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REVIEW

Strategies based on organ decellularization and recellularization

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SUMMARY

Transplantation is the only curative treatment option available for patients suffering from end-stage organ failure, improving their quality of life and long-term survival. However, because of organ scarcity, only a small number of these patients actually benefit from transplantation. Alternative treatment options are needed to address this problem. The technique of whole-organ decellularization and recellularization has attracted increasing attention in the last decade. Decellularization includes the removal of all cellular components from an organ, while simultaneously preserving the micro and macro anatomy of the extracellular matrix. These bioscaffolds are subsequently repopulated with patient-derived cells, thus constructing a personalized neo-organ and ideally eliminating the need for immunosuppression. However, crucial problems have not yet been satisfyingly addressed and remain to be resolved, such as organ and cell sources. In this review, we focus on the actual state of organ de- and recellularization, as well as the problems and future challenges.

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Introduction

Solid organ transplantation is currently the only curative treatment option available for patients suffering end-stage organ failure. Although transplantation has improved the quality of life and survival of patients, unfortunately, only a small group of patients benefits from this procedure because of organ scarcity. Indeed, 14 773 patients were on the waiting list for organs in the Eurotransplant region on January 2018, whereas only 6636 transplantations from deceased donors were performed in 2017 [1]. Moreover, in the same year, 1323 patients died on the waiting list [1]. Alternative treatment options are urgently needed to address the organ shortage problem. The technique of decellularization and recellularization has become an interesting experimental approach [2–4]. In a future clinical setting, a xenogenic human-scale (e.g. porcine) or allogenic organ will be used for decellularization, followed by recellularization with cells from the patient suffering from organ failure [e.g. induced pluripotent stem cells (iPSC)] are isolated, organ-specific differentiated and expanded). After the recellularization and maturation of this neo-organ, the personalized graft will be implanted without the need for long-term immunosuppression [5].

In this review, we will discuss the state of the art of this technique and the unsolved problems and future challenges (Fig. 1).

Decellularization procedure

Currently, no universal definition for the term decellularization has been established. Crapo *et al.* [3] have

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provided the only statement addressing this issue, concluding that (i) the ECM should not contain more than 50 ng of DNA per mg dry weight, (ii) DNA fragments should not be longer than 200 bp and (iii) no nuclear components should be visible within the ECM in to achieve optimal decellularization. Decellularization is characterized by the removal of the cellular and immunogenic components (e.g. alpha-Gal) from an organ, while simultaneously preserving the native ultrastructure and biochemical and biophysical properties of the extracellular matrix (ECM) [6,7]. Various decellularization techniques and agents have been described and are classified into biological, chemical and physical agents [3]. Biological agents mainly include enzymes, such as nucleases, trypsin and chelating agents (e.g. EDTA). However, chemical agents are most commonly used and are classified into acids and bases, hypotonic/hypertonic solutions, and ionic, nonionic and zwitterionic detergents. On the other hand, temperature regulation is the method of choice to achieve physical decellularization (e.g. freeze–thaw cycles before decellularization and temperature adjustment to enzymatic activity) [8,9]. Moreover, physical decellularization is performed using mechanical forces and pressure, as well as nonthermal irreversible electroporation, but these methods are inadequate for application in whole-organ decellularization [3].

Indeed, the construction of functional and perfusable neo-organs requires an intact vascular tree and an optimally preserved ECM. Therefore, pressure-controlled or flow-controlled perfusion decellularization was the most adequate method to homogenously expose the whole organ to the decellularization agents [6,10,11].

Moreover, the application of different environmental conditions potentially improves the quality of decellularization [12]. Decellularization protocols often combine different techniques and agents to achieve the optimal depopulation of the organ. However, only a few nonsystematic investigations exploring the effect of different decellularization protocols on the organ-specific ECM have been published [13–16]. The effects of various decellularization protocols on recellularization and implantation remain poorly investigated).

The ECM forms a noncellular three-dimensional macromolecular network composed of collagens, proteoglycans, fibronectin, laminins and other glycoproteins [17]. The ensemble of extracellular matrix and ECM-associated proteins (e.g. growth factors) is called the matrisome [18]. The ultrastructure and composition of the ECM is essential for cell differentiation, proliferation, survival and migration [17,18]. However, each decellularization agent and technique exerts a specific altering or disruptive effect on the ECM components. This method-specific impact must be studied in more detail [3]. Indeed, the application of an improper detergent, method or time may readily induce extensive damage to the 3D structure and its components. Investigations of the integrity of the ECM are currently mostly limited to immunohistochemical examinations, scanning electron microscopy and measurements of the total collagen, glycosaminoglycans (GAG) and DNA contents. More detailed approaches were first published in 2014 and 2016 by Gilpin *et al.* [16] and Li *et al.* [19], who performed proteomic analyses of decellularized lungs and livers respectively. Li *et al.* observed that 517 proteins remained intact after decellularization, only 58 of which where matrisome proteins. Because the matrisome is composed of hundreds of proteins, the disruptive effect of decellularization becomes concretely apparent [19]. Several other groups (e.g. Park *et al.* [20]) reported the removal of some of the previously present growth factors after decellularization, while still preserving the ability to support iPSC differentiation. A more extended overview of the deleterious effects of decellularization agents exceeds the scope of this review and is presented elsewhere [3].

Since balance is the key to optimal decellularization, inappropriate detergent concentrations or inadequate exposure times lead to an unsatisfactory removal of residual cells, threatening the lack of immunogenicity of the scaffolds. These residual cellular proteins potentially function as damage-associated molecular pattern (DAMPs) and lead to an undesired inflammatory response after implantation [21]. Other ECM components, such as nanovesicles and microvesicles, have only recently been discovered [22]. The first study published in 2016 identified miRNA-containing matrix-bound nanovesicles [22]. However, the study was limited to dermal, urinary bladder and small intestinal submucosa ECM, while these investigations are pending in decellularized solid organ ECM.

The previously mentioned, poorly explored topics considering the optimization of decellularization methods with the goal of optimal ECM preservation should be the focus of future studies. Nevertheless, questions remain regarding whether the existing approaches are sufficient to overcome these challenges.

Immunogenicity and its implications

A recellularized ECM scaffold remains a structure with a native composition, and the immunogenicity it elicits

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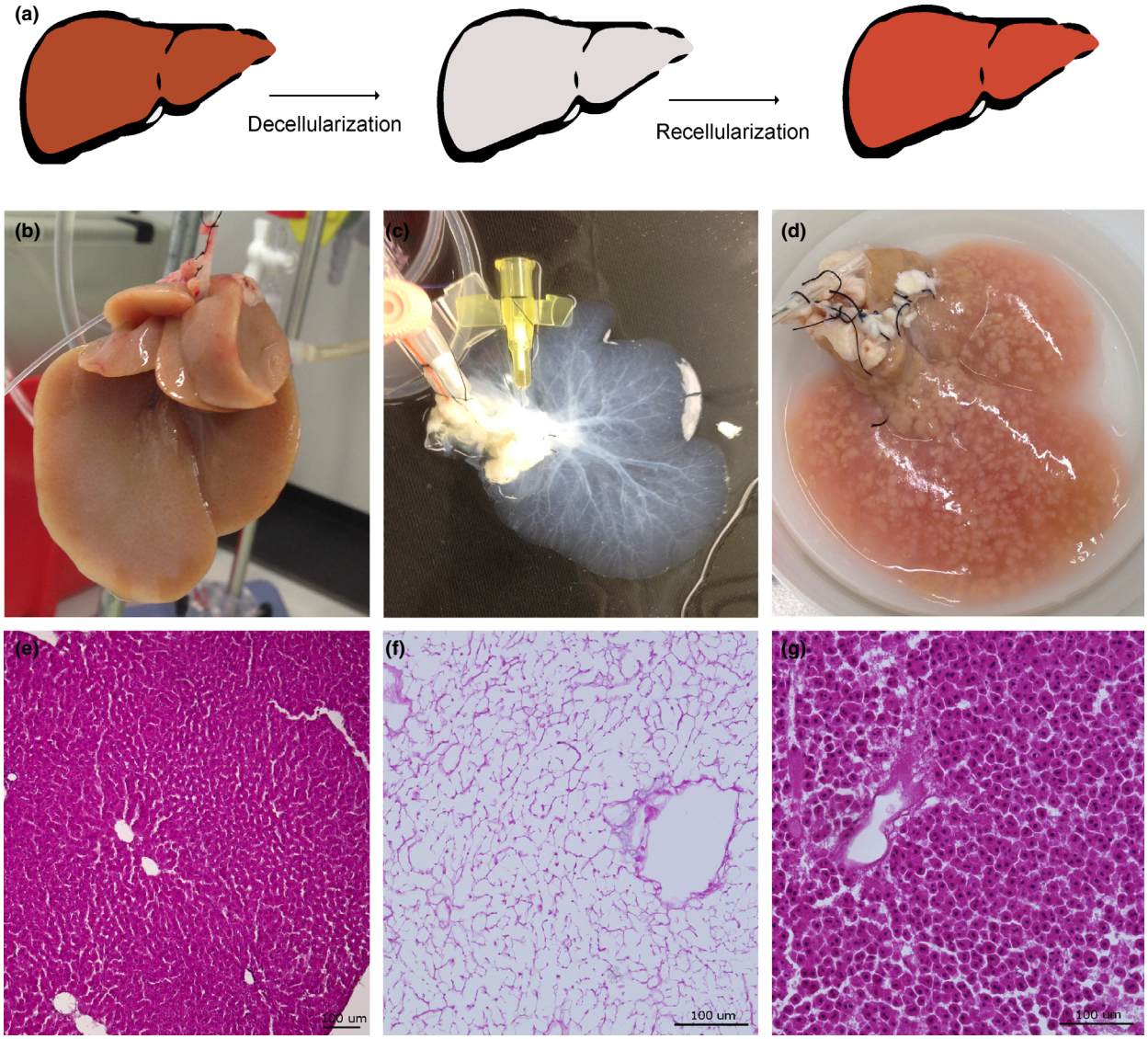

Figure 1 Concept of de- and recellularization (an example is shown for the liver). (a) Schematic showing de- and recellularization. (b) Native rat liver. (c) Decellularized rat liver. (d) Recellularized rat liver. (e) H&E staining of the native rat liver. (f) H&E staining of the decellularized rat liver. (g) H&E staining of the recellularized rat liver.

upon implantation is correspondingly anticipated. Indeed, the host response to the neo-organ may vary, depending on different factors. The activation of the host immune system remodels the implanted tissue, a process that is either destructive or constructive. The latter occurs when the scaffold is thoroughly decellularized, free of endotoxins or bacterial contamination and is implanted into a healthy surrounding tissue [23–26]. The response this structure evokes is multifold and complex. Although multiple studies have attempted to simplify the host response to biological scaffolds, the topic remains poorly investigated and the knowledge is quite limited to very few studies.

The immune response to implanted biological scaffolds is classified into innate and adaptive responses. Mast cells, dendritic cells, basophils, eosinophils, natural killer cells, neutrophils and macrophages all compose the innate immune system and are responsible for the acceptance of these scaffolds. The isolated study of the activity of only one of the aforementioned cell types upon implantation is challenging, since the immune response is generated by the interplay among these cell types [27]. Moreover, the innate immune response activates the adaptive immune system [28], which consists of B and T lymphocytes, also called memory cells, capable of remembering the pathogen and later reacting in

a pathogen-specific manner [29]. Indeed, the innate immune system is first mechanism of the host that reacts upon the implantation of an ECM scaffold. The first 24–48 h after implantation are characterized by the infiltration of neutrophils into the scaffold, followed by infiltration of mononuclear cells [30–32]. In this regard, macrophages and macrophage polarization from an M1 pro-inflammatory phenotype towards an M2 pro-remodelling phenotype must be mentioned. Successful decellularization elicits the constructive remodelling characterized by neo-matrix deposition produced by stem cells and results in a newly formed, site-appropriate tissue devoid of chronic inflammatory processes that is mediated by M2-phenotype macrophages. The M2 macrophages persist on the scaffolds for a long time, whereas their depletion correlates with a failure to degrade the implant. On the other hand, the opposite reaction is characterized by chronic inflammation and scar tissue formation [21]. Importantly, this simplistic depiction of the classification of the host response and the macrophage polarization into only two types of cells actually consists of much more complex events [27].

In 2001 and 2002, Allman *et al.* [33,34] reported the role of the adaptive immune system in remodelling decellularized ECM. T helper (Th) cells play a key role in the adaptive immune system. These cells are divided into Th1 cells, which are responsible for a pro-inflammatory response, and Th2 cells, which promote a regulatory immune response [35]. Allman *et al.* [33] studied the Th cell immune response by implanting porcine small intestine submucosa (SIS) into mice and observed a Th2 cytokine expression profile and antibody production, potentially indicating the acceptance of these xenogenic grafts. However, the immune response to decellularized ECM scaffolds is a far more complex orchestra and exceeds the scope of this review.

Crapo *et al.* defined a residual DNA concentration of less than 50 ng of dsDNA per mg of ECM with a fragment size of less than 200 bp as threshold indicators for a successful decellularization process. These values have become the most frequently used references [3]. Macrophages are capable of recognizing DNA fragments of 24 bp [36]. Residual DNA, when fragmented, converts into a DAMP and leads to inflammasome activation through Toll-like receptors (TLR) and subsequent microRNA activation and IL-6, IL-1, TNF and IFN-γ secretion [37,38]. Indeed, residual DAMPs that remain intact even after decellularization have become an increasing concern [27]. Both cellular molecules that are commonly known to function as DAMPs and residual ECM components such as hyaluronic acid, heparan sulphate and fibronectin that are converted into DAMPs upon fragmentation may affect the immunogenicity of the decelluarized ECM [39]. Although some of these DAMPs possess a constructive function, they mostly exert a destructive effect on the ECM [40].

Attempts to increase the organ pool and developments in the field of tissue engineering have led to an increased interest in xenogeneic materials as potential organ sources [41]. Simultaneously, the problem of the host immune response against the carbohydrates composing the porcine-derived xenografts has been noted. Indeed, the production of antibodies against the α-Gal epitope, such as anti-Gal IgM, IgG and IgA, leads to severe hyperacute rejection and coagulation within minutes [42–45]. Moreover, successful removal of this antigen is not achieved through decellularization alone, since it is expressed on ECM components such as laminin and fibrinogen, glycoproteins and glycogen [46,47]. Therefore, researchers have attempted to simultaneously remove the Gal epitope and decellularize the scaffolds. Stahl *et al.* studied the immunological effects of the decellularized αGal knock out porcine lung implants and compared them with native and decellularized wild type porcine lung implants for 8 weeks. The authors did not observe any significant differences between decellularized WT and KO scaffolds, although the removal of the αGal epitope delayed immune cell infiltration and reduced chronic T-cell-mediated reactions to the scaffolds [41].

In conclusion, the results from recent studies suggest that an immunological analysis of the decellularized scaffolds must be performed prior to implantation to assess the host response postimplantation. Current knowledge on the topic is based on the subcutaneous implantation of decellularized whole organs, and researchers are unsure whether this accumulated knowledge is also translatable to *orthotopic* implantation.

Bioreactors for de- and recellularization

Successful whole-organ recellularization requires a specific environment that mimics the physiological conditions of the specific organ. Usually, the process of recellularization is divided into two phases: the static cultivation of cells followed by the dynamic process of recellularization. Perfusion recellularization is required to achieve reendothelialization of the vascular tree and the even dispersion of the cells into the parenchyma. The dynamic recellularization phase includes the perfusion of the scaffold with a medium-based cell suspension [48,49]. Bioreactors must fulfil certain

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requirements, such as perfusion flow adjustment or access to the applied cells, to achieve recellularization *in vitro*. Bioreactors are designed in such a way that organ-specific biological and technical demands are met and optimal conditions for the engineering of a particular organ are supplied [50]. Although the body itself theoretically serves as the ideal bioreactor, an environment that allows an engineered neo-organ to mature under *in vivo* conditions until it can resume its function is required. However, several steps must be performed prior to *in vivo* maturation, steps that might be fatal to humans if performed *in vivo*. For instance the complete reendothelialization of the vascular tree must be subjected to organ-specific shear stress prior to implantation. Otherwise, the abrupt subjection to blood flow after static cultivation would lead to cellular rupture and thrombotic complications, the most minimal side effect of which would be graft dysfunction [51]. Moreover, the isolated *in vitro* incubation of the recellularized scaffold provides the cells with sufficient time to settle. Intraportal islet cell transplantation is an example of the crucial role this process plays, a procedure that requires large amounts of cells, since half of them die shortly after injection. The explanation for this cell death is the disruption of the vascular niche supplying islets with oxygen and nutrients during isolation and a lack of sufficient time for the islets to settle and restore this vascular component [52].

Thus, bioreactors are essential to adjust and maintain physiological conditions during decellularization and recellularization. Various determinants have been described to influence the results of decellularization, such as pH, perfusion pressure, surrounding pressure or perfusion flow rates. For instance a comparison of the effects pH values of 8, 10 and 12 on the optimal removal of residual DNA and simultaneous preservation of crucial components of the ECM in porcine lung matrices indicated major variability, where a lower pH suppressed the loss of GAG components and elastin but did not effectively remove all the residual DNA [53]. Moreover, Struecker *et al.* [11,54] showed a beneficial effect of oscillating surrounding pressure conditions on liver decellularization. Unfortunately, the observation and regulation of these parameters has only rarely been reported. Bioreactors play an even more important role in recellularization, as briefly mentioned above. Real time monitoring of certain parameters, such as pH, pO_2, pCO_2, temperature, electrolyte levels, glucose or lactate concentrations, and perfusion parameters, such as perfusion pressure and flow rates, is vital and these conditions must be maintained stable and adjustable, particularly during long-term culture [55]. This monitoring further allows researchers to calculate other important parameters, such as vascular resistance, which are essential for a controlled recellularization process. Furthermore, Ott *et al.* [56] successfully developed a resazurin-based assay to investigate cellular viability and proliferation during reendothelialization, a novel noninvasive method for a more controlled recellularization process. As mentioned above, bioreactors must fulfil organ-specific requirements. For instance recellularization of the lung requires tracheal access that facilitates the application of cells and medium via the tracheobronchial system or ventilation of the lung to observe gas exchange. On the other hand, tissue engineering of the heart requires electrical and biomechanical stimulation, such as the system built by Hülsmann *et al.* [57–59]. In the tissue engineering of organs such as the liver, monitoring of the levels of albumin and coagulation factors is of crucial importance.

Although the concept of using bioreactors to construct implantable functional whole organs is very promising, currently, these goals have been only partially fulfilled. A bioreactor is unlikely to reflect the complex mechanisms that occur *in vivo* during organ development and regeneration. Moreover, all bioreactors are single-organ systems, thus lacking the complex interactions to which tissues are exposed in the human body. Therefore, more extensive cooperation between biologists and engineers is required for advances in this field, not only by designing bioreactors that more accurately imitate *in vivo* conditions but also by obtaining a better and more concrete understanding of the *in vivo* conditions that are required to engineer these organs [50].

Recellularization

Recellularization is defined as the repopulation of acellular ECM scaffolds of tissues or organs with organ-specific cell types or stem cells [e.g. iPSCs or embryonic stem cells (ESCs)], aiming to reconstitute the microanatomy of the organ and thereby recreate the organ-specific function. The cells used for recellularization need guidance for their rearrangement and maturation, a task that is better performed by the ECM and its residual components. Indeed, as mentioned above, the preservation of the organ-specific ECM components such as growth factors and various structural proteins facilitates an optimal recellularization process [20,60–64]. However, an organ might be reconstructed by

cultivating organ-specific cells in an ECM derived from another organ (e.g. spleen and hepatocytes or hepatocyte-like cells) [65–68]. Although this concept promises to be very practical, it only appears to be suitable for organs possessing an endocrine function (e.g. the endocrine pancreas) that do not require excretion or a specific anatomy for their function [69,70].

In the ideal setting of the implantation of a recellularized neo-organ into a patient suffering from end-stage organ failure, the ECM scaffold would be obtained from a deceased nontransplantable human organ or from a human-scale animal organ (e.g. pig). This scaffold would subsequently be repopulated with specifically differentiated patient-derived induced pluripotent stem cells (iPSCs), rendering immunosuppressive therapy futile [5,7].

However, this setting only appears to be applicable to patients with chronic organ failure, since the generation of a human-scale neo-organ will require a long time to complete its *in vitro* or possible *in vivo* maturation until it can resume the function of the whole organ. Thus, acute organ failure would unfortunately outpace the extent of this technique.

Recellularization is typically performed using two different techniques. Depending on the cell type, the first technique involves recellularization via the vascular network or another hollow structure (e.g. bile duct, ureter, intestinal lumen or airway) [62,71–73]. Alternatively, recellularization via direct cellular injection into the parenchymal compartment is also possible. Additionally, combinations of both techniques have been described [74]. One major concern in recellularization experiments is the *black box* approach, which is performed in most studies examining repopulation, and different outcome parameters are subsequently evaluated. Biomechnical aspects during recellularization have not been considered, such as whether the parenchymal cells cross the basal membrane of the vessels during recellularization and how the ECM is damaged during direct injection. Moreover, the spatiotemporal resolution of recellularization dynamics remains poorly understood. A first study exploring the field of recellularization mechanics was published by Remuzzi *et al*. In their experiments, the authors systematically studied the cellular distribution at different time points after changing various parameters (e.g. cell number, flow rate and recellularization access). They used a theoretical model to study medium velocity during repopulation and observed low medium velocities within the afferent arterioles, which may explain the accumulation of cells within the glomerular capillaries. However, at high and medium velocities, cells formed clusters in the tubular space, which may be related to the disruption of the tubular membrane. This study highlights the importance of an in-depth analysis of the recellularization process [75].

For a successful recellularization process, the repopulation of the vascular ECM network with endothelial cells is necessary to prevent the thrombogenicity induced by the exposed vascular ECM and potential subsequent loss of the neo-organ graft. None of the reendothelialization studies published to date have shown the full coverage of the vascular framework from the main branches to the capillaries [10,56,76]. Hence, additional measures have been described with the aim of covering residual exposed ECM areas with different antithrombotic chemical cross-linking and covering agents [78–82]. Although these agents have reduced thrombogenicity, long-term prevention of clotting *in vivo* was not achieved.

Heart

The unique characteristics of the heart arise from interactions between autonomous electrical stimulation and its biomechanical function. The challenge of bioengineering a heart is to restore this perfectly orchestrated motion. Since the first clinical implantation of a tissue-engineered heart valve in 2000, cardiovascular surgery and tissue engineering have been close relatives [83,84]. Thus, unsurprisingly, the first report on whole-organ de- and recellularization was published by a cardiovascular research group.

Ott *et al*. described the first whole-heart de- and recellularization in 2008. In this study, decellularized rat hearts were subsequently recellularized with rat neonatal cardiomyocytes (rNCs) and rat aortic endothelial cells (RAECs), and the bioengineered heart scaffolds showed electrical and contractile responses to electrical stimulation and corresponding left ventricular function [6]. Since the publication of this ground-breaking study, many decellularization protocols for mouse, rat, porcine, ovine and human hearts have been developed [6,59,61,85–91]. The most commonly applied decellularization technique is the modified Langenhoff perfusion of the detergents via the ascending aorta and into the coronary arteries (flow- or pressure-controlled). However, a new approach reported by Nguyen *et al*. [92] describes a decellularization process through the venous and arterial system. Many recellularization and reendothelialization studies have been conducted since 2008 [6,58,59,61,74,87,91–94], with direct injection [6,57–59,91] (multiple injections) into the ECM and the

infusion of the endothelial cells via the coronary arteries or venous inlet [61,95,96] (e.g. multistep infusion and recirculation) being the most commonly applied methods. Combinations of both techniques have also been described [74,93,94]. Whole-heart recellularization in the rat model is commonly performed using rNCs and RAECs [6,57,58,74,96]. Various research groups have observed electrical signalling and corresponding contraction *in vitro*, where recellularized hearts were maintained for up to 30 days [6,57,61,91,94–96]. Xenogenic cell-scaffold combinations are possible (e.g. mouse ECM and human cells) [59,61,92,94,95], as described in the study performed by Lu *et al.*, who showed that mouse heart scaffolds were recellularized with human multipotent cardiovascular progenitor cells derived from iPSCs or ESCs. The authors further studied cellular differentiation within the matrix, their functional interaction and the scaffold reactions to inotropic drugs [61]. Surely, scale up of this technique is imperative for the translation of these findings to the clinic. To that end, porcine and human hearts have been recellularized, and Guyette *et al.* [91,93,94,97] used iPSC-derived human cardiomyocytes (500 million) to repopulate the parenchyma. Moreover, the mechanical properties of the decellularized tissue have also been studied [86,89,91,98,99], and the beneficial effects of mechanical and electrical stimulation during *in vitro* maturation have been confirmed [57–59,91]. Although de- and recellularized heart grafts have been heterotopically implanted in rat, porcine and bovine models for up to 60 days [6,74,87,93,97], none of these transplantation experiments showed *in vivo* functionality and long-term perfusion of the implants. As shown in the study by Robertson *et al.* [74], reendothelialized hearts exhibit less blood clotting in a heterotopic rat transplantation model than acellular scaffolds.

Lung

The first experiments of whole-lung de- and recellularization were published in 2010 [10,100]. The mechanical properties of the recellularized lung scaffolds should be similar to the native lung for proper function *in vivo*. Therefore, the development of harmless but effective decellularization protocols is crucial. Different techniques and detergents (e.g. SDS, SDC, Triton-X 100 and CHAPS) have been investigated in different species, such as mouse, rat, pig and human [16,76,101–105]. Unfortunately, the optimal decellularization protocol remains undefined.

Recellularization was initially performed by injecting primary pulmonary and endothelial cells, as well as cell lines, via the trachea and pulmonary artery [10,100]. The most basic function of a bioengineered lung tissue is gas exchange. Restoration of gas exchange, the most basic function of the lung, involves the reconstruction of the microstructure of the epithelialized airways and endothelialized vessels and is of utmost importance. Endothelial cells have been seeded via the pulmonary artery and the pulmonary vein [10,76,100,101,106]. The airway epithelium is repopulated through the bronchial system to rebuild the gas exchange surface, whereas the recellularization of the endothelium is performed via a multistep technique with various cells to mimic the native vessel, with perivascular cells supporting endothelial development [76,107]. The most recent large animal study was published in 2018 by Zhou *et al.* The authors recellularized porcine lungs with human airway progenitor cells and human umbilical vein-derived endothelial cells. After an *in vitro* maturation period of 6 days, the bioengineered lungs were implanted into pigs and perfused for 1 h. However, the gas exchange and the compliance of the repopulated lungs were significantly lower than the native lung. Different groups have established bioreactor-based *in vitro* cultures of recellularized grafts, in which conditions such as vascular perfusion and bronchial system ventilation were continuously supplied to either facilitate cellular differentiation, in the case of iPSCs or ESCs, or maturation of the neolungs. The main goal is to mimic the physiological environment and allow the cells to construct an intact neo-organ that resembles the native lung. Here, induced pluripotent stem cell-derived cells are favoured because of their expansion potential and autologous origin [16,76,103,105,108]. For recellularization, iPSC-derived pulmonary cells are favoured because of their expansion potential and autologous origin [76,109].

The first implantation of a recellularized lung scaffold in rats was published in 2010 [10,100]. Since the publication of these studies, sufficient gas exchange and perfusion of the recellularized, implanted lung scaffold were successfully reported, and these results have been translated to a humanized porcine model [76,101,107]. Furthermore, the first decellularization of human and human-size grafts showed the feasibility of upscaling this concept. However, the size of human lungs makes experiments costly. Therefore, recellularization experiments are mainly performed in smaller animal models.

Liver

Although many alternative treatment options have been investigated for patients with end-stage liver diseases,

such as a (bio-)artificial liver support system or hepatocyte transplantation, none of these approaches represents a viable alternative to orthotopic liver transplantation in the clinical setting [7].

Due to its outstanding regenerative capacity, the liver has been extensively studied in the fields of tissue engineering and regenerative medicine. Since the first description of whole-liver de- and recellularization in 2010, great achievements in the field of liver bioengineering have been reported [110,111]. Various decellularization protocols have been established in mouse, rat, ferret and human-scale animals, such as sheep and pigs [11,54,69,112–114]. Furthermore, the first protocols for decellularization of deceased human livers became available in 2017 in studies published by Verstegen *et al.* [115].Nevertheless, no systematic comparisons of the damage different decellularization protocols exerts on the extracellular matrix or on recellularization results are available. Recently, researchers have focused not only on optimizing decellularization conditions through the surrounding oscillating pressure or controlling the pressure during the process but also on the reendothelialization and recellularization of whole livers in different preclinical trials. Perfusion decellularization is typically performed via the portal vein (PV), but the hepatic artery (HA) or combinations of either HA, PV or hepatic veins (HV) are also used [8,11,54]. Recellularization has been described via PV or HA, or combinations of PV and HV. The common bile duct may offer researchers the opportunity to guide the hepatocytes to their natural position, bas the *Canaliculi biliferi* are formed by the hepatocytes. After decellularization, the space formerly inhabited by hepatocytes should be accessible via the bile canaliculi. Studies investigating whether this approach is feasible are lacking. Several studies have successfully achieved reendothelialization (with or without coating the remaining ECM) and parenchymatous recellularization of liver scaffolds *in vitro* [8,20,64,73,112,113,116–123]. Various cell types, such as hepatocytes (e.g. primary, hepatic stem cells or iPSC-derived hepatocyte-like cells), endothelial cells (e.g. HUVECs or endothelial progenitor cells) and combinations with biliary epithelial cells or mesenchymal stromal cells have been used for recellularization. The existing studies have achieved an *in vitro* cultivation of the neo-livers for up to 28 days and revealed restored functionality (e.g. albumin secretion, urea production, ammonium metabolism and enzyme regulation), drug metabolism and viability of the seeded cells [8,20,64,73,79,81,112,113,116–119,121]. According to Robertson *et al.*, recellularized livers produce albumin and metabolize midazolam until the end of their study at 28 days. However, the greatest challenge in liver bioengineering is the re-orchestration of the liver parenchyma, the vascular network and the biliary system, because an implantable neo-liver has must restore hepatic function, biliary excretion and, thereby, detoxification. No recellularization protocol has achieved the combination of all of these components. Several studies have reported the successful implantation of repopulated decelluarized liver grafts [20,64,80,112,113,120,124,125]. At 40 days, Yang *et al.* [64] showed the longest period of graft survival. Although these studies represent a great success, none of the implanted neo-livers showed sustained function.

Kidney

Although dialysis serves as a clinically applicable renal replacement therapy, kidney transplantation is the gold standard for the treatment of chronic kidney diseases, with respect to patient morbidity, survival and health economics [126]. Due to the general organ shortage, alternatives are urgently needed.

The first report of the de- and recellularization of renal scaffold was published by Ross *et al.* in 2009 [63]. Subsequently, different decellularization protocols were applied to kidneys from different species, such as mouse, rat, goat, pig, monkey and human [77,82,127–130]. Decellularization of whole kidneys is generally performed via the renal artery. Peloso *et al.* [131] reported the effective decellularization of 40 discarded human kidneys, bringing us a step closer to the possibility of a greater organ pool. Zambon *et al.* [132] addressed a further common concern regarding decellularization, which is the preservation of the vascular structures, by showing the disruption of the glomerular microarchitecture following the perfusion of detergents through the renal artery, and he modified the flow rate, detergent concentration and decellularization time to solve this problem. The kidney contains more than 26 types of cells organized in a complicated structure consisting of thousands of nephrons [2]. Thus, the identification of an unlimited cell source is another factor limiting the success of tissue engineering. Embryonic stem cells are pluripotent stem cells that are capable of generating all renal cell types. Ross *et al.* were the first group to show the proliferation and differentiation capacity of these cells in a kidney scaffold. The Batchelder group reported similar observations [60,63]. Bonandrini *et al.* supplied these cells through renal artery at a concentration of 12×10^6 cells and

dynamically perfused the scaffold with a recirculating medium for a maximum of 72 h. Adult-derived iPSCs represent another inexhaustible but costly alternative [133]. Abolbashiri *et al.* [134] isolated primary porcine renal cells to repopulate decellularized scaffolds and showed that these cells were capable of electrolyte absorption and erythropoietin production. Furthermore, endothelial cells have been used for the repopularization of the vascular tree in bioengineered kidneys to prevent the thrombosis of the otherwise exposed ECM [71,79,133]. Song *et al.* showed that the repopularization of a decellularized kidney scaffold with HUVECs through the renal artery and with neonatal kidney cells (NKCs) through the ureter was effective and functional *in vitro* by measuring urine production and *in vivo* by the orthotopic implantation of scaffold in rats. Orlando *et al.* [77] implanted decellularized porcine kidney scaffolds in pigs without previous recellularization to measure the mechanical properties of the vascular tree and showed that the scaffolds and the vasculature were able to withstand the physiological blood pressure after implantation.

Pancreas

Other therapeutic options, such as simultaneous kidney–pancreas transplantation (SPK) or islet transplantation (ITX), have evolved over the last few years to treat type I diabetes mellitus without requiring the administration of exogenous insulin. Graft survival rates and insulin independence have improved, but patients are still suffering from possible life-threatening complications, such as post-transplantation pancreatitis after SPK or portal vein thrombosis after ITX [135–137]. Furthermore, patients are confronted with the requirement for lifelong immunosuppressive therapy, which has many side-effects [138].

The technique of decellularization and recellularization might be able to generate an autologous endocrine pancreas and overcome these problems.

Only a few publications have examined the decellularization of whole pancreas and the subsequent repopulation with islets of Langerhans. Protocols for perfusion-based decellularization of the mouse, rat, pig and human pancreas have been published [15,72,78,139–148], but use different detergents and times to decellularize the pancreas. Detergents include nonionic tensides such as Triton X-100 and enzymatic agents such as DNAse. Despite these differences, the protocols all generate a cell-free ECM with a preserved ultrastructure.

Similarly, only a few publications have examined the decellularization and repopulation of the whole pancreas with islets of Langerhans [72,140]. Napierala *et al.* repopulated their decellularized rat pancreatic tissues with islets of Langerhans. After infusion, the islets were viable and functional, as evidenced by the results of a glucose stimulation-insulin secretion (GSIS) test [72]. Yu *et al.* were even able to implant a neo-pancreas repopulated with endothelial cells and insulinoma cells into diabetic rats. After transplantation, rats were euglycemic for a week [139]. Based on these findings, a repopulated pancreas scaffold is able to control blood sugar levels.

Other studies have mainly assessed cellular survival in human-scale pancreas-derived decellularized scaffolds. Mirmalek *et al.* decellularized parts of the porcine pancreas and repopulated scaffold-cubes with either human amniotic-fluid derived stem cells (hAFDSCs) or islets of Langerhans. The authors observed *in vitro* proliferation of hAFDSCs and the secretion of insulin by islets [142]. Katsuki *et al.* even repopulated a completely decellularized porcine pancreas with islets via the pancreatic duct. The concentration of insulin secreted in response to the GSIS test was comparable to islets cultivated in normal cell culture medium. Furthermore, islets survived for 4 days after repopulation. The compatibility of decellularized human pancreatic tissues and islets was shown by Peloso *et al.* The authors reported the first decellularization of the human pancreas. Furthermore, they seeded islets on scaffold cubes and observed insulin secretion after stimulation with high glucose concentrations. The authors also showed a reendothelialization with human pancreatic endothelial cells *in vitro* over a culture period of 6 days [145].

The last study emphasizes the importance of endothelial cells and the subsequent revascularization of decellularized scaffolds. Guo *et al.* decellularized whole-rat pancreas, repopulated it with endothelial progenitor cells (EPCs), and cultivated it for 3 days. The authors observed the accumulation of cells around the vessels. When those repopulated scaffolds were subcutaneously implanted into rats, new blood vessels formed [141].

Considering the idea of generating patient-specific endocrine pancreas, ongoing research on stem cell-derived islets is attracting interest. Several groups were able to transform skin fibroblasts from patients with DMT1 into insulin-producing cells (IPCs) that responded adequately to glucose stimulation [149,150]. In a recently published study from Wan *et al.*, decellularized pancreatic tissues were recellularized with mouse iPSC-derived ß-like cells and cultured for 5 days.

Furthermore, the authors showed increased expression of the insulin gene compared to a 2D culture [147]. An as yet neglected point is the importance of the islet niche for recellularization. The islet niche is the specific microenvironment characterized by high vascularization (approximately five arterioles per islet) and supplies the islet with sufficient oxygen levels, nutrients and hormones. Researchers have not determined whether this assembly can be rebuilt using recellularization. In 2019, Citro *et al.* addressed this problem using decellularized rat lungs scaffolds and observed a similar vascular hierarchy compared to the pancreas. The authors successfully observed the function of their vascularized islet organs following subcutaneous implantation into diabetic rats and observed better function than isolated implanted islets over the study period of 30 days.

Intestine

In the setting of intestinal failure caused by short bowel syndrome, the technique of de- and recellularization could be used to engineer a personalized part of intestine to extend the length of the small bowel. With this extension, normal intestinal function could be achieved. The decellularization of small intestinal submucosa is already a well-established procedure. These decellularized scaffolds have been investigated in various studies to recreate different tissue defects. The decellularization of whole parts of the intestine has been reported previously [62,151–153]. Dew *et al.* showed the successful reendothelialization of the rat small intestine with human endothelial cells under different culture conditions. The most outstanding study was published by Kitano *et al.* The authors decellularized a part of the rat small intestine and further recellularized the scaffold with HUVECs via the vasculature and intestinal spheroids via the lumen. They showed the *in vitro* maturation and function of the graft (e.g. glucose and fatty acid absorption). Moreover, the recellularized grafts were heterotopically implanted in a cervical position. The grafts remained viable for 2 weeks *in vivo* and showed measurable absorption [62].

Problems and future challenges

Although substantial achievements in the field of de- and recellularization have been reported during the last decade, we actually face great challenges in the translation of this technique from an experimental approach to a clinically relevant treatment (Fig. 2).

The cell source remains the greatest challenge. None of the previously published studies on recellularization produced a relevant cell mass with respect to the model. Uygun *et al.* [111] only recellularized a liver with 5–20% of the native liver mass in their model, depending on the cell count used. Park *et al.* recellularized a rat liver with 20 million iPSCs that differentiated into hepatocyte-like cells, which corresponds to approximately 2%

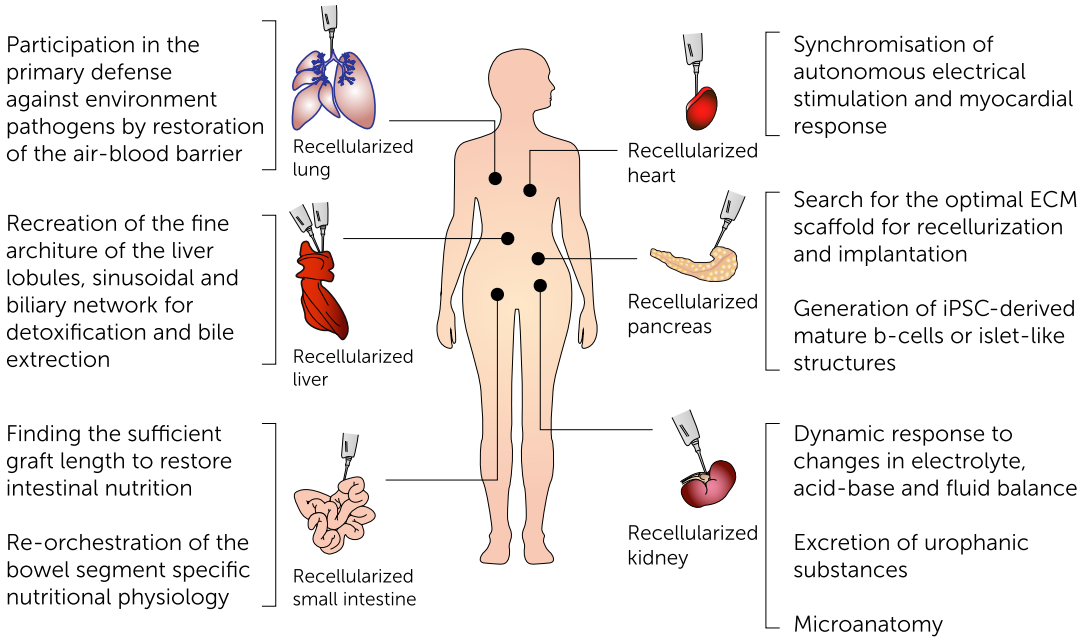

Figure 2 Obstacles in the de- and recellularization of solid organs.

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of the native rat hepatic cell count. The cost for this cell mass was 6000 dollars [20]. The actual techniques (e.g. cell differentiation and expansion) must be scaled to an industrial level to generate clinically relevant cell masses. This issue is subject of current discussions [154].

Thrombogenicity induced by the uncovered ECM is another crucial point. Although many studies have reported the successful reconstruction of the endothelial network and a reduction in thrombosis in various *in vitro* or *in vivo* tests, none of these studies achieved sustainable long-term survival of their grafts *in vivo*. Another approach to reduce or prevent thrombosis is the crosslinking or covering of the surface of ECM using different techniques. Furthermore, combinations of both techniques have been described. However, none of these experiments provides evidence for long-term graft function after implantation.

The composition of the culture and perfusion medium is still a disregarded issue that should be addressed in the future. The media composition appears to be important, because a neo-organ consists of different cell types with different requirements, which will change over the maturation period. Currently, growth factors are often added to the media to support cell growth or differentiation. The *Ott* group emphasized the importance of different media compositions in their recent work, showing that an angiogenic medium is initially required, followed by a stabilization culture medium. Moreover, investigations of the optimal medium composition or if native components, such as cell-free plasma, should be added are necessary. In case of the liver bioengineering, plasma obtained from organisms during liver regeneration (e.g. after partial hepatectomy) might promote the maturation of recellularized livers *in vitro*.

As mentioned above, the immunogenicity of decellularized tissue has been investigated in various studies by performing allogenic or xenogenic implantation at different sides [41,155,156]. These studies have provided proof of concept evidence. Nevertheless, *in vivo* investigations of the long-term immunogenicity of repopulated neo-organs will be needed prior to a clinical application.

Conclusions

The technique of de- and recellularization has achieved substantial advances in the field of organ bioengineering. However, the approach is still at an early stage and not yet applicable to clinical translation.

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Conflicts of interest

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