet resulted in satisfactory modification patterns of collagen. Even in eukaryotic cells, which possess the endogenous genetic program to carry out intensive post-translational modification, it is challenging to obtain the native hydroxylation and glycosylation patterns. Thus, recombinantly produced collagen may inadequately reflect the native molecule and may differ in functionality. In addition, yields of recombinant production of collagen need to be improved for its wide-spread application, particularly in mammalian cells. Once these hurdles have been overcome, it will be a promising approach to use recombinant human collagen in bioprinting approaches.

6.3 Matrigel

As outlined in detail in Section 3.1.2, Matrigel is increasingly added as a component of advanced bioinks. However, despite some desirable properties, such as support of cell growth and good printability in blended inks, the use of Matrigel has severe scientific limitations and is encumbered by ethical concerns. As outlined above, Matrigel contains multiple intrinsic growth factors as constituents, which were shown to influence cellular behavior (Vukicevic et al., 1992). This feature was impressively confirmed for bioprinting approaches by comparing the influence of the ECM on melanoma cells (Schmidt et al., 2019). When printed in Matrigel, the melanoma cells were able to spread, proliferate and form dense networks; in contrast, they did not proliferate at all in alginate-based bioinks and proliferated in clusters in gelatin-methacrylate bioinks. The choice of the bioink composition should therefore depend on the intended application. While tumor-studies may benefit from proliferative signals in tumorigenic ECMs such as Matrigel, these may be detrimental for other types of applications. For models of healthy organs, e.g., a liver model for toxicology studies, the high levels of growth factors of the tumor-derived ECM will generate the microenvironment of a tumor and provide unphysiological signals for cell proliferation and migration.

Another problem associated with the use of Matrigel is its organic source, as a result of which it is not well-defined and suffers from substantial lot-to-lot variation that can be a source of variability, complicating the interpretation and reproducibility of experimental results. Last but not least, bioprinted tissues usually aim at generating humanized organ models. By adding an ECM of murine origin, the consistency of the approach is thwarted by creating a chimeric system composed of human cells in a rodent extracellular environment.

In addition, bioprinting technologies not only aim at producing humanized organ models for more disease-relevant research, they are also supposed to develop alternative research tools to replace animal experiments. These efforts, however, are counteracted by using Matrigel, which is harvested from murine tumors. For the production of Matrigel, EHS sarcomas are propagated in live mice. Three weeks after injection of tumorous cells, the tumor reaches a mass of ~4 g (which equals almost 20% of the body weight of a female C57BL/6J mouse) from which approximately 6 mL Matrigel can be prepared (Kleinman, 2001).

Matrigel is a commercially distributed product, therefore the amount of Matrigel used worldwide is not readily available in the public domain. It is, however, obvious that Matrigel is a widely used supplement for advanced cell culture techniques. According to a literature search of the PubMed database, more than 12,000 entries contain the term "Matrigel" as of September 2020. Within the period 2010-2019, 600-800 published papers per annum include the phrase "Matrigel". Matrigel is sold in sizes of 5 to 100 mL. As the tumors can be up to 4 g and each gram of tumor mass yields approximately 1.5 mL Matrigel, a single large package requires more than 16 mice to produce. It also has to be taken into consideration that it is not possible to harvest the complete tumor mass for Matrigel-production, as the sarcoma must be maintained by passaging. Furthermore, some tumors that have grown too large and have become necrotic have to be discarded (Kleinman, 2001). It is thus obvious that a very large number of animals are needed to cover the annual demand for Matrigel. As the tumor, which grows to one fifth of the body weight, can also be expected to cause severe suffering of the animals, the development of alternative strategies avoiding the use of Matrigel can substantially contribute to the implementation of the animal welfare principles.

Various approaches have been taken to replace Matrigel. One example is the use of ECM formed by the cells *in vitro* (Hoshiba, 2017). This procedure, however, is comparatively demanding, as multiple factors, including the culturing conditions and media composition, influence the composition of the ECM. It is also difficult to prepare matrices that mimic the native ECM's composition, mechanical properties and structure. Despite these challenges, cell culture-derived ECM can simulate the tumor microenvironment, as demonstrated by Scherzer et al. (2015). The authors used fibroblast-derived matrices to culture various lung epithelial cells and found the fibroblast ECM to be well-suited to recreate and study the tumor microenvironment. In another study, ECM from human dermal fibroblasts maintained the *in vitro* expansion of keratinocytes in a stem-like state (Wong et al., 2019b).

An advanced strategy to replace animal-derived ECM in bioinks is the application of ECM from human donors (hECM) (Choudhury et al., 2018; Eo et al., 2017). The use of hECM has several obvious advantages: First of all, it creates an allogenic system, i.e., cells and ECM originate from the same species, in contrast to xenogenic systems produced by culturing human cells in Matrigel or related ECM material of animal origin. At the same time, the use of hECM avoids the need to harvest ECM from animals. These advantages, however, are linked to some intrinsic disadvantages, including poor mechanical and viscoelastic properties of ECM for bioprinting approaches that make optimization of bioinks containing hECM for a given application necessary. In our attempts to generate an organ model in an alginate-gelatin bioink devoid of Matrigel, we used lung ECM from a human donor (Hiller et al., 2018). Systematic optimization of the bioink revealed 0.5 to 1 mg/mL hECM to provide high stability of the printed constructs and to support viability and metabolic functions of the printed HepaRG cells. The most critical limitation of this approach is the need for clinical material, so a steady sup-

ply of ECM from human donors in sufficient quantities cannot be guaranteed. An alternative may be the use of basal membrane proteins from human placenta (Hackethal et al., 2019), a clinical waste material that can be used as a virtually unlimited source for the isolation of hECM. However, its usability for bioprinting approaches remains to be confirmed.

As all matrices of biological origin have batch-to-batch variations that may influence the outcome of experiments, well-defined synthetic alternatives have been proposed that are not only highly reproducible but may also be fine-tuned by altering the synthetic material (Aisenbrey and Murphy, 2020). Most of the synthetic scaffolds developed to date are derived from polyacrylamide (PAM) and PEG. They can easily be functionalized with peptides such as the above-mentioned RGD motif (see Section 3.2.1) to promote cell adhesion and proliferation. In addition, recombinant vitronectin, an ECM glycoprotein that is abundant in serum, may serve as a well-defined, biological matrix for 3D culturing of cells (Braam et al., 2008; Fan et al., 2017).

6.4 Fibrin

Activation of fibrin by thrombin is the key final step in the blood coagulation pathway. The resulting fibrin forms a hydrogel, which has also been used in bioprinting (de Melo et al., 2020). As the pre-polymer fibrinogen is incapable of maintaining the shape of a printed construct and the cross-linked fibrin is too viscous for proper extrusion, various strategies have been developed to use fibrinogen in the bioink, followed by rapid conversion into fibrin after the printing process. One common strategy is to add thrombin in PBS to the receiving Petri dish, which will cross-link fibrinogen to fibrin (Han et al., 2020). While fibrinogen used in bioprinting applications is commonly of bovine origin, fibrinogen from human plasma may also be used, as shown in the skin model discussed above (Derr et al., 2019), which, however, also used rat tail collagen and porcine skin gelatin as bioink components. Still, replacement of bovine fibrinogen by human plasma fibrinogen is a viable path to avoid the use of animal-derived material.

6.5 Synthetic and plant materials

Another alternative to the use of material of animal origin is its replacement with plant-derived or synthetic materials. As discussed in Section 3.1.3, alginate is the most widely employed plant-derived material in bioprinting (Abasalizadeh et al., 2020; Axpe and Oyen, 2016). It is usually isolated from brown seaweed. In addition to being readily available, alginate has good printing properties and can easily be cross-linked by Ca^{2+} ions. Another advantage of alginate is its biodegradability by sodium citrate or alginate lyase.

A further natural hydrogel with multiple examples for its application in bioprinting is agarose, a linear polymer consisting of repeating disaccharide units made up of D-galactose and 3,6-anhydro-L-galactopyranose. Agarose is extracted from red seaweed. Biocompatibility of agarose is lower than that of other hydrogels such as alginate or Pluronic F-127, as the cell proliferation rate and biosynthesis of cell components is limited (Hospodiuk et al., 2017). Furthermore, cell adhesion and spreading are low. Still, agarose is used in 3D cell culture and is increasingly used for bioprinting approaches, particularly in blended formulations. For example, López-Marcial et al. (2018) investigated the bioprinting properties of alginate-agarose hydrogels for additive manufacturing of biological materials for cartilage tissue engineering. The composites had good rheological and bioprinting properties and demonstrated excellent biocompatibility, as shown by high cell viability over a 28-day culture period and matrix production over the same period. Another comparative study revealed substantial differences of plant- and animal-derived hydrogels for the bioprinting of mesenchymal stem cells (Daly et al., 2016b). While alginate and agarose hydrogels supported the development of hyaline-like cartilage consisting of type II collagen, GelMa as well as a methacrylated PEG-based hydrogel predominantly supported development of a more fibrinocartilage-like tissue, which is characterized by a mixture of type I and type II collagen. To introduce protein components, a hybrid Matrigel-agarose hydrogel was developed, as depicted in more detail in Section 3.1.2, that demonstrated desirable rheological properties and good biocompatibility for long-term cultivation of cells (Fan et al., 2016). Human intestinal epithelial HCT116 cells encapsulated in the printed Matrigel-agarose constructs had high cell viability and proper cell spreading morphology. This approach, however, is connected to the problems outlined for Matrigel above.

Another natural macromolecule with some promising properties for bioink preparation is *chitosan*, a β-1,4-linked glucosamine polymer that is formed by deacetylation of chitin. Chitosan is a non-toxic, biocompatible polysaccharide that is mostly obtained from shellfish, shrimp waste, and crab and lobster processing and thus replaces material from higher animals with that from lower ones. From the 3R (replacement, reduction, refinement) perspective, however, it would be desirable to fully substitute animal-derived materials, which can be achieved by isolating chitosan from fungi (Ghormade et al., 2017). The degree of deacetylation and the molecular weight of chitosan directly affect its physical and mechanical properties, whereby the degree of acetylation is directly proportional to its viscosity, biocompatibility and biodegradability. Dependent on pH and temperature conditions, chitosan can be used to form a hydrogel that is liquid at room temperature and transitions to a gel as the temperature increases (Domalik-Pyzik et al., 2019; Kean and Thanou, 2019). Cross-linking occurs physically by non-covalent interactions. Although thermosensitive hydrogels thus overcome the typical problems associated with the use of chemical cross-linkers, such as cytotoxicity and reduced cell viability, they possess weak mechanical strength as well as slow gelation properties. These disadvantages can be overcome by physically blending chitosan with other polymers, as well as by chemical cross-linking with natural agents like genipin or tannic acid (Hafezi et al., 2019; Rivero et al., 2010). In a systematic study, different gelling agents for chitosan were compared, and $NaHCO_3$ was found to be more amenable to bioprinting than β-glycerophosphate or K_2HPO_4 (Ku et al., 2020). Some disadvantages of chitosan, however, are its unstable mechanical properties and unfavorable characteristics regarding bioprintability (Hospodiuk et al., 2017).

From the animal welfare perspective, it is generally desirable to replace material obtained from higher animals (mam-

mals) with material that can be extracted from lower animals such as crustaceans and – even better – by plant material. From a scientific point-of-view, hydrogels containing animal-derived substances, e.g., ECM, collagen or gelatin, may have substantial influence on human cells. These chimeric systems of human cells embedded in environments of animal origin may therefore have unphysiological properties. As plants are evolutionarily more distant from humans than animals, their polymers usually have a lower impact on human cells. Still, the combination of plant material with human cells produces highly unnatural chimeric systems. To make the bioprinted organ models physiologically more relevant, however, it is possible to degrade the plant material over time and replace it with ECM deposited by the human cells.

Synthetic materials have been used in bioprinting to a lesser extent as they are, in many cases, less biocompatible than natural materials. Fully chemically synthesized substances are desirable from the animal welfare perspective, as they obviously do not require materials of animal origin; however, they are sometimes cytotoxic (as natural substances may be as well) and do not support adhesion of cells, which is required for spatial distribution of cells in 3D organ models. One important advantage of synthetic substances is that their properties can be adjusted by fine-tuning their synthesis. As discussed in Sections 3.2.1 and 6.3, PEG is an example of a synthetic material with good biocompatibility. Its interaction with human cells, however, needs to be improved by the addition of chemical groups to ensure cellular adhesion. Pluronic F-127 is another artificial substance that is frequently used to produce organ models by bioprinting. When using Pluronic-127, however, it should be noted that effects on the transcription of genes have been reported, mainly through the activation of certain stress signaling pathways (Sriadibhatla et al., 2006). It is therefore wise to investigate possible unintended side effects when including Pluronic-127 in a bioink formulation. One of the main fields of its application is to exploit its melting properties to produce vascular structures. For this application, Pluronic F-127 is a fugitive ink, which initially fills tubes lacing through the construct and is then liquefied and extracted by cooling the construct to 4°C. These examples demonstrate that synthetic bioinks also hold great potential for bioink optimization and should be developed further.

Another promising approach is the use of synthetic peptides as a bioink. These materials form highly printable hydrogels that are reminiscent of the native ECM (Boyd-Moss et al., 2017). An example is the synthetic peptide RAD16-I, which consists of 16 amino acids, comprised of four repetitive units of arginine (R), alanine (A), aspartic acid (D), and alanine (A). This self-assembling peptide can form a hydrogel structure that is permeable to gases, nutrients, and growth factors. In a recent study, a RAD16-I bioink, supplemented with methylcellulose to increase its viscosity, was used to culture human mesenchymal stem cells in a predefined 3D structure (Confino et al., 2019). The bioink displayed good shape fidelity and stability, and cell viability was high. Furthermore, the cells were able to differentiate into the adipogenic lineage, confirming the potential of peptide-based bioinks for tissue engineering applications.

7 Conclusions

For decades, two-dimensional cell cultures and animal models have been the dominating tools in biomedical research. Currently, 3D tissue models are about to revolutionize life sciences, as they allow the study of natural cell-cell and cell-matrix interactions in humanized systems. Bioprinting is an upcoming technology that produces organ models with unprecedented precision. As it allows use of human cells, it can help to improve human-relevant biomedical research, avoiding the problem of species-specific differences inherent in pre-clinical animal studies. At the same time, bioprinting contributes to the 3R principles, as organ models can replace *in vivo* experiments. However, many hurdles must still be overcome before biomimetic systems will be generally accepted as an alternative to animal testing. The main challenges include production of multi-cell type models with high spatial resolution, the inclusion of immune cells, and the vascularization of the organ model. Therefore, a great effort is being made to further improve the quality of existing models, e.g., by producing a vasculature by coaxial bioprinting (Wu et al., 2020). Despite continuous development of systems of higher relevance, some studies have already demonstrated the potential of bioprinted organ models to serve as tools for toxicological investigations, cancer studies, and experiments with infectious pathogens, as outlined in Section 5.

A closer inspection of currently available bioprinting approaches, however, reveals that substances of animal origin are widely used at various stages of the production and cultivation process. These include components that are generally used in eukaryotic cell culture, such as FBS, as well as materials that are specific to advance culturing technologies, including animal ECM (Matrigel), gelatin and related compounds. The often-hidden use of animal material in bioprinting of humanized organ models counteracts both attempts to reduce animal use for research purposes and to increase reproducibility and relevance of research to human health and disease. It generates chimeric systems of human cells in an environment consisting of components of animal origin. Throughout our research, we have found that virtually all published bioprinting studies use animal-derived material at some stage of the experimental process. An exception is the study of Mendes et al. (2019) that produced a xeno-free model, though it compared the newly developed bioink to hydrogels consisting of animal material.

It is thus obvious that bioprinting still has a long way to go before it can develop its full potential of replacing animal use and producing completely humanized systems. For models with maximal physiological relevance, the ultimate goal will be a 3D construct consisting of all organ-relevant human cell types embedded in their own ECM. This can be achieved with degradable hydrogels that stabilize the 3D structure during the printing procedure and can then gradually be replaced by ECM deposited by the cells. The study of Langer et al. (2019) took a major step in this direction, as the hydrogels of the bioink provided tensile strength and rigidity during tissue fabrication but were removed during subsequent culture, leaving a scaffold-free structure of cells that deposited its own ECM and self-organized. A

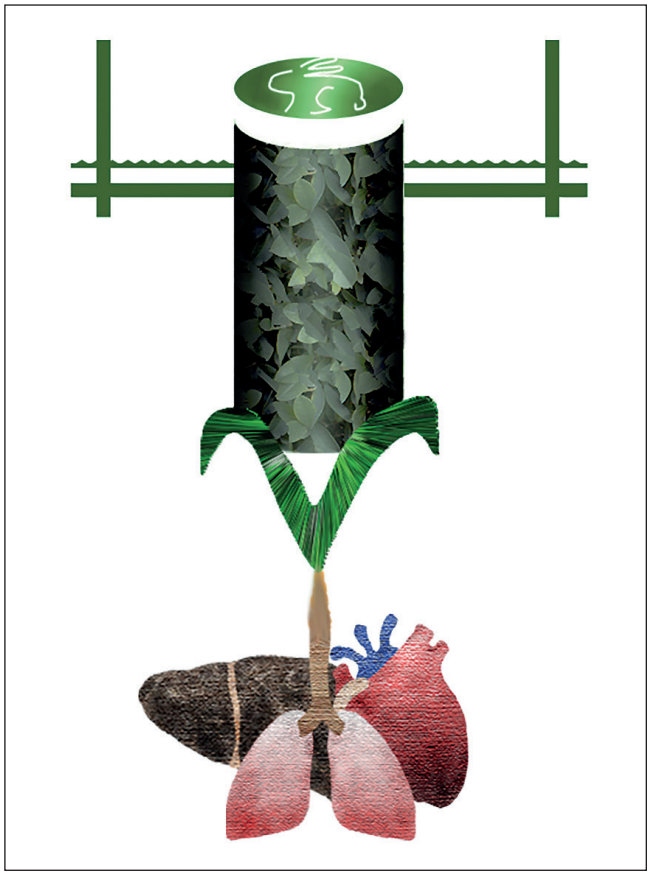

Fig. 5: Logo for clean bioprinting
The logo symbolizes that clean bioprinting of organ models uses only bioinks that are devoid of animal components.

drawback of this study, however, was the use of porcine gelatin for the hydrogel that should be replaced with material from a non-animal source. Nevertheless, the development of degradable bioinks supports the upcoming concept of 4D bioprinting, which includes 3D bioprinting of tissue structures that are then allowed to self-organize or respond to stimuli and adopt novel structures over time (Yang et al., 2019).

Although researchers are increasingly trying to implement 3R aspects in their research strategies, the higher costs and greater effort involved are inevitably weighed against animal welfare and therefore seldom result in replacement of all components of animal-origin used in bioprinting technologies. We believe that the substitution of animal components, such as with recombinant human proteins or synthetic hydrogels and media, will improve the significance of research in bioprinted organ models for human (patho-)physiology. Thus, implementing 3R principles in bioprinting will not hinder research, but rather promote its relevance.

Based on these arguments, we encourage researchers, regulatory bodies and government organizations to promote the implementation and spread of these approaches. The development of advanced skin models has shown that political measures such as the ban of animal experiments in the cosmetics sector can inspire the search for alternatives. Similar regulations in biomedical research for replacement of animal materials such as FBS or ECM obtained from live animals with adequate deadlines to adapt to alternative technologies may help to advance new developments. In addition, a financial reward or compensation system for the use of expensive serum-free media may be implemented for publicly funded research, similar to funds for open access publishing that have been established in many countries in recent years. Finally, we suggest calling xeno-free approaches clean bioprinting and propose a logo that can be used to mark organ models produced without any animal materials (Fig. 5).

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Conflict of interest

The authors declare that they have no conflicts of interest.

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