Bioengineering Organs for Blood Detoxification

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For patients with severe kidney or liver failure the best solution is currently organ transplantation. However, not all patients are eligible for transplantation and due to limited organ availability, most patients are currently treated with therapies using artificial kidney and artificial liver devices. These therapies, despite their relative success in preserving the patients' life, have important limitations since they can only replace part of the natural kidney or liver functions. As blood detoxification (and other functions) in these highly perfused organs is achieved by specialized cells, it seems relevant to review the approaches leading to bioengineered organs fulfilling most of the native organ functions. There, the culture of cells of specific phenotypes on adapted scaffolds that can be perfused takes place. In this review paper, first the functions of kidney and liver organs are briefly described. Then artificial kidney/liver devices, bioartificial kidney devices, and bioartificial liver devices are focused on, as well as biohybrid constructs obtained by decellularization and recellularization of animal organs. For all organs, a thorough overview of the literature is given and the perspectives for their application in the clinic are discussed.

1. Introduction

The kidney and liver are complex organs possessing vital functions to the body. The kidney has an essential blood purification function and a critical role in maintaining the body homeostasis.[1] In severe kidney diseases, from chronic kidney disease (CKD) up to end stage kidney disease (ESKD), a break-down in renal function leads to the accumulation of waste solutes/toxins in the body, which subsequently results in disease progression and eventually to patient's death. A rather sudden failure, called acute kidney injury (AKI), can also lead to patient's death or progress toward CKD.[2]

The liver also possesses important functions for digestion, metabolism and immunity. Often considered as the factory of the body, it can be affected by many

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chronic or acute diseases. Long term alterations of liver tissue follow different steps, from steatosis to cirrhosis. Acute liver failure (ALF) comes from massive necrosis mainly provoked by intoxication (drugs, food) or from huge decompensation of cirrhotic state (acute on chronic liver failure) and results, among other symptoms, in a sudden increase of intracranial pressure that can lead to brain edema and death, for the most fulminant cases.[3]

For all patients with severe kidney and liver diseases, the best solution would be organ transplantation. However, due to shortage of donor organs or specific clinical state, most of these patients are treated with rather incomplete therapies focusing mainly on life preservation rather than cure. The current treatments for severe AKI and ESKD patients are either dialysis (peritoneal dialysis, PD, or hemodialysis, HD) which covers only a small fraction of the physiological renal functions and achieves limited removal of uremic toxins.[4]

For the ALF, a temporary support, based on toxins removal,[5] can help liver regeneration. It is obvious that there is strong need for new concepts, which include devices, extracorporeal or implantable, that could better mimic and/or replace the kidney and liver functions.

In the last years, it has been widely recognized that regenerative medicine can offer innovative solutions for reconstruction of functional kidney and liver tissues.[6] In this review paper, after presenting the classical artificial organs, we discuss in detail the progress in this field, including the development of:

- bioartificial kidney (BAK) and liver (BAL) devices;
- scaffolds for bioengineering of kidney and liver organs, by decellularization and recellularization of animal organs.

In these fields of research, the (scientific and technological) challenges are big. There is need for interdisciplinary research efforts focusing on improved biomaterials, advanced cell biology, better understanding of the biomaterial tissue interaction and of their safety. The organ complexity increases from artificial via bioartificial to tissue engineered, and the regulatory demands increase from extracorporeal to implantable organs.

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2. Kidney and Liver—the Natural Organs

2.1. The Kidney: Structural and Functional Aspects, Pathologies

The kidneys are highly specialized organs that play a central role in the regulation of water, electrolyte and acid base balance (**Figure 1**).[1c,7] They control the volume and the ionic composition of body fluids, their pH and osmotic concentration. They are also responsible for the production of hormones[8] and reabsorption of nutrients, ions and water from the plasma ultrafiltrate.[9] An important function of the kidneys is excretion of waste solutes by filtration (via the glomeruli) and active secretion (by the tubules). The waste solutes include endogenous metabolic waste products and exogenous compounds like drugs and environmental pollutants and toxins.

The nephron is the functional unit of the kidney. It is divided into several segments that have specific roles. First, blood travels through the glomerulus where water and small and middle-sized solutes (up to ≈60 kDa) pass the capillary walls due to the high-pressure present in the capillaries. The resulting glomerular filtrate (≈120 mL min^{-1} or ≈170 L d^{-1} in healthy situation), or ultrafiltrate, travels through the proximal tubule where the majority of water and essential components are reabsorbed. In addition, the proximal tubule is responsible

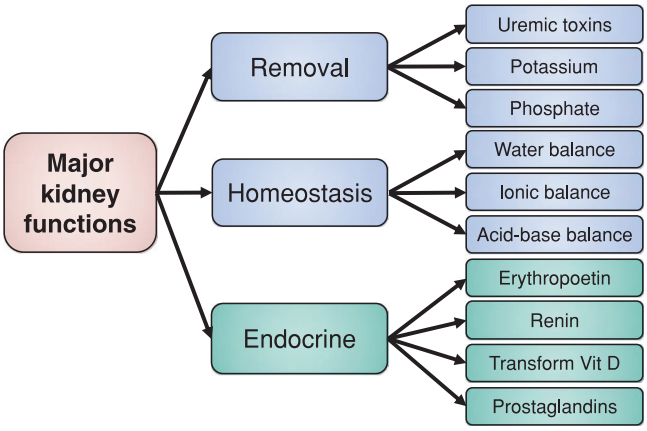

Figure 1. Important kidney functions contributing to body homeostasis.

for active solute/toxin secretion, hormone production and metabolic activation. Proximal tubule epithelial cells (PTEC) have a wide variety of specialized transporters that coordinate the basolateral uptake and luminal release of, among others, protein-bound solutes with a high capacity and selectivity.[10] These unique characteristics make PTEC particularly sensitive to xenobiotic-and ischemia induced toxicity and subsequent AKI.[11] It is, therefore, not surprising that many kidney diseases are initiated by proximal tubule damage.[12] Finally, downstream of the proximal tubule, an additional amount of water and solutes (primarily electrolytes) is reabsorbed from the filtrate to the blood within the loop of Henle, the distal convoluted tubule and the collecting duct system, thus concentrating the urine and finalizing the fluid and electrolytes homeostasis. The urine is transported and eliminated via the renal pelvis, ureter and urinary bladder.

It is estimated that more than 10% of the worldwide population suffers from a more or less severe form of kidney disease. With the increased prevalence in risk factors, such as hypertension, cardiovascular disease and diabetes mellitus in the aging population, the prevalence of CKD is rising. The kidney function of these patients may progressively and irreversibly decline until total loss, called ESKD, which leads to the accumulation of a variety of endogenous metabolites with life-threatening consequences. One of the main indicators of kidney function is glomerular filtration rate (GFR), defined as the volume of the plasma ultrafiltrate formed by glomerular capillaries per unit of time (mL min^{-1}).[13] Based on the GFR values there are five distinguishable stages of CKD (**Table 1**).

Table 1. GFR-based classification[14] and global prevalence[15] of CKD.

Stage	Description	GFR [mL min^{-1} per 1.73 m^2]	Prevalence [%]
1	Kidney damage with normal or increased GFR	>90	3.5
2	Kidney damage with mildly decreased GFR	60–89	3.9
3	Moderately decreased GFR	30–59	7.6
4	Severely decreased GFR	15–29	0.4
5	Kidney failure	<15	0.1

2.2. The Liver: Structural and Functional Aspects, Pathologies

The liver is the second organ, after skin, in size and weight (1.5–2 kg in adults). It is one of the most complex organs of the human body and it is located in the upper region of the abdominal cavity. The liver receives ≈25% of the cardiac blood output via two main distinct vascular systems: the portal vein (with high concentration of nutrients and poor oxygenation) and the hepatic artery (with high oxygen content). Blood from both vessels mixes and flows through an interconnected network of specific hepatic capillaries, called sinusoids. Hepatic acinus is the structural and functional unit in the liver, constituted of millions of them. The blood is drained from the portal area into the central hepatic vein via the sinusoids. The acinus is arbitrarily divided into 3 zones, corresponding to the periportal, to the midzonal parenchyma, and to the centrilobular zone of the hepatic lobule, respectively. Exchanges (nutrients, oxygen, metabolites, waste products) take place between liver cells and blood in this area. The functions of hepatocytes, the most active cells in the liver, depend on their position in the acinus and are mainly affected by local partial pressure of oxygen. This phenomenon is called "zonation." The blood, finally collected in the central vein, exits the liver and returns to the systemic circulation. Hepatocytes also facilitate bile secretion into the canaliculi. Bile streams in canaliculi are parallel to blood flow in the sinusoids, but in the opposite direction toward the bile duct. Then, bile leaves the lobule and is conveyed to the gall bladder.

The complete description of the liver microstructure is beyond the scope of this review (interested readers can find more details elsewhere).[16] Briefly, at least 15 different cell types can be found in the normal liver. Hepatocytes compose the parenchyma of the liver and are the major cellular components of the organ. Liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs), hepatic stellate cells (HSCs) and pit cells are collectively identified as the major nonparenchymal cells (NPCs) of the tissue. Cholangiocytes are epithelial cells delimiting intrahepatic bile ducts and adjust the content of primary bile secreted by the hepatocytes. According to physiologists and clinicians, the human liver possesses more than 500 physiological functions, not all are well identified, however, they can be classified in three major classes: biotransformation, storage and synthesis (**Figure 2**).

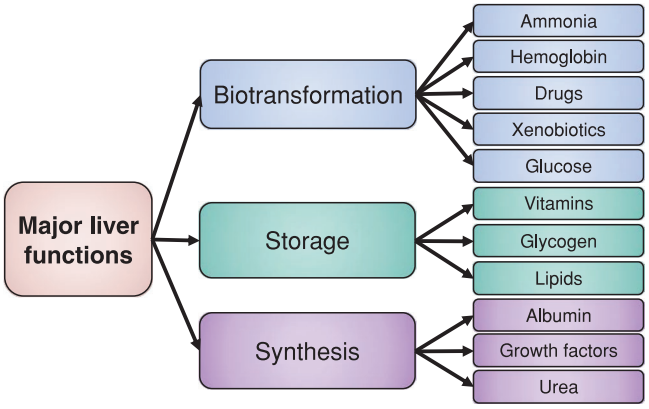

Figure 2. Major liver functions in the body.

The World Health Organization estimates that over 650 million people worldwide are affected by some form of liver disease and worldwide 1–2 million deaths are accounted to liver related diseases annually. In case of major liver failure, several disorders can be observed: an elevated ammonia level, partially responsible for the increase of intracranial pressure, leading to cerebral edema and coma, increased coagulation time, hyperbilirubinemia, etc. In case of acute or fulminant liver failure (ALF), the only treatment currently available is orthotopic liver transplantation. However, recurrent organ shortage leads to a constant increase of the number of patients on the waiting list (17 000 individuals in the US for the liver). For some specific cases, artificial liver can support life until transplantation can be performed.

3. Artificial Organs

3.1. Artificial Kidney

3.1.1. Current Therapies

According to the European Uremic Toxin Work Group (EUTox; www.uremic-toxins.org/), a working group within the European Society for Artificial Organs (ESAO), uremic toxins can be classified into three main categories[17]:

- small sized water soluble (Mw < 500 Da): such as urea (60 Da), creatinine (113 Da);
- middle sized (Mw > 500 Da): such as β_2-microglobulin (11 800 Da), parathyroid hormone (9225 Da);
- protein-bound (PBUTs): such as indoxyl sulfate (251 Da, > 93% bound to protein), p-cresol sulfate (188 Da, > 95% bound to protein), hippuric acid (179 Da, > 39% bound to protein).[18]

Current detoxification strategies can be classified into peritoneal and extracorporeal, depending on where it occurs inside or outside the body, respectively (**Table 2**, presenting the main concepts; the interested reader can find more information elsewhere).[19]

During PD, the toxins and excess water from the blood is removed via diffusion across the peritoneal membrane into the dialysate which is placed in the abdominal cavity. The dialysate is exchanged 4–6 times per day via an abdominal catheter. Approximately 10% of patients with ESKD in the world is using this treatment.[20] PD is relatively simple and can be performed at home, contributing to a relative maintenance in quality of life. Furthermore, it is generally cheaper than HD done in the hospital; however, it has lower toxin removal rates than HD and higher risks of peritoneal and catheter related infections.[21]

In HD, hemofiltration (HF), hemodiafiltration (HDF), hemoperfusion and their combinations, the blood returns to the patient after cleaned from uremic toxins, without introducing foreign blood or plasma. In HD, the driving force for solute removal is the concentration gradient across the membrane. The highly concentrated toxins in blood diffuse through the HD membrane to the dialysate. It is very effective for the removal of the small, water-soluble toxins but it has limitations for the removal of the middle-sized uremic toxins and of the PBUTs. During HF treatment, toxins can be removed via

Table 2. Summary of renal replacement therapies, adapted from.[19,26]

Therapy	Method	Toxin removal			Advantage	Disadvantage	Duration
		Small	Middle sized	PBUT			
PD	Catheter	Yes	Partially	Difficult	Cheaper and simpler than hemodialysis	Infection risks, less toxin removal than that of HD, recommended for patients with partial kidney failure[27]	4–6 exchanges per day with dialysate
HD	Membranes	Yes	Partially	Partially	Removal of the small-sized uremic toxins	Insufficient removal of middle sized uremic toxins	4 h d^{-1} 3–4 times per week
HF	Membranes	Yes	Partially	Partially	High removal of middle and large sized toxins comparing to HD and dialysate is not used	Need for susbtitution fluid to maintain blood volume. Clearance of small molecules lower than in HD	4 h d^{-1} 3–4 times per week
HDF	Membranes	Yes	Partially	Partially	Better removal of the small water soluble, middle, protein-bound toxins with the synergy effect of HD and HF	Need for substitution fluid (sterile solution or high quality dialysate)	4 h d^{-1} 3–4 times pe week
Hemoperfusion	Sorbents	Partially	Partially	Yes	Effectively removes the liposoluble toxins and PBUT.	Complications including hypotension, thrombocytopenia, and electrolyte disturbances.	
HD or HDF with MMMs	Membranes and sorbents	Yes	Partially	Partially	Advantages from HD (or HDF) and adsorption. Safe from thrombogenesis caused by sorbents, higher removal of middle and protein-bound toxins	Need to correct electrolytes and blood volume	less or similar to HD (or HDF)
CPFA	Membranes and sorbents	Yes	Yes	Yes	Advantages from plasmapheresis, adsorption, and HF, minimal the risk of thrombogenesis caused by sorbents, better toxin removal	Need to correct electrolytes and blood volume	

convection (volume flow through the membrane) due to applied transmembrane pressure. Convective transport there improves clearance of middle sized uremic solutes. In HDF, the diffusive and convective transports are combined. In HF and HDF, a large amount of ultrafiltrate passes through the membrane and, therefore, a susbstitution fluid (either sterile physiological solution or filtered dialysate) needs to be reinfused in the blood lines to maintain the hemodynamic stability.[19,22] Recently, the clinical implementation of HDF increases, reaching more than 10% of the European patients.[22]

During hemoperfusion, the patients' blood passes through a cartridge containing sorbents (charcoals or synthetic materials like resins, etc.), which adsorb and remove some uremic toxins. Hemoperfusion can effectively remove the molecules that are liposoluble, like PBUTs, or have high molecular weight and poorly eliminated by HD membranes.[23] However, it is not suitable for removing small and water-soluble compounds like urea. Obviously, the combination of HD and hemoperfusion could be advantageous for removing a broad range of uremic toxins. In fact, the concept of Mixed Matrix Membranes (MMM) combines the benefits of filtration and adsorption in one membrane.[24] The MMM consists of two layers: a porous polymeric layer with embedded activated carbon particles and a porous, polymeric particle-free layer (**Figure 3**). The adsorptive particles on the outer layer can increase the removal of the toxins, including PBUTs, by keeping the concentration gradient of the toxin at the maximum level.[25] The particle free layer prevents direct contact between patient's blood and the particles and it is responsible for the selectivity of the whole membrane.

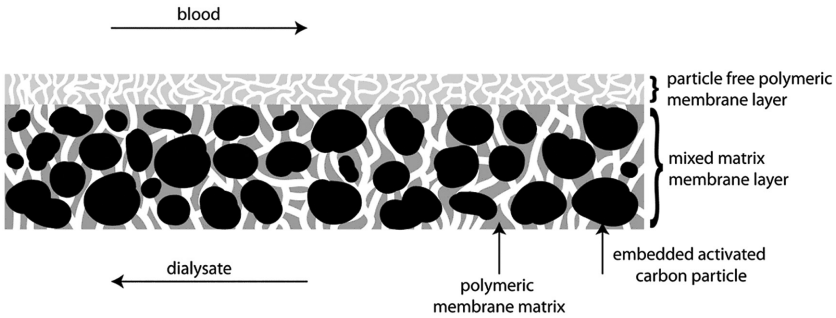

Figure 3. A) The concept of MMM. Reproduced with permission.[24d] Copyright 2012, Elsevier. B) SEM image of a mixed matrix hollow fiber membrane. Adapted with permission.[24c] Copyright 2016, Nature Publishing Group.

More examples of therapies combining diffusion and adsorption can be found elsewhere.[19]

3.1.2. Materials for Artificial Kidney

The first membranes applied for dialysis treatment were made of regenerated cellulose. However, they were later replaced by modified cellulosic membranes (cellulose triacetate (CTA); cellulose diacetate; and cellulose acetate (CA)) due to blood incompatibility concerns, especially complement activation.[26,31] Nowadays, the majority of the market is dominated by synthetic membranes fabricated from polysulfone (PSf), polyethersulfone/polyamide (PES/PA), polyethersulfone (PES), polymethylmethacrylate (PMMA), polyester polymer alloy (PEPA), ethylene vinyl alcohol copolymer (EVAL) (**Table 3**). In comparison to cellulose-based membranes, the PSf- and PES-based membranes, have higher ultrafiltration coefficient and very good selectivity. Besides, they can be sterilized with various methods and they are mechanically stable.[26] Current artificial kidneys contain ≈7000–17 000 hollow fibers with diameter of about 0.2 mm and thickness of 15–50 µm. The typical fiber packing density of the device (volume percentage covered by the fibers) is ≈50 to 60% to achieve optimal liquid flow distribution within the device.[32]

3.1.3. Wearable Artificial Kidney

The healthy natural kidney filters the blood for 24 h d^{-1}/7 d a week, in contrast to the current therapy of 4 h treatment/3 times a week. As the healthy normal kidney does, it has been indicated that the slower, more frequent and prolonged HD could achieve better removal of the middle-sized and large-sized uremic toxins.[33] The portable and/or wearable artificial kidney (WAK) are intended for prolonged, if possible, continuous therapy in order advance patient homeostasis, better removal of solutes, reduce health costs, enhance patient mobility and improve their quality of life.[34]

The first conceptual model for the portable artificial kidney was reported by Kolff et al.[35] In recent years, three different devices have been under development: the wearable ultrafiltration systems, WAK,[36] and the peritoneal-based artificial kidney such as the Vicenza wearable artificial kidney.[37] These devices are facing important technical and clinical challenges, including the need for a safe vascular access, optimal blood anticoagulation, minimum amount dialysate (<500 mL) and/or a dialysate regeneration system, adequate safety sensors (for air bubble detection, pressure, and alarm), a power source independent from an electrical outlet, lightweight and ergonomic design.[34,36,38]

Table 3. The properties and performance of artificial kidney compared to natural kidney. Data taken from the literature[28–30] and from industrial sources.

	Natural kidney[28]	Modified cellulose	PSf	PES/PA	PES	PMMA	PEPA	EVAL	PAN
Filtering area (m^2)	1.5	1.1–2.1	0.7–2.3	0.6	0.9–2.5	1.3–2.1	0.8–2.1[29]	1.0–1.8	1.05–2.15
Number of capillaries	≈1 000 000	≈6500–≈13 000	≈9000–≈15 000	≈7000	≈10 000–≈15 000	10 700–16 900	≈9500–≈12 000	≈7000–≈12 500	≈10 000–≈12 000
Capillary inner diameter (μ)	8	200	185–200	215	200–215	200	210	175	210
Capillary thickness		15	35–40	50	30–40	30	30	25	42
Blood volume (mL)		55–125	30–140		60–150	70–130		85–140	
Blood flow rate (mL min^{-1})	1200	200–500	100–500	50–200	200–500	100–500	200	200–400	200–400
Operate time (h per week)	168	≈12–16							
Ultrafiltration coeff. (mL/h/mmHg)	GFR > 90 mL min^{-1} per 1.73 m^2	31–47	8–124	33	42–93	26–41	24–63	9–15	33–65
Sieving coeff. (clearance, mL min^{-1})									
Albumin	0		<0.003	<0.01	<0.01	<0.01		0.04	<0.01
Urea	1[28b] (125)[30]	1(90–380)	1 (165–300)	1 (50–167)	1 (190–460)	1 (171–184)	1 (170–198)[29]	1 (174–288)	1 (173–310)
Creatinine	1[28b] (125)[30]	1 (75–363)	1 (140–281)	1 (50–146)	1 (171–431)	1 (157–180)	1 (155–194)[29]	1 (153–247)	1 (156–269)
β_2-microglobulin	>0.95[28b] (125)[30]		0.65–0.8	0.63			0.58–0.68	0.65	
Sterilization	–	Gamma irradiation, Ethylene oxide	Steam or Gamma irradiation	Steam	Steam or Gamma irradiation	Gamma irradiation	Gamma irradiation	Gamma irradiation	Gamma irradiation
Manufacturer	–	- Baxter - Toyobo	- Asahi Kasei Medical - Fresenius - Toray - B Braun	- Baxter-Gambro	- Toyobo - **3M**	- Toray industries	- Nikkiso Co	- Asahi Kasei Medical	Baxter/Gambro

During the past decades the technology concerning the artificial kidney (membrane, dialysis machines, anticoagulation, etc.) have been remarkably developed, however, still the artificial kidney therapy cannot mimic the function of the natural kidney.

3.2. Artificial Liver

The first applications of membrane processes for liver support were attempts of using HD/HF or plasmapheresis techniques, already dedicated to treatment of kidney failure or to therapeutic plasmapheresis. Many trials with humans have been described since the late 50s, but did not achieve significant improvements in the patients' state, although in some cases, encephalopathy was alleviated.[39] Further, pre-clinical and clinical research has turned to the combination of several artificial devices (membranes + nonspecific ion-exchangers and activated charcoal adsorption columns) to increase the efficiency of the overall extracorporeal detoxification system. As encephalopathy is associated with an accumulation of toxic molecules (not all of them being identified), the hypothesis for the treatment was the removal of a large spectrum of substances: lipophilic, albumin-bound ones such as bilirubin, bile acids, metabolites of aromatic amino acids, medium-chain fatty acids and cytokines, etc. The application of full blood through these columns is limited due to biocompatibility issues. In general, these columns are applied in the filtrate/dialysate compartment as a secondary circuit. The artificial livers currently on the market are summarized in **Table 4**. Further details, including clinical outcomes, can be found elsewhere.[40]

4. Bioartificial Organs

4.1. The Bioartificial Kidney

The therapies using artificial kidney can only partially substitute the renal filtration function, as only small and some middle-sized solutes can be removed.[45] Besides, among the filtered solutes are also essential molecules (amino acids, vitamins), which, in healthy kidney, would be intrinsically reabsorbed

Table 4. Summary of commercially available artificial livers.

Device	Provider	Primary circuit	Removal process in the secondary circuit	References
Plasma Adsorption	Asahi Kasei Medical	Plasmaflo pore size 0.3 µm	anion exchange column Plasorba (bilirubin removal)	[41]
MARS	Baxter	MARSFlux hemodialyser (albumin aided transport) MWCO < 70 kDa	Albumin bound toxins fixed on ion-exchange and charcoal columns. Hydrophilic substances removed by dialysis	[42]
Prometheus	Fresenius Medical Care	Plasma fractionation membrane MWCO : 300 kDa	2 adsorption columns (ion exchange and charcoal)	[43]
HepaWash	ADVOS	Hemodiafilter MWCO : 70 kDa	changes in pH and temperature and dialysis to regenerate circulating albumin and remove toxins	[44]

by PTEC. Their loss during dialysis significantly contributes to comorbidities associated to ESKD.[46] Additionally, despite recent progress in dialysis membranes, PBUTs still remain difficult to clear due to albumin-binding, leading to their progressive accumulation.[47]

In the healthy kidney, the core of all active processes (secretion, reabsorption and endocrine, metabolic and immunological functions) lies to the cellular components. Thus, for a successful RRT, cells governed functions of the kidney should be targeted. This may be achieved via the development of a (self-sufficient) BAK that combines the capabilities of the inanimate dialysis systems with the inherent biological renal functions of PTEC. In practice, BAK combines a hemofilter used in conventional dialysis with a bioreactor unit containing renal PTEC, termed as a renal assist device (RAD).[48] Additionally, a compact portable or even an implantable BAK device would confer patients with greater mobility, improving their quality of life.[49]

4.1.1. Cell Sources for BAK: Replicating the Proximal Tubule Function

A key challenge for developing a BAK is finding a robust cell source for the device. A choice for an autologous versus a non-autologous approach should balance the requirement for highly functional cells with sustained viability and activity when cultured in the device. Besides, since these cells would be constantly exposed to uremic conditions, their long-term performance is mandatory. Herein, we review the major cellular options with potential for RAD (**Figure 4**) and the cell-based BAK systems developed thus far (**Table 5**).

Primary Renal Proximal Tubule Epithelial Cells: The combination of living cells and artificial devices has raised vigorous debate about the cell source, type and expansion procedures, but also concerns regarding cell phenotype modifications over time, their safety and stability.[48,50] Although being an attractive cell source at first, xenogeneic origin of cells has been abandoned due to serious potential risk of endogenous retrovirus

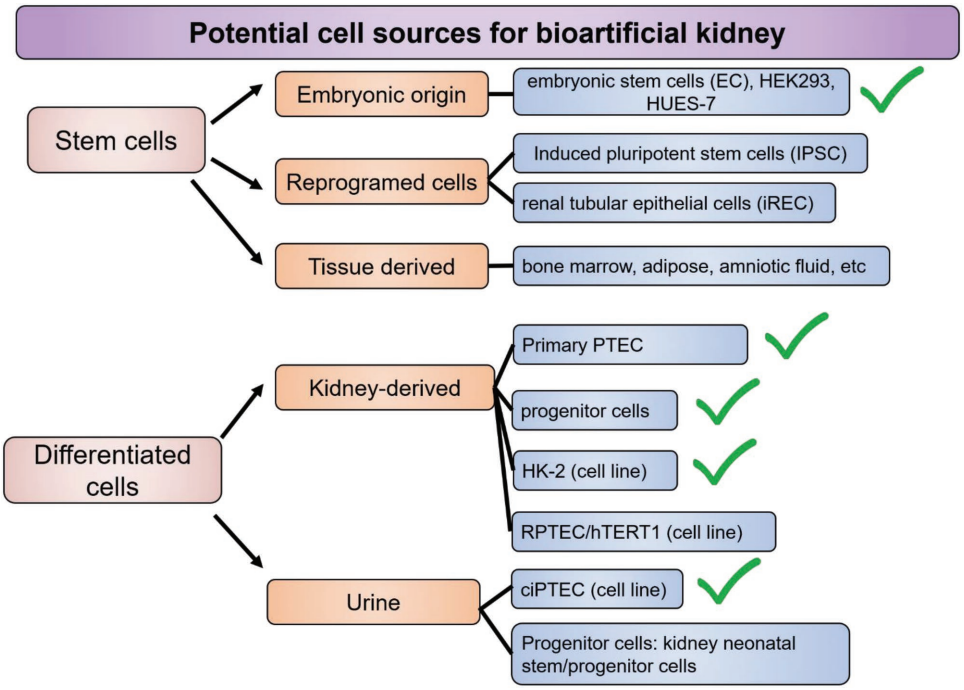

Figure 4. Cellular sources for use in bioartificial kidney. Functional renal tubular cells suitable to be loaded in the BAK can be obtained by either differentiation of stem cells or direct isolation of mature cells from kidney tissue or urine as source for cell line development or organoids. Green ticks indicate where a BAK/RAD has been already tested in vitro.

Table 5. Currently developed BAK/RAD systems using human cell sources.

Source	Type	Cell	Advantages	Disadvantages	BAK/RAD system	Reference
Kidney tissue	Primary	Hate	- Excretion of ammonia - Metabolic and endocrine behavior - Clinical phase I and IIa: rapid recovery of kidney function	- Relative scarcity - Loss of metabolic function after few passages - No reports on clearance capacity	- PSU coated with laminin or collagen IV - Intraluminal seeding of cells	[53,56]
			- Epithelial phenotype (markers) - Active transport of anionic compounds	- Phenotype loss after few passages - Partial differentiation	- PES/PVP/coated with L-DOPA and collagen IV - Intraluminal seeding of cells	[57]
			- Epithelial phenotype (markers) - Metabolic activity - Immunomodulatory effects	- Partial differentiation - No demonstration of active transport	- Hemofilter hollow fibers - No coating - Extraluminal seeding of the cells	[58]
			- Epithelial phenotype (markers) - Immunoprotection and metabolic activity - Awaiting clinical trials	- Partial differentiation - No reports on clearance capacity	**BRECS** - Wearable design - Carbon disks	[59]
			- Immunoprotection and metabolic activity - Continuous hemofiltration for 100 h - Awaiting clinical trial	- No information on clearance capacity	**iRAD** - SNM	[54,60]
	Cell line	HK-2	- Epithelial phenotype (markers) - Erythropoietin expression	- No functional activity	PSF coated with laminin Internal seeding of cells	[61]
Urine	Cell line	ciPTECs	- Active uptake of organic cations/anions - Metabolic, endocrine and immunomodulatory behavior - Preliminary evidence on lack of oncogenicity and tumorigenicity	Potential alterations of phenotype at high passages	**Living membranes** - microPES coated with L-DOPA and collagen IV - Extraluminal seeding of cells	[62]
Fetal	Cell line	HUES-7 Embryonic stem cells	- When differentiated, similar in phenotype with hPTEC	- Tumorigenic potential - Donor to donor variations - Partial differentiation	- PES/PVP or PSf/PVP - Matrigel coating - Extraluminal seeding	[63]

infections.[51] For clinical applications, human origin of cells is highly desired. However, very few cell models are currently available. Human PTEC (hPTEC) display most accurately the physiological and functional demands of the kidney by expressing various transporters essential for uremic toxin handling, concomitantly with the re-uptake of useful substances.[52] In the first RAD prototype, primary hPTEC isolated from potential donor kidneys that proved unsuitable for transplantation, were loaded on the device.[53] In preclinical evaluation, the cells remained viable and functional for 24 h. Later on, Fissell et al. introduced human cortical epithelial cells as the cellular components of an implantable renal assist device (iRAD, see details later). Upon interaction with silicone nanopore membrane (SNM), the cells formed a confluent monolayer and their polarization and differentiation was confirmed by transepithelial resistance measurements.[54] Another approach is to isolate cells based on their surface marker profiling. Van der Hauwaert et al. identified a cellular subset among cells isolated from healthy kidneys, namely a $CD10^+/CD13^+$ population (≈4% of the total cell population), as a pure, functional, and stable PTEC population, that displayed proximal tubule markers (aquaporin-1, *N*-cadherin and MUC1) and epithelial characteristics (barrier functions).[55] However, these characteristics were present for up to five passages, after which signs of dedifferentiation were identified,[55] thus limiting their applicability for BAK.

Stem Cells: Embryonic or Induced-Pluripotent Tissue-Derived Stem Cells: In the quest for an unlimited cell source for the BAK, stem cells or cells with a stem-like signature received special attention due to their potential to expand and evolve into diverse renal cell subsets.[64] Noteworthy are the results reported by Narayanan et al. about the successful differentiation of human embryonic stem cells (hESC) into PTEC in a reproducible manner. Under in vitro settings, differentiated cells formed an epithelial layer with tight junctions and showcased a polarized morphology with apical microvilli. In addition, they were able to recapitulate some of the tubular structures both in vitro and in a rodent model.[63] When cultured on coated polymeric membranes, they were able to maintain a differentiated epithelium.[63] Although unquestionably promising, the use of hESC requires a thorough investigation in terms of functionality and stability. Besides, the use of hESC raises serious bioethical and biosafety concerns, as these cells have the potential to form teratomas, too. Obviously, the FDA will not approve the clinical applications of these cells,[50] thus alternative routes are currently being developed.

The use of induced pluripotent stem cells (iPSC) as a cell source for tubule epithelium could revolutionize the field. Based on a Nobel Prize-winning technology, the iPSC can be derived from any somatic cell of the patient, bypassing cell shortage limitation.[65] By the precise manipulation of signaling, the direct differentiation of stem cell niched toward

a variety of renal lineages is attainable, which can subsequently be developed into PSC-derived renal organoids.[66] The generation of a wide variety of renal progenitor cells, would enable the reconstitution of the kidney cellular complexity, and, potentially, of its functions.[66b,67] These cells form an easily accessible source of PSC without the ethical issues of ESC. However, viral transfection poses a risk for oncologic derailment. Therefore, new methods for induction of iPSC are being explored in rodents and humans, including transfection with nongenome-integrating adenoviruses, injection of recombinant proteins and usage of plasmids, micro RNAs and synthetic messenger RNAs.[68] Currently, these protocols vary in efficiency and many use feeder layers that restrict clinical applications. Moreover, although it is possible to envision the use of patient-derived iPSC to develop a clinically functional BAK, up to date, no iPSC-based RAD has been developed.

A shorter route of obtaining renal tubular cells from fibroblasts could be their direct reprogramming by forced expression of transcription factors involved in tissue development. Recently, induced renal epithelial cells (iREC) of mouse and human origin have been generated. The iREC exhibit epithelial features and a global gene expression profile resembling that of the native cells. Besides, they function as differentiated renal tubule cells and have sensitivity to nephrotoxic substances.[69] It is though too premature to estimate the potential use of iREC for the RAD.

Alternatively, cells with a lower differentiation potency, such as tissue-derived stem/progenitor cells could also be considered. Whether adipose,[70] bone marrow,[71] amniotic fluid,[72] or kidney-derived,[73] they are an attractive alternative to obtain large cell numbers as they maintain self-renewal characteristics under prolonged expansion and can differentiate and acquire an epithelial phenotype, stable for only a few passages.[70–72] However, a confirmation of epithelial-specific markers is not convincing enough for their potential application in a RAD device.[74] These findings reiterate the demand for an unlimited and phenotypically and functionally robust source of hPTEC in the context of BAK application.

Cell Lines with Active Transporters and Metabolic, Endocrine, Immunomodulatory Functions: Despite the promising potential of primary and stem cells-derived hPTEC, it is still questionable if these are indeed the most useful cell type for BAK. Not only the limited cells source, but also the limited lifespan of the cells, interdonor variability as well as the lack of standardized isolation procedures are serious stumbling blocks for their use. With the high surface area requirements of the bioreactor unit of BAK (0.7–1.0 m^2),[56,75] it is questionable whether sufficient numbers of cells can be obtained at affordable prices for regular use in clinical practice. In response to this setback, (conditionally) immortalized human PTEC (ciPTEC) have been developed.[76] The immortalization procedures enable to obtain sufficient cell numbers and stable expression and function upon prolonged expansion.[76a] In comparison to other cell lines,[76b,d] the ciPTEC line developed by Wilmer et al.[76a,c] showed a wide variety of relevant transporters known to mediate the active excretion of PBUT.[77] The interaction of uremic toxins with metabolic enzymes, UDP-glucuronosyltransferases activity and mitochondrial activity was confirmed in ciPTECs, too.[78] The stability of relevant organic anionic (OAT1, OAT3) and cationic (OCT2) transporter expression at gene, protein and functional levels, significantly prevail over other cells lines.[62a,76a] The ciPTEC were also shown to secrete an active form of vitamin D when exposed to a mix of uremic toxins at concentrations that match those found in CKD patients.[79] Considering the progressive reduction of active vitamin D in these patients, this feature could be an exquisite addition to the function of the BAK system. Moreover, it has been reported that conventional hemodialysis removes vitamin D,[80] thus ciPTEC could become an important source of this metabolite. In the last decade, a new system for the expansion and differentiation of human autologous epithelial tissue has been developed, the organoid culture system.[81] Originally, it was developed for colon epithelium, but later was set up for the expansion and differentiation of less proliferative epithelia like the liver and pancreas. Some of the authors are currently working on the development of renal organoids, too.[82]

4.1.2. Development of BAK Devices

Initially, the extracorporeal device comprised of the in-series combination of a conventional hemofilter and a specialized bioreactor. While the hemofilter would provide filtration, the cell-loaded bioreactor would assure reabsorption, secretion and other essential metabolic and endocrine functions. The first attempts to create such device were made by Aebischer et al., who demonstrated the feasibility of attaching and growing kidney epithelial cells on semipermeable hollow fiber membranes.[83] Proceeding work of Humes and colleagues led to a bioreactor that consisted of porcine primary renal cells cultured on the inner surface of hollow fibers (**Figure 5**).[84] In combination with conventional hemofilter, the system was shown to significantly increase the survival rate of patients with AKI, when compared to those treated with conventional RRT only.[56,85] Unexpectedly, an interim analysis of a follow-up phase IIb study showed a high survival rate in patients treated with a cell-free sham device. Adding the difficulties in the manufacturing process, the study has been suspended. Notwithstanding its historic significance, this BAK remains the only one approved for clinical trials by the US Food and Drug Administration (FDA). Further, the knowledge acquired with this study has catalyzed the development of two therapeutic alternatives, the BRECS and the iRAD which are reportedly entering clinical trials soon (see details later).[86]

In recent years, some of the authors of this review have developed a BAK system containing "living membranes" based on ciPTEC[76a,87] cultured on PES membranes.[2,62d,88] To achieve reproducible, good quality cell monolayers, a dual coating of 3,4-dihydroxy-l-phenylalanine (L-DOPA) and collagen IV (Col IV) was applied to the fibers, following earlier reports[57,89] (**Figure 6**). For this BAK system, the transepithelial transport of both cationic and anionic uremic toxins has been confirmed.[62a,d]

Further studies on cell performance when exposed to patient-derived blood, as well as, dialysate fluids and flows usually applied in conventional HD, are required. Additionally, to counteract the immunostimulant, oncogenic, tumorogenic potential generally associated with immortalized cell

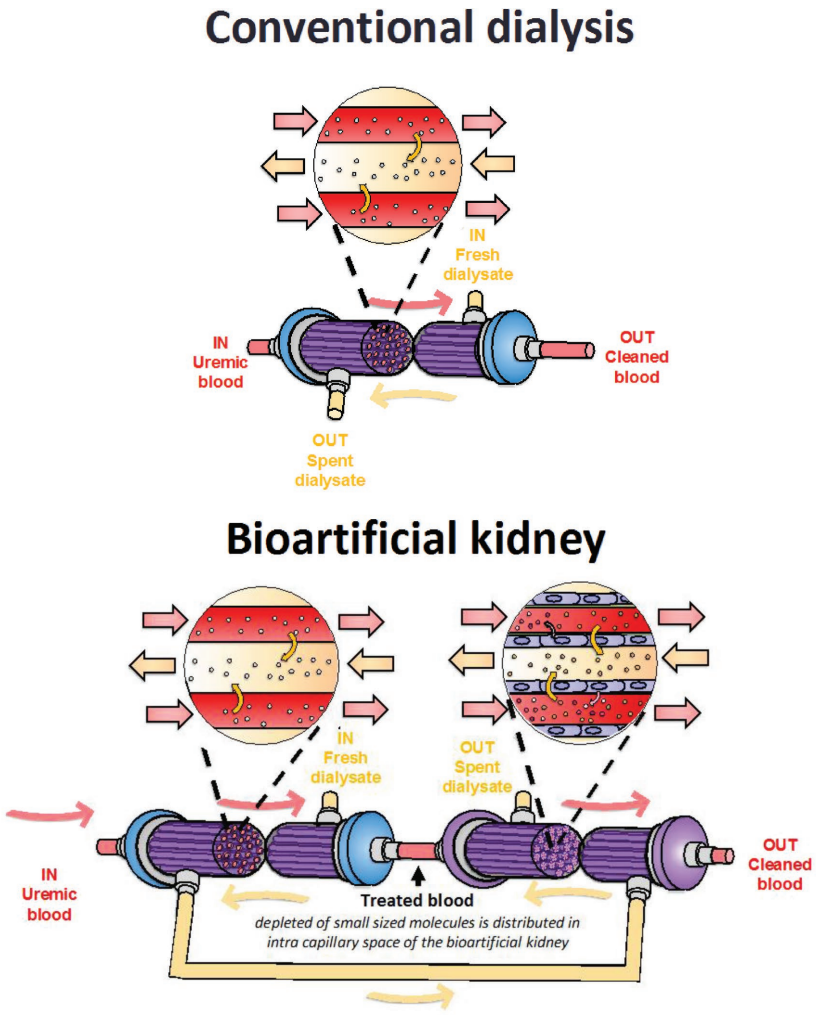

Figure 5. The conventional dialysis therapy via an artificial kidney filter is coupled in series to a bioreactor having hollow fibers coated with hPTEC. The latter can active transport of uremic toxins and nutrients and secretion of bioactive molecules.

lines, extensive research needs to be performed. To this end, encouraging preliminary results reported by Mihajlovic et al. suggest a lack of ciPTEC induced alloimmune response in vitro[62c] and no tumorigenic potential.[90] Accordingly, a comprehensive risk assessment becomes pivotal before considering a clinical trial.

BRECS is a cell therapy system for point of care treatment of AKI.[59] Approximately 10^8 renal epithelial cells are cultured onto porous niobium coated carbon disks, after which the device is cryopreserved for storage. Upon reconstitution 1 to 3 months later, the cells maintain viability, phenotype and metabolic activity (lactate production, oxygen consumption, and glutathione metabolism). Designed to be used with ultrafiltrated blood or in a peritoneal dialysis setup, BRECS does not rely on an extracorporeal continuous source of filtrate,[59b,91] which could be a significant step toward a wearable and even an implantable application. The first preclinical testing suggested that BRECS delivered from an extracorporeal circuit exhibits therapeutic efficacy with improved cardiovascular outcome and prolonged survival rate when compared with cell-free controls.[59c,92]

A further extension of the wearable RAD system is the implantable one, or iRAD, proposed by Fissell and Roy.[60a] This iRAD utilizes microelectromechanical systems to miniaturize the original RAD design into a compact (0.1 m^2), implantable, self-monitoring, and self-regulating device. It comprises of two compartments, both containing SNM, which would provide immunoisolation and a high ultrafiltration performance, enabling the iRAD to be powered exclusively by blood pressure. The first compartment would act as long-life hemofilter, removing toxins, excess water and salts, while the second one would act as bioreactor based on SNM seeded with renal PTEC.[54] These cells would selectively reabsorb water and essential substances, allowing the discharge of only toxins in the bladder.[60c,93] Although the development of iRAD is unquestionably significant, the majority of reported studies tackle the technical aspects concerning its manufacturing and miniaturization rather than the performance of the cellular components. Thus, an extensive confirmation of how the concept would replace the renal function has yet to be provided. Meanwhile, the FDA acknowledged its potential impact to clinical practice and selected the system to pilot a new regulatory approval program for bringing medical device technologies to patients faster and more efficiently.[94] This iRAD is targeted to enter clinical trials in 2018.[86a]

4.1.3. Outlook and Perspectives of BAK

The development of BAK devices is currently mainly in preclinical stage and future work will focus on confirming its safety and efficacy in a relevant animal model of ESKD (e.g., nephrectomised rat, uremic goat) to provide enough information for ethical committees and regulatory agencies to decide for further development and eventual clinical trials. One of the critical questions that has to be addressed is whether the BAK should be perfused with blood or plasma following a plasma filtration procedure, and whether the device could be re-used. The latter would mostly depend on cell viability and functional recovery after a single treatment session. In addition, prior to clinical testing, the manufacturing process should be determined in order to ensure consistent, reproducible and high-quality final product for safe use in patients. Regarding this issue, the mode of storage and shelf-life of the final product need to be established. In particular, the optimal cryopreservation conditions have to be determined in order to ensure a safe and functional device with viable cells after thawing and reconstitution. This is an extremely important point to evaluate as it might affect the manufacturing procedures and future supply

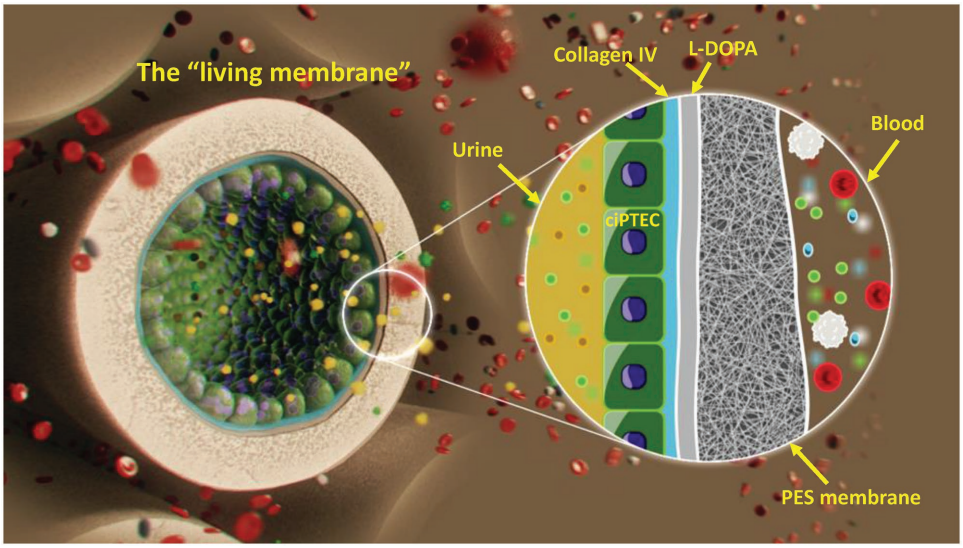

Figure 6. The BAK system containing ciPTEC cells cultured on PES follow fibers.

chain strategies.[95] The possibility to develop and store a safe and high-quality device would allow the production of an off-the-shelf product which could be manufactured in large-scale manufacturing facilities, in a stable and standardized manner, from where it could be distributed to specialized medical centres. Nonetheless, very careful transport conditions would have to be ensured in order to avoid any damage of the final product.

Another important issue remains the optimal cell population(s) and cell sources used. Although proximal tubular cells are important in the excretion of PBUT, other renal cells, both epithelial and mesenchymal contribute to the different renal functions (e.g., interstitial cells produce erythropoietin). Whether the cells in the RAD need to be derived from the patient (autologous) depends on the design of the BAK and whether these cells will be exposed to the (immune system of) the patient. The latter will also define the amount of genetic manipulation and subsequent genetic instability allowed, as the carcinogenic risk of manipulated cells will make it impossible to use these cells in devices were these cells are in direct contact with the host. Finally, it should be noted that the manufacturing costs, market size, risk/benefit profile and reusability will influence the price of the device and/or treatment sessions, which are extremely important challenges that advanced therapies are facing nowadays.[95]

4.2. The Bioartificial Liver

The artificial extracorporeal liver systems described in previous section have shown interesting outcomes for some types of patients. However, they only replace the direct detoxification functions of the liver and do not achieve biotransformation or synthesis ensured by the hepatocytes. Alternately, BAL aims at recreating all the liver-specific functions, by using metabolically active liver cells. The term BAL was first employed by Matsumura et al. in 1987[96] who proposed to perfuse a suspension of porcine hepatocytes in an extracorporeal bioreactor based on a Kill flat dialyzer.

4.2.1. Liver Cell Sources

One of the major challenges to solve in the BAL support devices is the cell source that will be used to replace liver functions. Thus, different cell types are being explored, such as, primary human hepatocytes, primary porcine hepatocytes, tumor-derived and immortalized cell lines, embryonic stem cells, and stem cell-derived hepatic cells (**Table 6**).

Primary Human Hepatic Cells: Ideally, primary human hepatic cells, such as hepatocytes, but also potentially Kupffer cells, liver sinusoidal endothelial cells, stellate cells, as well as cholangiocytes, should be employed for clinical application of the BAL, since their presence in the tissue ensure livers physiological functions in vivo.[97] However, their use faces many pitfalls. First, the irregular access to human liver, aggravated by the competing demand of whole-organ transplantation, obstructs the planning of sudden treatment. Long-term cultures or cryopreservation could alleviate the problem, but the loss of differentiated metabolic cell functions in time together with the associated cost of their maintenance resulted in logistic issues rather than solutions in their use.[98]

Other important issue in the use of human primary cells isolated from liver tissue is the transmission of malignancy or infection to the patient.[98b] Therefore, primary hepatic cells from human origin have not been widely used in BAL. The group of Guo-Zheng Chen developed an extracorporeal bioartificial liver support system (EBLSS) using cultured primary human hepatocytes and nonparenchymal liver spheroids within hollow fiber cartridges to study its support effect for fulminant hepatic failure. Compared with the control group, i.e., dogs with the EBLSS without the primary cells, the study group showed the ability to compensate the functions of the liver.[99] Millis et al. used human primary hepatocellular carcinoma cells in a BAL for a clinical treatment of more than 100 h, during which clinical parameters improved the hepatic functions of the patient.[100] Baccarani et al. developed a protocol to isolate, cryopreserve and thaw human hepatocytes.[101] The optimization of these 3 steps allowed obtaining

Table 6. Summary of relevant cells used in BALs.

Source	Type	Cell	Advantages	Limits	System	Reference
Hepatic Primary cells	Human primary cells	Hepatocytes Nonparenchymal liver cells	- Recreate liver function	- Relative scarcity; - Loss of metabolic functions in time - Possible transmission of infections/ malignancy	EBLSS BAL	[99–102]
	Porcine primary cells	Hepatocytes	- close to human physiology - Availability	- Loss of metabolic functions in time - Possible immune responses	HepatAssist BAL	[106,107]
					AMC-BAL	[108]
Tumor-derived and immortalized cell lines	Cell line	HepG2 C3A	- Unlimited expansion potential	- Low hepatic activities and functions - The chance of transferring tumorigenic products	ELAD	[109]
	Cell line	HepaRG			AMC-BAL	[110]
Pluripotent cells	Primary	Fetal hepatocytes	- Higher proliferation capacity compared to adult hepatocytes	- Low capacities for ammonia elimination and urea production - Possible tumorigenicity - Incompletely differentiated nature - Low Availability	Not available	[111]
	stem cells	hESC	- High availability	- Production a large-scale - Possible immune-compatibility - Risk of teratoma formation	BAL	[112]
	stem cells	iPSC	- High availability - Not immune compatible		BAL	[103,113]

a large number of hepatocytes for treating patients affected by ALF.[102]

Porcine hepatocytes: Porcine hepatocytes present functions close to human ones, in terms of metabolism and ammonia removal.[103] This promoted their deployment in BAL, and before 2000, this cell source was the most frequently used.[104] Porcine hepatocytes were indeed readily available and just one porcine liver could provide enough hepatocytes for several BAL treatments, a significant advantage compared with the use of human ones.[105]

In 1994 Demetriou and collaborators started the clinical trials, approved by FDA, with a porcine hepatocyte-based BAL system called HepatAssist.[106a] Porcine hepatocytes, cultured on microcarriers, maintained differentiated hepatic functions.[106b] Based on this premise, Sakai and coworkers tried to obtain a large number of porcine hepatocyte spheroids to be used in a BAL through a rotational culture in a spinner flask fitted with a silicon tubing apparatus for oxygen supply.[107] In 2002, Van de Kerkhove et al. started a phase I trial with a liver support device called AMC-BAL system that consisted of an extracorporeal bioreactor which could be filled with at least 10×10^9 viable porcine hepatocytes.[108]

Although the attempts for using porcine hepatocytes have been relevant in the last decades, due to the several concerns of using xenogeneic cells (transmission of zoonotic diseases, protein-protein incompatibility between species, the possible immune responses during treatment), most of the groups working on BALs have now switched to human cells to avoid these issues.

Tumor-Derived and Immortalized Cell Lines: Tumor-derived hepatocyte cell lines and immortalized cells have an unlimited expansion potential; however, these cells lines present relatively low hepatic activities and functions. The C3A cell line is one of the immortalized adult human hepatocytes mostly used in BAL system. Derived from a human hepatoma cell line named HepG2,[109a] it demonstrated high albumin and alpha-fetoprotein-synthesizing capacity and a nitrogen-metabolizing ability. The developers of the Extracorporeal Liver Assist Device (ELAD), as well as Selden's group in UK, used this cell line for providing enzymatic functions and improving bilirubin and ammonia levels, and hepatic encephalopathy.[109b–d] Alternately, immortalized human hepatocyte cell lines are constructed by transfection of primary hepatocytes with Simian Virus 40 T antigen.[114] Therefore, the risk of transferring tumorigenic products combined with their low functions are the major concerns.[115] Hence, before being employed in BAL, hepatoma or hepatocellular carcinoma-derived liver cell lines need severe evaluation of specific hepatic functions and safety aspects. It would be essential to create systems whose growth can be regulated to avoid malignant transformation such as the Cre/LoxP system that guarantees a reversible immortalization.

To date, one of the most promising approach seems to be the immortalization of fetal hepatocytes by overexpressing hTERT.[116] Human fetal hepatocytes exhibit a higher proliferation capacity compared to adult hepatocytes. In some studies, the use of these cells have shown modest clinical improvements in ALF patients.[111a,b] Although they can be immortalized[111c] to increase their availability, they are not suitable in clinical situation due to their low capacities for ammonia elimination and urea production. In addition, their possible tumorigenesis and incomplete differentiated nature needs to be addressed before they can be used clinically.[111d]

In 2004, Parent et al.[110a] reported a bipotent liver progenitor cell line (HepaRG) from a patient with a liver tumor and chronic hepatitis C. This cell line was able to coexpress hepatocyte and bile-duct markers and hepatocyte-specific markers

due to a progressive acquisition of hepatocytic phenotype, thus it could be a promising candidate for BAL application.[110b] By employing these cell lines, Nibourg et al. designed a human cell-based BAL showing a high level of hepatic functionality and efficacy in a rat model of ALF.[110c]

Embryonic Stem Cells and Induced Pluripotent Stem Cells: The use of these renewable cells could overcome all the limitations of the different hepatic cell sources used in BALs mentioned earlier.[117] The ESCs are obtained from the inner cell mass of preimplantation embryos and have the ability to self-renew and differentiate into cells of all three germ layers.[118] Up to date, many protocols have been proposed to generate ES-derived hepatocytes for BAL systems.[119] Soto-Gutiérrez et al.[112a] differentiated mouse ES cells into hepatocytes by coculture with a combination of human liver nonparenchymal cell lines and in the presence of different growth factors, sorting functional hepatocytes with albumin expression. The treatment of hepatectomized mice with a BAL implanted subcutaneously with these cells improved liver function and prolonged survival. Although multiple literature reports have adopted ES-derived hepatocytes, there are still some lingering ethical issues for some people and religious groups,[120] and more importantly, they are concerns with the robustness of their hepatic functions.[121]

The iPS cells can be differentiated toward the hepatic lineage, improving the prospects in hepatology field and consequently their potential use in BAL devices. We mention hereafter only the works performed in the field of BAL. More details on biologics can be found in other recent reviews.[113b,122] In 2015, Ren et al. developed a BAL with iPS-derived hepatocytes (iHeps) arrayed on the extracapillary space of hollow fiber membranes[123] and in 2016, Shi et al. produced iHeps at clinical scales to be seeded in a BAL system. Then, in a porcine ALF model, hiHep-BAL treatment led to attenuated liver damage, resolved inflammation and enhanced liver regeneration. These results are promising,[103] however, the use of viral vectors, the modifications in the cell cycle, and the risk of teratoma formation[124] are major concerns for application of these cells to BAL devices. They are however limited in extracorporeal systems. Therefore, ESCs and iPSCs remain the most promising approach to be explored for extracorporeal BAL.[121]

4.2.2. Scaffold Free Approach and Coculture

Highly efficient cells can be obtained via tissue engineering approaches that better mimic the in vivo structure or microenvironment.[125] Since in the liver, the ECM is not predominant, this part focuses on 3D culture of hepatocytes, alone or associated with other cell types.

Spheroids/Hepatospheres Formation: Spheroids or so-called hepatospheres are based on the capacity of single suspended cells to form aggregates by cellular self-assembly. The process involves three steps[126]: 1) a rapid aggregation of suspended cells by establishment of integrin binds, 2) a delay-period with an E-cadherin expression and accumulation, 3) homophilic cadherin-cadherin interaction and compaction of the aggregate shape. These constructs behave as an avascular tissue. Therefore, spheroids with a diameter greater than 250 µm commonly have nutrient limitations and waste accumulation inside the core that led to a necrotic core surrounded by a viable rim.[127] The first development of hepatic spheroids was described by Koide.[128] This 3-D culture achieves extensive cell-cell contact, polarity, bile canaliculi,[129] and transcriptional change in comparison to 2D culture.[130] Part of this difference is due to transcriptional regulator Hnf4α.[131] All these elements mimic better hepatic tissue, leading to better cell viability and the maintaining of many differentiated liver functions for a prolonged time.[125,129,132]

Tissue engineering has provided different protocols to produce spheroids, with its advantages and limitations (**Table 7**). Up to now, there is no gold standard for a production system. In general, the 3D cultures provide many benefits compared to 2D culture but they are more laborious. However, new optimizations or techniques are being drawn up to facilitating different aspects including methodology of analyzing, scaling

Table 7. Advantages and limitations of methods for cell aggregation and spheroid formation.

Method	Principle	Advantages	Drawbacks	Reference
Liquid overlay	Nonadhesive support +/agitation	Low cost, simple Easy to scale up	Not homogenous, no size control	[133]
Pellet culture	Centrifugal force	Low cost, simple	Not homogenous, no size control	[134]
Microwells	microfabrication	Low shear stress Size control Control ratio in coculture	Need specific equipment	[135]
Hanging drop	Inversion of lid	Low cost Low shear stress Size control Control ratio in coculture	Difficult to scale up for mass production	[136]
External forces	Electric, magnetic field or ultrasound	Low shear stress	Need specific equipment Not homogenous, no size control	[137]
Rotary systems	microgravity	Simple	Need specific equipment Not homogenous, no size control	[138]
Spinner flasks/ bioreactors	Suspended cells + stirring	Simple Easy to scale up	Need specific equipment Not homogenous, no size control	[139]
microfluidics	Microrotational flow	Low shear stress Size control Control ratio in coculture	Difficult to scale up for mass production	[140]

up, or manipulation. In the pursuit of in vivo-like 3D environment mimicking better the native tissue, culture 3D can be combined with coculture.

3D Culture Combined with Coculture: Various studies have shown that coculture of hepatocytes or hepatocyte-like cells (target) with supporting cells is a way to maintain/improve or induce hepatic functions.[141] In the BAL context, 3D coculture systems could help reducing the high request biomass by enhancing hepatocytes functions. In these constructs, two parameters are critical:

The Cell Choice: Actually, coculture systems were performed with different type of human or mammalian (pig or rodent) from hepatic origin (nonparenchymal cells ex Kupffer cells, hepatic stellate cells or sinusoidal endothelial cells) or not (fibroblasts, endothelial cells mesenchymal stem cell) (**Table 8**). Coculture can influence negatively (e.g., activate Kupffer cells) or positively (stellate cells) the hepatic functions or differentiation. The majority of studies used xenogeneic primary hepatocytes because human cells are scarce and cells line fail to perform all hepatic functions associated with a tumorigenic potential. The utilization of xenogeneic source raises questions of security and probably these cocultures would not reach the clinic. However, these studies illustrate their potential. Proliferative and stem cell of human source are probably the promising alternative to primary cells for clinical application.

Coculture Conditions and Cells Ratio: To respect the native organization liver, physiological ratio can serve as a strong indicator. However, there is no consensus within the literature regarding the optimum cell ratio. The analysis of the different studies revealed that the optimum ratio depends on the origin of supporting cells and does not systematically coincide with the physiological cell proportion. Although, direct comparisons are difficult due to various approaches of 3D coculture conditions. Indeed, the choice of 3D protocol or the coculture condition (cells mix or by successive covering) affect considerably the result.

Organoid Approach: Another solution to get scaffold free highly organized structures is the development of organoids derived from few cells from a tissue, embryonic stem cells or induced pluripotent stem cells, which can self-organize in three-dimensional culture owing to their self-renewal and differentiation capacities. The most promising results there were generated by Takebe's group with the production of liver "buds" obtained on a soft gel by condensation of hepatic like cells derived from hiPSCs, HUVEC, and human mesenchymal stems cells.[142] Very recently, this group describes a combined platform allowing cell screening and high yield of buds (up to 10^8 cells per batch), which is still under the requirements for a full BAL.[143]

4.2.3. Membrane-Based BAL Systems

Membranes with suitable molecular weight cutoff (MWCO) (ranging from 70 to 100 kDa) act as selective barrier for the transport of nutrients and metabolites and immune-isolation of cells. Indeed, membranes allow protection of hepatocytes from adverse immune reaction by patients' hosting cells, and protection of hosting cells from potential oncogenic risks or zoonosis. Moreover, the cell compartmentalization preserves hepatocytes from shear stress of dynamic perfusion.

In the first membrane based BAL, hepatocytes were used in free suspension[96,155] and, to prolong their lifespan and activity, in adhesion to microcarriers,[156] entrapped in collagen gel,[157] in basement membrane matrix (i.e., Engelbreth-Holm-Swarm-EHS gel),[158] or encapsulated in agarose microdroplets.[159] Successively, membranes made by natural or synthetic polymers were used as scaffolds for cell adhesion mimicking the ECM with which cells interact in natural environment. This is important for the polarization of cells and organization in a 3D architecture.[160] In this way, membranes perform a dual task, ensuring the selective transport of metabolites, nutrients and specific products to and from cells, and modulating their adhesion and functions.[161]

The surface chemistry and topography of membranes strongly affect and influence cell-material interactions, and thereby cell response and tissue formation. In fact, physicochemical and morphological membrane surface properties (i.e., charge, free energy parameters, wettability, roughness, topography, pore size, pore shape and

Table 8. Overview of coculture methods to produce heterospheroids or organoids.

Target	Supporting cells	3D methods	Cell ratio	Coculture method	Reference
			target: supporting		
Primary hepatocytes rat	IH 3T3 NIH 3T3–HUVEC	Liquid Overlay	NC	Covering	[144]
Primary hepatocytes rat	Hepatic stellate cells	microfluidics	10: 1	Mix	[145]
Primary hepatocytes rat	Pancreatic islet cell	microfluidics	From 7:1 to 1:7	Mix	[146]
H35s (cell line)	Fibroblasts (p H)	microfabrication	3:1	Covering	[147]
Primary hepatocytes rat	Hepatic stellate cells	microfabrication	3:1	Mix	[148]
Primary hepatocytes rat	Hepatic stellate cells	microfabrication	3:1	Mix	[149]
Primary hepatocytes rat	Hepatic stellate cells	Liquid Overlay	2:1	Mix	[150]
Primary hepatocytes rat	Stellate cell Kupffer sinusoidal endothelial cells	Liquid Overlay	1:2	Mix	[151]
Primary hepatocytes rat	NIH 3T3 Mouse fibroblasts Human Fibroblasts	Spinner Culture	1:2 1:1 2:1	Mix	[152]
Primary hepatocytes rat	sinusoidal endothelial cells	Spinner Culture	1:3	Mix	[153]
Primary hepatocytes rat	Nonparenchymal cells	Rotary culture	2:1	Covering	[154]
Primary hepatocytes rat	Nonparenchymal cells	Liquid Overlay	8:2	Mix	[133b]
HLC derived from hIPSCs	HUVEC, hMSC	Liquid overlay	10(iPSC):5(HUVEC):1(MSC)	Mix	[142]

distribution) influence cell adhesion, affecting and modulating the cytoskeleton organization for the formation of focal adhesion complexes and cell motility and shape.[162] It is also shown that membranes with high Young's modulus and strength promoted capillary development by endothelial cells in a BAL systems.[160b]

Different strategies have been undertaken to improve hepatocyte-membrane interactions including the functionalization of membrane surface with ECM biomolecules or peptides, such as, the RGD sequence and galactose moiety,[163] which are recognized by hepatocyte receptors. An alternative approach involves the grafting of functional groups, such as $-COOH$ and $-NH_2$ over the membrane surface that has been found to enhance the membrane polarity, and thus the cell adhesion and functions.[164]

Membranes can be fabricated in different configurations (e.g., flat, tubular, hollow fiber, or capillary) giving rise different type of systems and devices. Membranes in flat configuration are largely used in small-scale devices based on their operational simplicity. There, long-term maintenance of cell functions can be achieved, thanks to 3D culture and adapted oxygenation, making them useful for toxicology studies and drug screening.[165] Hollow fibers are indeed preferred and largely developed because they provide a great surface area for cell adhesion and proliferation in a small volume with respect to other configurations, as well as, scalability perspectives. The requested volume of HF configuration is 0.1% the capacity of a T-flask, or 0.5% the size of a stirred tank to grow an equivalent number of cells.[166] **Table 9** provides an overview of the set ups developed for BAL. Although they are not at human scale, one can extrapolate that the improvements achieved at a lower scale can be translated to human size module. Several configurations have been investigated (**Figure 7**).

In most of the HF membrane devices, cells are cultured in the extra capillary space, and nutrients fed through the lumen of the fibers and transported to extra capillary space across the membrane. A wide range of solutes concerning molecular size (from small electrolytes to large proteins) and physicochemical properties (hydrophilic, hydrophobic molecules) are transported through the membrane. In particular, oxygen is one of the most important limiting nutrient for precariously vascularized systems owing to its relatively low solubility and high uptake rate of hepatocytes. One of the critical issues for keeping hepatic cell functions is the molecular mass transfer (which depends on the diffusion/convection) and kinetic mechanisms considering that molecules are simultaneously transported and consumed/produced by cells. The efficacy of hollow fiber membrane BALs is still limited due to the lack of information that might lead to an improved operation. Appropriate mathematical models can help solving this issue. Attempts have been made by using computational methods that allow to solve the set of differential equations describing the mass transport across hollow fibers with high degree of accuracy and in short time.[127,167]

An oxygenating hollow fiber bioreactor (OXY-HFB) developed by Jasmund and coworkers consists of two mats of HF membranes arranged crosswise with a constant distance of 200 µm. In this device polyethylene (PE) heat exchange and polypropylene (PP) oxygenating fibers, provide temperature control and oxygenation, respectively. Primary hepatocytes were cultured at high cell density in direct contact with the perfused medium.[168] Mizumoto and Funatsu developed two different hybrid artificial liver support systems (HALSSs), a liver lobule-like structure (LSS-HALSS), and a multicapillary polyurethane foam (PUF-HALSS). LSS-HALSS consists of a housing containing PE HFs coated with ethylene vinyl alcohol (EVAL), regularly arranged close together, among which hepatocytes were cultured. PUF-HALSS consists of a macroporous structure in which hepatocytes were inoculated among many capillaries arranged to form a channel for the culture medium. In both the systems, hepatocytes either from porcine origin or derived from ES spontaneously formed organoids.[169] A multibore membrane consisting of seven capillaries grouped in a foamy porous and highly permeable support structure was applied for the culture of human hepatocytes. Cells were compartmentalized in the lumen of modified PES multibore capillaries that were connected to each other.[170] S. Ren et al. hosted iHeps in the extracapillary space of semipermeable fibers. This work showed that the iPS-hepatocytes in the BAL device maintained the secretory function and exhibited cell maturation.[171] Mizumoto et al., packed differentiated ESCs with liver specific functions in the lumen of CTA HFs woven in a textile sheet.[172] A peculiar configuration was realized by cross assembling in alternating manner two bundles of HFs with specific physicochemical, morphological and transport properties for medium inflow and outflow. This bioreactor was able to maintain functionally active human hepatocytes up to 19 d, and to differentiate liver progenitor cells.[173,174] The same concept was utilized to culture under an efficient oxygenation and nutrient supply human liver spheroids and an organotypic coculture system.[175,176] Gerlach et al.,[180] used three independent interwoven capillary mats for medium inflow, medium outflow (two separate bundles of PES HF membranes) and oxygen/carbon dioxide exchange (hydrophobic multilaminate HF membrane). Primary hepatocytes were cultured in the extra capillary space of a 3D network with a counter-current medium perfusion and internal oxygenation.[177–179]

Several of the devices discussed earlier have also been tested in clinical trials, see **Table 10**. The first larger clinical trial utilized an LSS constituted of porcine hepatocytes loaded in polyvinylchloride (PVC) membrane cartridges.[155] The next BAL devices prolonged their lifespan by using hepatocytes in adhesion on membranes with different MWCO and configurations. The Academic Medical Center Bioartificial Liver (AMC-BAL) utilized a spirally wound nonwoven polyester matrix on which hepatocytes were cultured among polypropylene (PP) HF membranes for oxygen delivery.[181] A phase I clinical trial utilizing the AMC-BAL reported successfully bridged patients to liver transplant.[108,182]

The only two BALs that have undergone the most extensive clinical trials up to phase III, are the Extra-corporeal Liver Assist Device (ELAD, Vital Therapies, Inc.) developed by Sussman et al.,[183] and the HepatAssist Circe Biomedical, now renamed HepaMate, developed by Demetriou et al.,[156] ELAD consists of four HF cartridges of CA with MWCO of 70 kDa that separates the cells from patient's plasma. In this device, 200 g of human HepG2/C3A hepatoblastoma cell line, are loaded in the extra capillary space of each cartridge, with plasma flowing in the membrane lumen. Successively ELAD was modified in

Table 9. An overview of the hollow fiber set ups developed for BAL.

HF configuration	Membrane	Cell position	Cell source	Cell capacity	Culture technique	Reference
BAL, parallel assembled	PSf (MWCO 100 kDa)	lumen	Primary rat and porcine hepatocytes	$3–10 \times 10^5$	Spheroids, collagen entrapment	[157a,b]
BAL,parallel assembled	PSf (pore size 0.2 μm)	extrafiber	Primary rat hepatocytes and HepG2	9×10^7	Spheroids encapsulated in agarose microdroplets	[159]
BLSS, parallel assembled	PE (pore size 0.3 μm)	extrafiber	Primary porcine hepatocytes	5.4×10^9	Monolayer, entrapment in collagen gel	[157c]
BAL, parallel assembled	polyolefin (pore size 0.4 μm)	extrafiber	Primary rat hepatocytes and HepG2	2×10^7	Monolayer, entrapment in EHS gel	[158]
EBLSS, parallel assembled	cellulose nitrate and cellulose acetate (pore size 0.2 μm)	extrafiber	Primary porcine hepatocytes with 50% nonparenchymal cells	1×10^8	Aggregates	[99]
MBR, parallel assembled	Modified PVDF (pore size 0.5 μm)	extrafiber	Primary rat hepatocytes	5×10^7	Aggregates	[163d]
OXY-HFB, crosswise alternating mats	PE (pore size 0.2 μm) mats for internal heat exchange; PP mats for internal oxygenation	extrafiber	Primary porcine hepatocytes	2.5×10^9	Monolayer	[168]
LSS-HALSS, parallel assembled	PE coated with EVAL	extrafiber	Primary porcine hepatocytes	0.5–10 g	Centrifugal inoculation, organoids formation	[169]
PUF-HALSS, multicapillary	PU	porous foam		6.5 g	Spheroids	
MBR, multibore fibers	PESM (pore size 0.2 μm)	lumen	Primary human hepatocytes	7.5×10^6	Monolayer	[170]
BAL, parallel assembled	PSf (pore size 0.21 μm)	extrafiber	iPSC-derived hepatocytes	$9–10 \times 10^6$	Entrapment in Laminin coated beads	[171]
ES-BAL, woven in a textile sheet	cellulose triacetate (pore size 0.2 μm)	lumen	Mouse embryonic stem cells	6.6×10^6	Packed cylindrical organoids	[172]
MBR, crossed alternating bundles	PEEK-WC (MWCO 190 kDa) bundle for inlet medium; PES (pore size 0.2 μm) bundle for outlet medium	extrafiber 3D network	Primary human hepatocytes	13×10^6	Monolayer	[173]
			Rat Progenitor Liver Cells	$1–25 \times 10^6$	Aggregates	[174]
	PES (pore size 0.2 μm): two independent bundles for inlet and outlet medium		Primary human hepatocytes	8×10^6	Spheroids	[175]
			Primary human hepatocytes with sinusoidal and stellate cells	11×10^6	Multilayer organotypic coculture	[176]
MBR, multiple interwoven sets	PES (MWCO 400–500 kDa, two independent sets for inlet and outlet medium; hydrophobic multilaminate for internal oxygenation	extrafiber 3D network	Primary porcine hepatocytes with 5–10% nonparenchymal cells	3×10^9	Aggregates	[177]
			Primary human hepatocytes with 5–10% nonparenchymal cells	1×10^8		[178]
				2×10^7–1.5×10^{10}		[179]

order to increase cell mass (up to 400 g for each cartridge) and MWCO of membranes (up to 120 kDa), as well as to improve the oxygenation and to prevent risk of cancer cell migration.[184] Notwithstanding concerns related to the use of hepatoma cell line, that exhibit besides decreased liver specific functions (i.e., ureagenesis and drug metabolism), over 250 subjects were treated in clinical trials with ELAD. Among these, the phase III VTI-208 was the largest, randomized, controlled, open-label trial to date, which has begun in 2013 and completed in 2015, enrolling 208 subjects. Although pre-specified subsets based on age and lesser disease severity showed promising trends toward efficacy, VTI-208 failed to achieve its primary and secondary endpoints. Therefore, a second phase III trial, VTI-210, and a phase II clinical trial, VTI-212, both begun in 2014, were discontinued. Currently, a new phase III clinical trial, VTL-308, is enrolling subjects from May 2016. The Company expect to

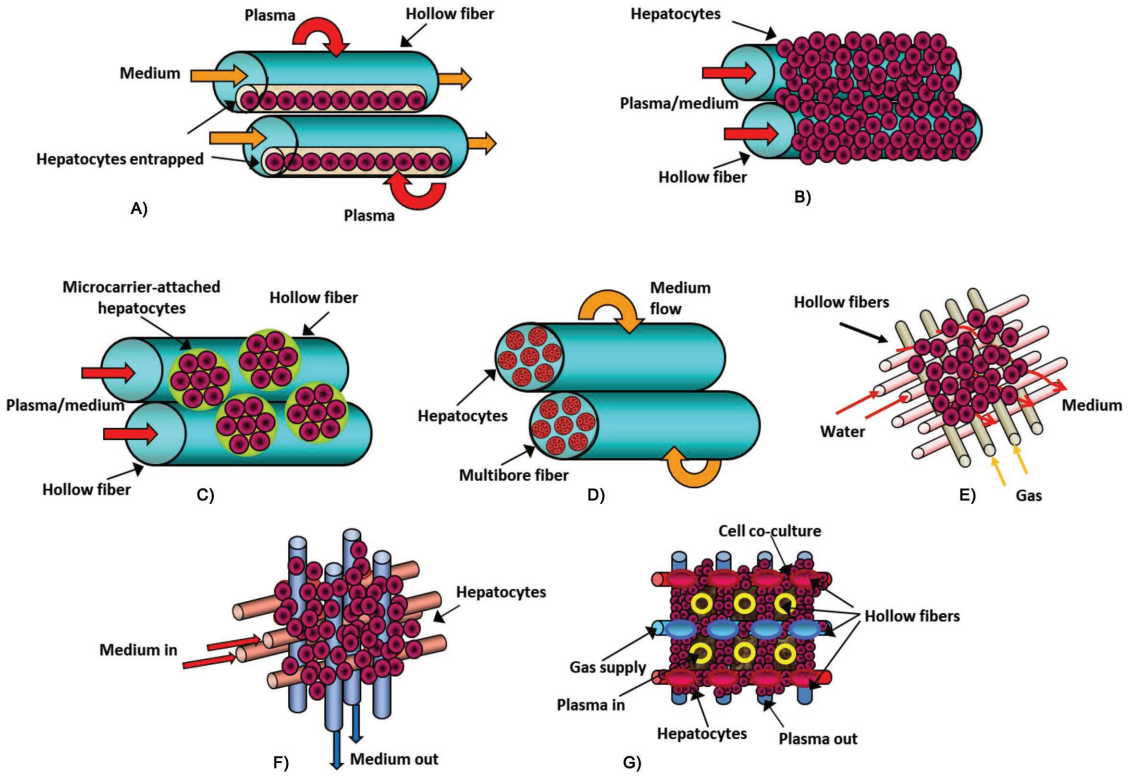

Figure 7. Configurations of HF membrane BALs using hepatocytes cultured: A) in a 3D gel matrix inside of HF membranes; B) outside of the HF membranes in a monolayer; C) outside of HF membranes attached to microcarriers; D) in the intraluminal compartment of a multibore fiber bioreactor; E) between mats of oxygenating and heat exchange fibers; F) among two bundles of cross-assembled HF membranes; G) in a network of three sets of independent interwoven HF membranes. Adapted with permission.[250] Copyright 2010, Wiley-VCH.

Table 10. Membrane based BALs under clinical trials.

BAL device	Configuration	Membrane	Cell source	Cell capacity	Clinical trial phase	Reference
Kiil dialyzer bioartificial liver	Flat	Cellulose (MWCO 20 kDa)	Primary rabbit hepatocytes	1×10^{10}	I	[96]
Liver Support System	Hollow fiber	PVC	Primary porcine hepatocytes	4×10^{7}	II	[155]
AMC-BAL	spirally wound	- Nonwoven polyester matrix - PP HF (pore size 0.2 µm)	Primary porcine hepatocytes	1×10^{10}	I	[108,181,182]
ELAD, Vital Therapies	Hollow fiber	CA (MWCO 70–120 kDa)	human HepG2/C3A hepatoblastoma cell line	200–400 g	III	[183,184]
HepatAssist, HepaMate Circe Biomedical	Hollow fiber	PSf (pore size 0.2 µm, MWCO 3000 kDa)	Primary porcine hepatocytes	5×10^{9}	III	[106b,156]
LSS	Interwoven HF	- PA (MWCO 100 kDa) - PSf (MWCO 80–300 kDa) - PP (pore size 0.2 µm) silastic	Primary porcine hepatocytes	2.5×10^{9}	I/II	[180,187]
MELS	Interwoven HF	- PES (MWCO > 400 kDa) - Hydrophobic multilaminate HF	Primary porcine and human hepatocytes	$2–4 \times 10^{10}$	I	[188,189a,b]
RFB	Packed bed	Polyester mesh	Primary porcine hepatocytes Primary human hepatocytes	200–230 g 6.5×10^{9}	I	[190,102]
Excorp Medical BLSS	Hollow fiber	CA (MWCO 100 kDa)	Primary porcine hepatocytes	70–120 g	I	[115]
TECA-HALSS	Hollow fiber	PSf (MWCO100 kDa)	Primary porcine hepatocytes	$1–2 \times 10^{10}$	I	[192a]

enroll at least 150 subjects at about 40 sites in the United States and Europe and to report top-line results in mid-2018.[185]

HepatAssist Circe Biomedical utilizes PSf HF membranes with pore size of 0.2 µm (MWCO 3000 kDa) and 5×10^9 primary porcine hepatocytes attached to collagen-coated dextran microcarriers and loaded into the extra capillary space. The blood plasma passes through a charcoal absorber and membrane oxygenator before entering the bioreactor, into the lumen of the HF membranes. This device was the first to be tested on a large clinical scale, with more than 200 subjects treated. In particular, in a phase III randomized controlled clinical trial, enrolling patients with fulminant and sub-fulminant liver failures from 20 sites in the United States and Europe, HepatAssist demonstrated safety and improved survival in a post hoc subgroup analysis,[106b] but failed to demonstrate improved survival after 30 d in the overall study population.[186]

The Liver Support System LSS, in which primary hepatocyte aggregates were cultured on and between independent interwoven hollow fiber membranes,[180] underwent phase I/II clinical trials.[187] Thereafter, it was integrated into a modular extracorporeal liver support system (MELS), and combined with DetoxModule for albumin dialysis.[188] MELS underwent phase I clinical trials[189] and, notwithstanding first encouraging results, the device never progressed in controlled, randomized clinical trial required for regulatory approval.

A different configuration was used in the radial flow bioreactor (RFB), developed at the University of Ferrara, in which hepatocytes in adhesion on a polyester mesh between two sheets of polyester layers, are perfused by the patient's plasma that flows from the center to the periphery of the device. RFB was tested in phase I clinical trials by using primary porcine hepatocytes[190] and human hepatocytes.[102]

The Excorp Medical bioartificial liver support system (BLSS), developed at the University of Pittsburg, utilizes primary porcine hepatocytes embedded in a collagen matrix in the extra capillary space of CA hollow fibers. This device was involved in phase I trial.[115,191] TECA hybrid artificial liver support system (TECA-HALSS) is another BAL tested in phase I clinical trials[192] that utilizes primary porcine hepatocytes loaded in the extra capillary space of PSf HF membranes with MWCO 100 kDa.

Recent advances in stem cell technology enable for differentiating hepatocyte-like cells (HLCs) exhibiting highly specific liver functions. The promise on the potential use of HLCs as a feasible alternative for the treatment of liver failures seems to be in the near future.[193] A radial flow bioreactor using HLCs induced from human fibroblasts, is the first BAL system that has been tested in a preclinical trial on a pig model.[103] To date, no stem cell-based BAL system has undergone clinical trial.

4.2.4. Microencapsulation and Bioreactors

Microencapsulation consists in forming beads or capsules in which hepatic cells are entrapped. This technique is also able to provide physical separation and protection of the cells from the recipient's immune system (**Figure 8** describes for main techniques used for cell microencapsulation). The most common

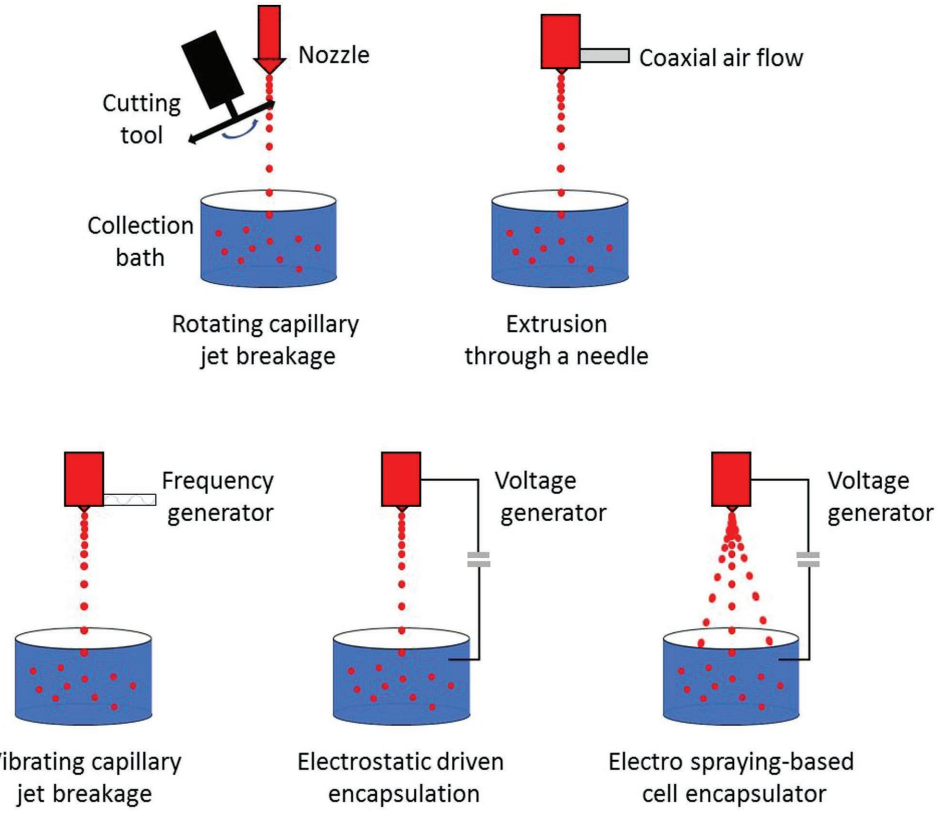

Figure 8. Main microencapsulation techniques.

natural hydrogel-forming polymer for biomedical applications is alginate, a linear copolymer containing blocks of (1,4)-linked β-D-mannuronate (M) and α-L-guluronate (G) residues, obtained from brown seaweed. This material is quite inert and not toxic regarding cells, ensuring adequate biocompatibility. In addition, its relatively low cost and gelation capacity by divalent cations such as Ca^{2+}[194] makes the process suitable for hepatocyte encapsulation.[151] Alginate is suitable for cell encapsulation because it has very limited inherent cell adhesion and cellular interaction (being a hydrophilic polymer, it promotes spheroid formation and thus enhances cell-cell interaction and hepatocyte functionality.[195]) This however can be a disadvantage for tissue engineering applications as there is a lack of specific cellular signals promoting adherence or differentiation. However, alginate can be modified by the addition of cell attachment peptides or other biologically active molecules (chitosan, fibrin gels, Matrigel, collagen).[195,196]

The technique to immobilize cells in calcium alginate matrices was originally developed by Lim and Sun.[197] Later, various techniques have been reported mainly using a two-step procedure: i) formation of droplets containing the cells mixed with the material achieved via extrusion through a needle ii) droplet solidification (gel formation) in the gelation bath composed of divalent cations (such as Ca^{2+}). The beads size is an important parameter and can be tailored depending on specific demands, such as, single cells' or spheroids' encapsulation. It depends on the diameter of pipette or syringe, the generation of additional forces to force the droplets to fall down (coaxial air flow, electrostatic generator, jet-cutter, etc.), the viscosity of the alginate solution and the rate of alginate flow. Generally, for BAL applications, beads with a diameter of 400–1000 μm are preferred to promote exchanges.[198]

Encapsulation of Hepatic Cells and Perfusion: **Table 11** presents an overview of studies of hepatic cell microencapsulation of BAL or other therapeutic applications. The beads are placed in a bioreactor allowing mass transfer between the surrounding fluid and the inner of the beads, where the biotransformation takes place. In vitro, rotating flasks can be used, but for BAL application in extracorporeal circuit, the favored configuration is the fluidized bioreactor, originally described by Legallais et al.[199] and also employed by Selden et al.[200] In most cases, a unique population of hepatocytes (primary or cell lines) were encapsulated. Recently, Song et al. investigated the potential of encapsulating hepatic like cells derived from human iPSCs aggregated with stromal cells in a hydrogel capsule. They were further implanted in immunocompetent mice.[201]

4.2.5. Outlook and Perspectives of BAL

Many attempts have been performed in the last thirty years to validate the concept of external BAL. They are therefore much more advanced than BAK, and are encouraged by the lack of efficiency of purely artificial organs to replace liver functions and properly take care of acute liver failure. However, a functional and effective solution was not found yet. Since 1987 more than 30 different BALs with different configuration, culture technique, cell source and capacity have been reported, and almost half of them have been evaluated in clinical trials. Unfortunately, only two have been involved in phase III trials, and even though some encouraging results in terms of safety and survival benefit have been reached, they did not show significantly improvements in comparison with controls, thus none of them have been approved by the FDA for marketing application so far.

One of their major drawbacks is their lack in efficiency that might be attributed to poor cell viability and/or functionality in the constructs, or limited mass transfer between the biological compartment and the patient's blood. Organoids formation from ipSC derived hepatic cells, considering not only hepatocytes but other liver cell populations could represent an innovative approach in the field, as well as the design of new matrices. Bioprinting, although limited in size at the moment, could also lead to new biohybrid organ design.[214] Other approach relies on liver decellularization/recellularization and is presented in the next chapter.

5. Organ Decellularization and Recellularization

The use of a native kidney and liver scaffolds repopulated with autologous cells can be an attractive approach for developing kidney and liver organs. The scaffolds are obtained there by removing all native cells from organs via a process called decellularization, leaving an ECM structure whose integrity is important to direct cell attachment and to generate a permissive environment for cell survival, proliferation and differentiation. The scaffold recellularization is the most difficult phase due to the complexity of the organs.

5.1. Kidney and Liver Decellularization

The most used decellularization protocols for the dense and complex structure of the kidney include the use of detergents such as the nonionic Triton X-100[215] and the anionic sodium dodecyl sulfate (SDS)[215a,216] or their combination.[215b,217] These protocols can produce acellular kidney or liver scaffolds from organs of various animals, see **Table 12**.

In 2009, Ross et al. first reported the production of a rat renal scaffold obtained by a protocol of decellularization based on 5 d of continuous perfusion through the renal artery (RA) with ionic and nonionic detergents, and enzymatic degradation of the cellular nuclear material. There, SDS was more effective than Triton X-100 to eliminate cells in the kidney.[215a] Good results in rat kidneys using SDS were also obtained by further shortening the infusion time up to 5 h.[216a,218] Recently, scaffolds have also been successfully obtained from pig kidneys[219,215b,216a,220] and rhesus monkey[221] which considered as relevant animal models to translate the decellularization technology to human-size organs. Nonetheless, the most clinically compatible scaffolds are those derived from kidneys discarded from human transplantation.[219c,222]

Similar to kidney, the liver decellularization techniques and treatments depend on the agents and the protocols used[231,232] (see Table 12). In contrast to kidney, perfusion is usually performed via the venous system, either anterograde through the portal vein (PV), or retrograde via the hepatic

Table 11. Microencapsulation of hepatic cells for therapeutic purposes.

Encapsulation technique	Reference	Material for beads (B)/Capsules (C)	Diameter [μm]	Cell type and initial density [cells mL^{-1}]	Biological activity
Rotating capillary jet breakage by JetCutter	[200,202]	Alginate (B)	500	HepG2 $1.5–1.75- 2. \times 10^6$	Lactate production Glucose consumption AFP production Bilirubin conjugation
	[203]	Alginate (B)	573–753	HepG2 $1.9–2.1 \times 10^6$	Glucose consumption AFP production
Extrusion through a needle (with or without coaxial air flow)	[204]	Alginate, chitosan, collagen (C)	2000	Primary pig hepatocytes 6×10^6	Ammonia removal Urea production
	[205]	Alginate (B)	400	Rat Hepatocytes $0.5–10 \times 10^6$	Albumin secretion Urea synthesis CYP450, UGT activity
	[206]	Hydroxyethyl methacrylate, methacrylic acid, methyl methacrylate (outer layer) and collagen (inner layer)	150	Rat hepatocytes $1–5 \times 10^7$	Albumin secretion Ammonia metabolism Urea production CYP450 activity
	[133c,207]	Alginate (B)	600–800	C3A, HuH-7 $5 \times 10^5– 10^6$	Albumin secretion Urea secretion Glucose consumption
Vibrating capillary jet breakage	[208]	Alginate (B)	582–584	Human hepatocytes or Rat Hepatocytes $2–3.5 \times 10^6$	Albumin secretion Urea secretion CYP1A/2 activity
	[209]	Alginate (B)	430–520	HepG2 1×10^6	CYP450 activity AFP
Electrostatic driven encapsulation	[210]	Alginate (B)	500–600	HepaRG 6×10^6	Albumin secretion Ammonia detox CYP3A4, 1A2, MRP2 activity
	[211]	Silk sericin–alginate–chitosan (C)	315–816	HepG2 1×10^6	Urea, albumin production Glucose consumption
	[212]	Alginate (B)	800	C3A, Huh7 3×10^6	CYP450 activity Urea secretion Albumin secretion
	[213]	Alginate-chitosan (C)	800	C3A 3×10^6	CYP1A2 and 3A4 activity Urea, albumin secretion
Electro spraying-based cell encapsulator	[201]	Alginate (C)	1000–2000	Primary human hepatocytes + stromal cells 5–10 cell aggregates (diameter of 150 μm)/capsule	Albumin secretion Urea secretion CYP450 activity

vein (HV) and inferior vena cava (IVC). For example, Uygun et al. succeeded in the decellularization of ischemic rat livers based on portal vein perfusion using different concentrations of SDS[227] and Baptista et al., decellularized several animal organs (mice, rats, ferrets, rabbits, and pigs) employing a different perfusion procedure depending on liver size and structure (**Figure 9**).[229,233] De Kock et al. achieved whole rat decellularized livers in 60 min by portal perfusion with a mild detergent[228] whereas Soto-Gutierrez et al. used a combination of enzymatic, detergent, and mechanical protocols to obtain decellularized whole-rat livers.[230] Their decellularization method preserved the 3D macrostructure and ultrastructure, the native microvascular network of liver, but also up 50% of growth factor content. More recently, Mazza and collaborators also reported decellularization of a single human lobe (left), completed in 14 d, or of the whole human liver, in 6 weeks.[231]

The success of the decellularization protocol must be confirmed in terms of analysis of residual cellular material, preservation of ECM proteins and growth factors and preservation of ultrastructure and vascular architecture of the decellularized organ. The residual DNA content should be below 50 ng DNA/mg tissue to avoid problems of cytocompatibility in vitro and to adverse host reactions, in vivo, following implantation of biological scaffolds.[223a,232a] The success of the decellularization process is also dependent by the preservation of the architecture and composition of the ECM.[215b,216b] This is mostly assessed via electron microscopy, including transmission electron microscopy (TEM)[216b,219a,223b] and SEM.[215,216b,217a] **Figures** 9 and **10** display representative

Table 12. Summary of relevant works in kidney/liver decellularization.

Organ	Decellularization protocol		Perfused vessel	Model	Reference
	Chemical agents	Biological agents			
Kidney	TritonX 100	Trypsin-EGTA	RA	Rat	[217b]
	TritonX 100	DNase	RA	Rat	[217a]
	TritonX 100	_	RA	Pig, rhesus monkey	[223]
	TritonX 100 and SDS	DNase	RA	Rat	[217,224]
	TritonX 100 and SDS	_	RA	Rat, pig	[217b,219b,221,223a,225]
	SDS	_	RA	Rat, pig, rhesus monkey, human	[220a,221,223,224,226]
	SDS	DNase	RA	Human	[219b]
	SDS	_	RA	Pig	[223a]
Liver	SDS	–	PV	Rat	[227]
	TX100 and SDS	–	PV	Rat	[228]
	TX100 and NH_4OH	–	PV	Mouse, rat, ferret, rabbit, and pig	[229]
	TX100	Trypsin–EGTA	IVC	Rat	[230]
	TX100 and SDS	Trypsin-EDTA	HV and IVC	Human	[231]

RA: renal artery; U: ureter; HV: hepatic vein and IVC inferior vena cava.

images to demonstrate the absence of cellular content and the integrity of ECM structures retained. In addition to the parenchymal structures, vascular patency is the basic requirement for successful organ recellularization by analysis with angiography or with micro computed tomography (Figure 9B).[215b,216b,219b,c,234]

Prior to implantation or for in vitro use, sterilization of the scaffolds is necessary to primarily eliminate endotoxins, intact viral and bacterial DNA. The most used procedures include incubation with acids or solvents,[235] ethylene oxide exposure, gamma irradiation, and electron beam irradiation.[220b,236]

5.2. Kidney Recellularization

To complete kidney regeneration, the reconstruction of nephron is essential, involving repopulation with endothelial cells in the vasculature and glomeruli and epithelial cells in the tubules and collecting ducts. Different cell types, in link mostly with those already used in BAK, as primary cells, embryonic stem cells, fetal cells, adult derived stem cells and adult derived inducible pluripotent stem cells have been used for kidney recellularization (see **Table 13**).

Evaluation of the seeded scaffold is often assessed via the presence of cell differentiation by immunostaining or histology,[215a,216b,219a,225,238] as well as, via TEM and SEM.[215b,219a] Moreover, the secretory function of the kidney is evaluated by different valuable indicators.[219a,237] Finally, investigations on the long-term

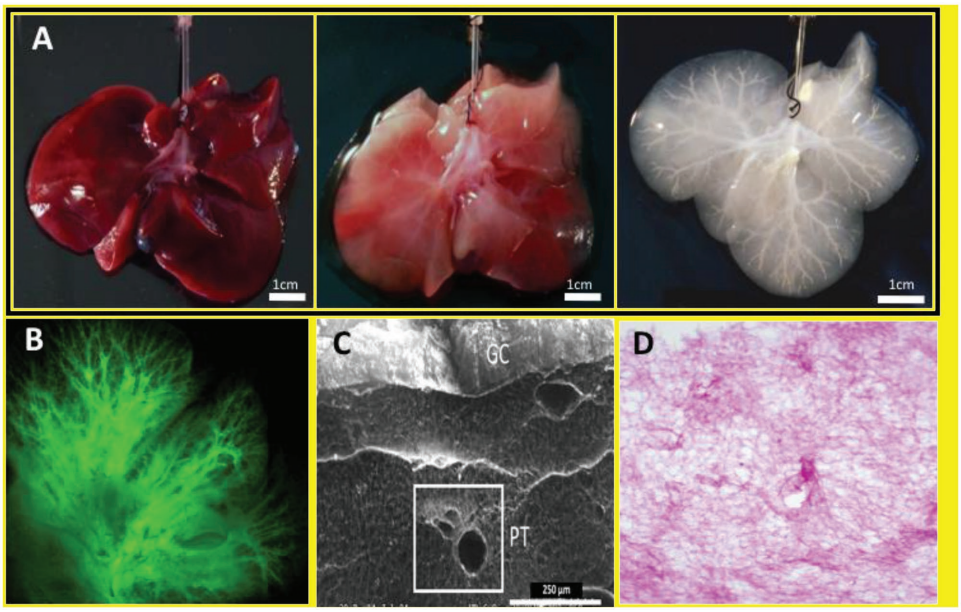

Figure 9. A) Macroscopic view of a ferret liver at 0, 20, and 120 min during the decellularization process. B) Decellularized mouse liver visualized under fluorescence microscopy revealing the native vascular tree of the liver after FITC-dextran injection through the portal vein. C) Scanning electron microscopy picture showing the Glisson's Capsule (GC) and portal triad (PT). D) Hematoxylin and eosin staining showing complete absence of cellular elements. Adapted with permission.[229] Copyright 2011, the American Association for the Study of Liver Diseases.

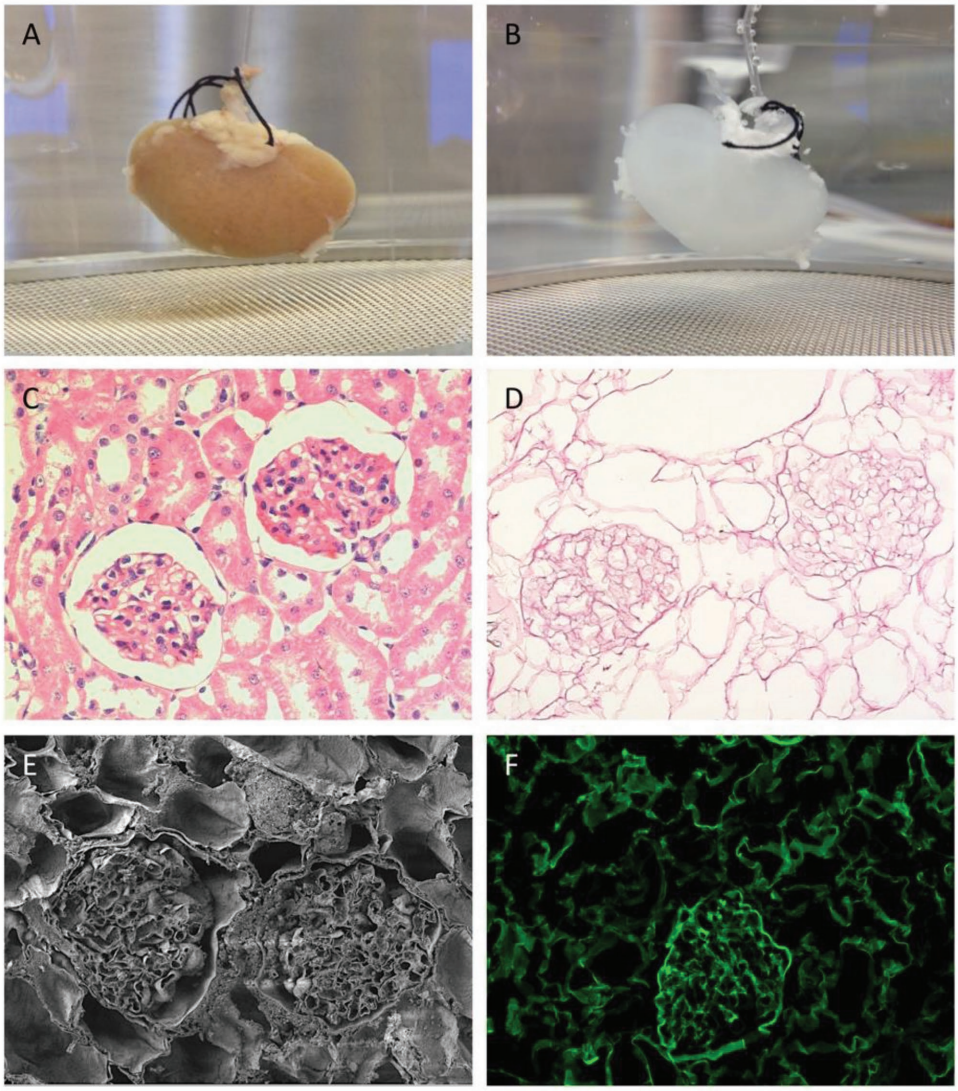

Figure 10. Rat kidney scaffold characterization. Representative photographs of rat kidney A) before and B) after decellularization showing that after detergent perfusion the scaffold retains the shape and macroscopic structure of native kidney. Hematoxilin and eosin images of C) native and D) decellularized kidney demonstrate the absence of any cellular content and the integrity of glomerular and tubular structures within the scaffold. E) SEM analysis and F) collagen IV immunofluorescence analysis demonstrate the preservation of ECM ultrastructure and composition.

Table 13. Protocols for kidney recellularization.

Type cells	Infusion method	Via	Cell number	Flow rate	Time	Reference
hPTEC	Pulsatile perfusion	RA	40×10^6	4 mL min^{-1}	7 d	[215b]
Primary renal cells	Continuous	RA	50×10^6	1.5 mL min^{-1}	7 d	[216a]
	Multiple injections	Cortical region	400×10^6	10 mL min^{-1}	28 d	[237]
Mesenchymal stromal cells	Continuous	RA	50×10^6	1.5 mL min^{-1}	7 d	[216a]
Neonatal kidney cells	Continuous	U	60×10^6	1.5 mL min^{-1}	5 d	[219a]
HUVEC	Continuous	RA	50×10^6	1.5 mL min^{-1}	5 d	[237]
iPSC	Pulsatile perfusion	RA	5×10^6	4 mL min^{-1}	7 d	[238]
ESC	Direct injection	RA, U	2×10^6	Static condition	10 d	[215a,216b,218a]
	Multistep	RA, U, V	15×10^6	0.4 mL min^{-1}	72 h	
	Continuous	RA	12×10^6	1.2 mL min^{-1}	72 h	

RA: renal artery; U: ureter; V: vein.

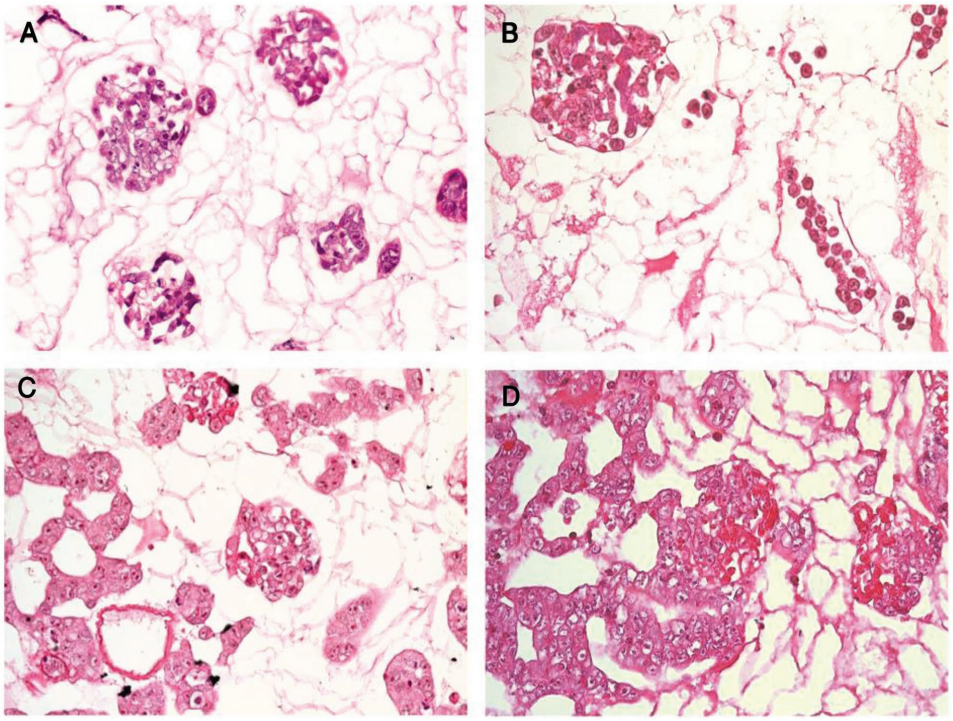

Figure 11. Rat kidney scaffold recellularization strategies. Hematoxilin and eosin images of kidney scaffold seeded A) via the renal artery, B) via renal artery and ureter, C) via renal artery, renal vein, and ureter, and D) via renal artery by high pressure and flow rate.

viability and functionality of the kidney should are done by analyzing the morphology and function after orthotopic scaffold implantation for a prolonged period of time.

For the cell seeding, kidney scaffolds can be perfused via renal artery, renal vein, ureter and for direct injection through the renal capsule.[215a,216b,218a,219a,237] For example, Ross et al.,[215a] manually perfused murine ESCs through the renal artery and the ureter observing that infused cells repopulated mainly small vessels, arterioles and glomeruli while retrograde injection of cells via the ureter ends to uneven cellular distribution with few cells reaching peritubular capillaries. Song et al.,[219a] injected neonatal kidney cells through the ureter and applied a negative pressure gradient to the organ chamber. This approach improved cell distribution throughout the kidney parenchyma. Recently, some of the authors of this review compared the potential of different recellularization procedures with mESCs (**Figure 11**).[218a] In addition to infusion from the renal artery and ureter, the effect of cell infusion into the renal vein to reach the venous circulation was evaluated. Combination of the three infusion routes (renal artery, vein and ureter) achieved an improved cellular delivery, however, the repopulation was limited to focal areas in the cortex and in the medullary volume. Perfusion of cells through the renal artery with high flow pressure resulted in better scaffold repopulation due to translocation of seeded cells out of the arterial circulation into peri-tubular space, but it did not result in a complete repopulation of the entire scaffold.[215b,218a] An alternative protocol for repopulation is the direct injection through the renal capsule using a needle.[237] This protocol could be useful for better repopulation of renal parenchyma, but it may cause damage to the renal tissue.

Besides obtaining uniform and efficient cell seeding, it is also very important to infuse in the scaffold a sufficient cell number. For example, the number of cells required for a rat kidney is in the order of a billion of cells, while, so far, the cell

Table 14. Protocols for liver recellularization.

Cell Type	Infusion method	Via	Cell number	Flow rate	Time	Reference
hUVEC hFetal Liver Cells (hFLC) MS1Endothelial cells	Continuous	PV IVC, PV, IVC+PV	30×10^6 70×10^6 100×10^6	3 mL $min^{-1} \rightarrow$ 0.5 mL min^{-1} 5 mL min^{-1}	7 d 3 d	[229]
Rat Hep Endothelial cells	Multistep	PV	200×10^6	15 mL min^{-1}	5 d 5 d	[242]
Rat Hep	Direct injection Continuous Multistep	PV	$10–50 \times 10^6$	2 mL min^{-1}	7 d	[230]
Pig Hep	Multistep	PV	$10^{[9]}$	4 mL min^{-1}	7 d	[243]
HepG2 hUVEC Neonatal liver slurry	Multistep	Bile duct PV	$100–200 \times 10^6$	25 mL min^{-1}	7 d	[244]

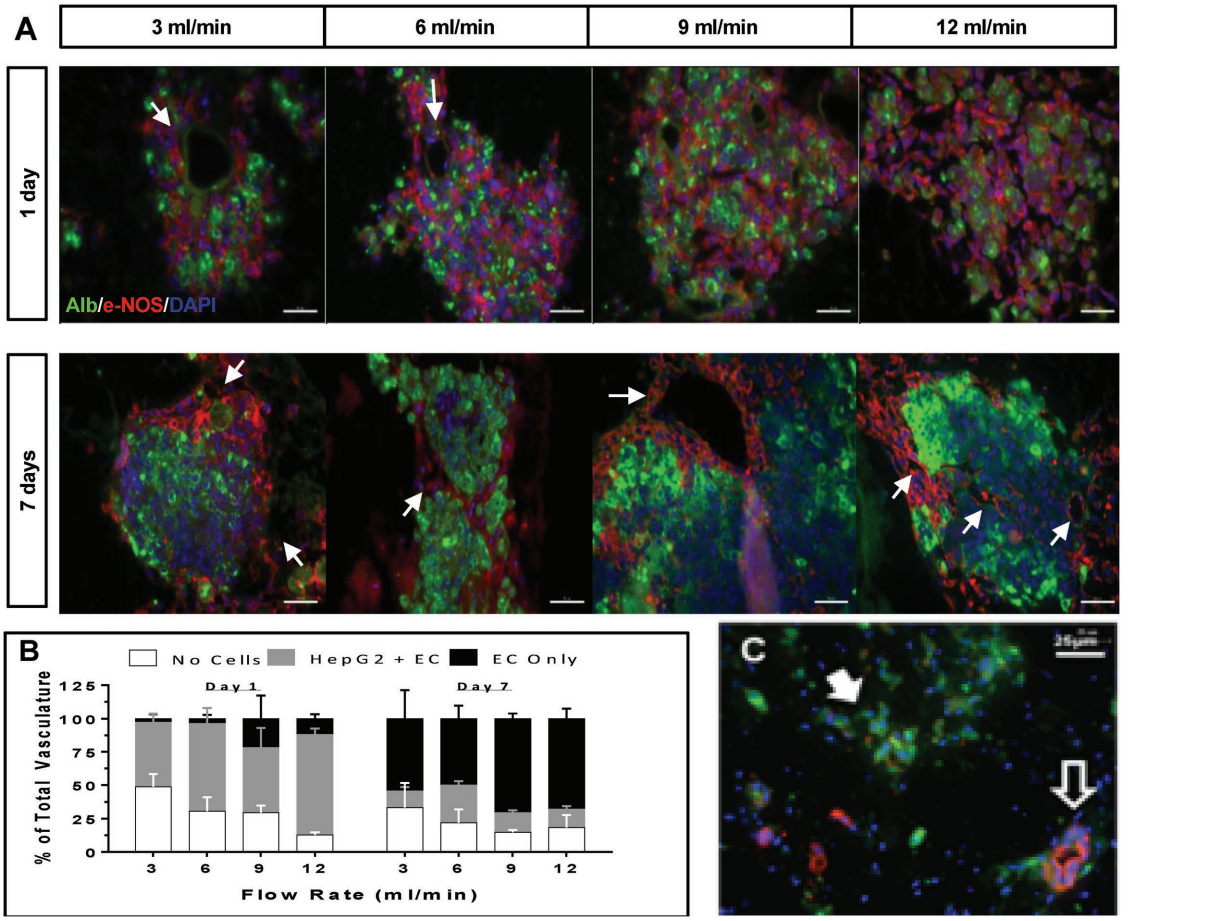

Figure 12. A) Representative images of albumin staining of HepG2 cells (green) and eNOS staining of endothelial cells (red) from days 1 (top) and 7 (bottom), at the indicated flow rates (white arrows point to vascular structures). B) Analysis of cell types (EC+HepG2, EC only, and no cells) covering the vascular structures at 3, 6, 9, and 12 mL min^{-1}, 1 and 7 d post cell seeding, showing better cell distribution and more efficient revascularization at a precise flow rate, 9 mL min^{-1}. C) Liver scaffold recellularized with human fetal liver cells showing parallel formation/differentiation of liver hepatoblasts into hepatocyte clusters expressing albumin (green) and biliary ductular structures expressing cytokeratin 19 (red). (A,B) Reproduced with permission.[241] Copyright 2016, Mary Ann Liebert, Inc. (C) Reproduced with permission.[229] Copyright 2011, the American Association for the Study of Liver Diseases.

proliferation after seeding is not sustained for a long time and only millions of cells (up to 40) have been achieved.[218a,237] Since the cell number for recellularization of a human kidney is estimated to be at least 300 billion cells, it is obvious that new methods are required for obtaining this massive cell population in vitro.

5.3. Liver Recellularization

In the case of liver recellularization, different cell seeding methods have been developed (see overview of **Table 14**) and in vitro growth of multiple liver cell types maintaining its functionality have been achieved (typical example given in **Figure 12**).[239] However, the current knowledge and technology is still limited, making the transplants viable for a few hours or at best a few days, due to blood clotting and poor revascularisation.[240] Cell seeding via continuous perfusion can deliver large cell quantities into the whole scaffold. However, long perfusion times may potentially damage the scaffold due to continuous exposure to elevated shear during the seeding.[229,241] Besides, one can obtain good cell engraftment when multistep cell infusion is followed by continuous perfusion of media.[230] Nevertheless, the efficiency at generating a functional vascular network has been similar, whatever the cell seeding method adopted.[232a] Furthermore, certain physiologic mechanisms of vascular response and adaptation to shear stress, like NO and prostacyclin secretion, seem to also play a part in the efficiency of the revascularization process of liver scaffolds (Figure 12).

5.4. Outlook and Perspectives of Organ Decellularization/Recellularization

Kidney and liver regeneration based on decellularized scaffolds could be a very attractive technology for replacement of renal and liver function in patients. However, we are still in the early phases of the development there and their clinical

implementation for blood purification is rather far in the future. In particular, the recellularization phase is still quite challenging and there is need for comprehensive cell seeding methods for enabling the homogenous recellularization of the whole scaffold volume and the creation of a vascular network. Nevertheless, currently recellularized renal and liver scaffolds can be used for the development of cellular assays for drug screening conditions.[245]

6. Future Perspectives

Although significant progress has been made in the development of bioengineered kidney and liver systems, several challenges still remain. The clinical translation of in vivo systems, as BAK or BAL, in preclinical tests is a real hurdle. The scaling up is an important issue which actually stopped many devices in the preclinical phase. Another important challenge is fulfilling several requirements and criteria, including detailed trial design, patient selection and diagnosis, as well as achieving Good Manufacturing Practices concerning the development and production of the components of the devices (biomaterials, allogeneic cell source, etc.). In fact, it is very important that the developers of bioengineered organs are aware about the all the above early on their research and be in continuous contact with the regulatory agencies to minimise potential hurdles early in the development process.

Besides the approaches presented here, there are other emerging ones, which in the future could potentially contribute to the development of bioengineered kidney and liver organs. One of the most promising is the development of organoids which are the self-organizing 3D in vitro cell cultures consisting of multiple cell types aiming to resemble in vivo organ structure and function.[246] Human adult stem cell-derived organoids have already been developed for many organs, including kidney, liver, the small intestine and colon, stomach, pancreas, prostate, fallopian tube, and taste buds,[247] and have been used for disease modeling, drug testing, and therapeutic application.[247b,248] Because of their autologous and easily expandable nature, they could constitute source of cells for renal and hepatic replacement strategies, including bioartificial (BAK, BAL) and decellularized organs.[249]

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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