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REVIEW ARTICLE

Hydrogel-Based Cell Therapies for Kidney Regeneration: Current Trends in Biofabrication and *In Vivo* Repair

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Abstract: Facing the problems of limited renal regeneration capacity and the persistent shortage of donor kidneys, dialysis remains the only treatment option for many end-stage renal disease patients. Unfortunately, dialysis is only a medium-term solution because large and protein-bound uremic solutes are not efficiently cleared from the body and lead to disease progression over time. Current strategies for improved renal replacement therapies (RRTs) range from whole organ engineering to biofabrication of renal assist devices and biological injectables for *in vivo* regeneration. Notably, all approaches coincide with the incorporation of cellular components and biomimetic micro-environments. Concerning the latter, hydrogels form promising materials as scaffolds and cell carrier systems due to the demonstrated biocompatibility of most natural hydrogels, tunable biochemical and mechanical properties, and various application possibilities. In this review, the potential of hydrogel-based cell therapies for kidney regeneration is discussed. First, we provide an overview of current trends in the development of RRTs and *in vivo* regeneration options, before examining the possible roles of hydrogels within these fields. We discuss major application-specific hydrogel design criteria and, subsequently, assess the potential of emergent biofabrication technologies, such as micromolding, microfluidics and electrodeposition for the development of new RRTs and injectable stem cell therapies.

Keywords: Proximal tubules, uremic toxin secretion, renal assist devices, injectable formulations, hydrogels, stem cells.

1. INTRODUCTION

CURRENT STATUS OF RENAL REPLACEMENT THERAPIES

1.1. The Limitations of Current Treatment Options

Nowadays, one in ten persons are estimated to have impaired renal function. In 10-20% of these cases, the gradual loss of renal function becomes progressive and chronic kidney disease (CKD) manifests in clinically advanced stages [1]. Substantial deterioration in renal function affects up to 30-40% of elderly, who have a high rate of comorbid diseases like diabetes and hypertension, that in turn are risk factors for disease progression [2]. When reaching end-stage renal disease (ESRD), patients depend on life-saving renal replacement therapies (RRTs). Kidney transplantation is the gold standard with the highest potential for increased longevity and quality of life, although acute or chronic transplant rejection is a high risk factor; life-long immunosuppressant therapy to lower this risk is inevitable. Unfortunately, the demand for donor kidneys by far exceeds the available sources, resulting in an average waiting time of three to five years [3]. According to the ERA-EDTA Registry Database, 1041 Europeans per million needed RRT at the end of 2014, but only 36 per million obtained a transplant [4].

The currently available alternative RRT is hemodialysis. Willem Kolff invented the extracorporeal device for blood filtration around 70 years ago, but progress in improving clinical outcomes stagnated during the last decades. Dialysis treatment is time-consuming and intensive, and has been shown to adversely affect all quality of life domains compared to kidney transplantation [5]. Although dialysis allows life sustainment as medium-term solution, metabolic and endocrine functions, electrolyte homeostasis and the regulation of the cardiovascular tone are not remedied at all [6, 7]. Moreover, excretion of waste products is only partly reached; the insufficient blood clearance capacity of larger and protein-bound solutes can degenerate into the uremic syndrome, in which accumulating solutes (*i.e.* uremic toxins) damage various organ systems [8-10]. As a consequence, 5-year survival rate for ESRD patients obtaining dialysis is only 36% compared to 86% after kidney transplantation [7]. Obviously, essential biological functions of the kidney cannot solely be replaced by current medical devices.

1.2. The Limitations of Kidney Regeneration

In comparison to organs like liver or skin, the mammalian kidney has a very limited regenerative capacity [11, 12]. Acute injury, especially in the proximal tubules, can be reversed *via* cellular repair processes involving dedifferentiation, migration and redifferentiation of remnant healthy tubular cells [11-15]. However, these repair processes can merely modify structure and function of existing nephrons; nephrogenesis has never been observed in mammals after completion of organogenesis around birth [14]. Moreover, the existence and contribution of endogenous stem or progenitor cells in adults are still under debate [14, 16, 17]. It is evident, however, that severe or repetitive injury exceeds the capabilities of renal repair processes. Chronic damage consequently results in maladaptive repair responses, including tubular hypertrophy, scarring and interstitial fibrosis, with irreversible loss of function [11, 17, 18].

While the role of adult renal stem cells *in situ* is rather controversial, new insights into embryonic development have led to a range of protocols for directed differentiation of embryonic or induced pluripotent stem cells (iPSCs) towards kidney lineages *in vitro* [19, 20]. Additionally, some research groups were able to create human kidney organoids that mimic embryonic development, including the formation of nephron structures [21-23]. This development forms an exciting basis for personalized cellular therapeutic applications. Moreover, increasing evidence suggests nephroprotective effects of injected stem cells in acute renal injury models, mainly *via* paracrine effects of renal trophic factors [24-27]. How-

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ever, major safety issues remain due to the risk of uncontrolled biodistribution of injected cells and possible tumorigenicity. The former could be tackled with improved delivery systems, but it remains to be evaluated whether therapeutic benefits can outweigh the risk of tumorigenesis [28].

1.3. The Intersection of RRT and Renal Regenerative Medicine

The progress in stem cell biology and concurrent advances in tissue engineering and biotechnology have created an intersection between RRTs and regenerative medicine, which opens up new possibilities to approach ESRD. The following paragraph provides an overview of emerging RRT strategies, which we summarized into three groups: (a) whole kidney engineering, (b) biofabrication of renal assist devices (RADs), and (c) *in vivo* regeneration (Fig. **1**). Within these strategic lines, hydrogels can be applied as an overarching biomaterial with different, application-specific functions, as outlined in paragraph 3. The fourth paragraph explores in more detail possible applications and associated requirements for hydrogels in the concept of regenerative RRT. In the last paragraph, we give an overview of various biofabrication techniques for hydrogel processing, and assess their potential for RRT development.

2. CURRENT TRENDS IN RENAL REPLACEMENT THERAPIES

2.1. Whole Kidney Engineering

2.1.1. Holy Grail of Kidney Engineering

As the structure of an organ is inherent to its function, a lab-grown copy of a kidney with its intact intricate architecture and function is considered the holy grail in kidney engineering. However, the required resolution and cellular complexity is currently far beyond our reach. Enabling technologies like micro-patterned scaffolding, advanced microfluidics, 3D bioprinting and sophisticated bioreactors allow for more defined cell deposition, tissue structure and function, and promise great advances in the future [29]. Nonetheless, on the short term, these techniques will mainly revolutionize engineering of less complicated structures. To date, most advances have been made in engineering bladder, skin, cartilage and bone tissues [30-33]. To recapitulate the kidney, with its fine vascularization, the cortico-medullary axis and more than 20 highly differentiated cell types, remains a supreme discipline.

2.1.2. Stem Cell-Derived Kidney Organoids

Yet, whole kidney engineering is being approached with remarkable progress. Aforementioned organoids can be built from scratch by self-organizing iPSCs, which closely resemble human embryonic kidney tissue in the first trimester [21-23,34]. When cultured on vascular membranes in chicken eggs, engineered kidneys from murine renogenic stem cells have been shown to attract exogenous blood vessel branches for blood supply and formation of glomerulus-like structures [34]. Unfortunately, these mini-kidneys are not on a desired scale for humans and lack a single coherent collecting duct system for concentrating function and urine drainage; therefore, clinical usage remains a distant goal.

2.1.3. Xenoembryos and Blastocyst Complementation

Two other interesting approaches to engineer whole kidneys bottom-up are xenoembryos and blastocyst complementation. Both techniques use the developing embryo of another species as natural niche for *de novo* organogenesis. The patient's stem cells are injected into the xenoembryo or blastocyst to borrow the signals for stem cell direction and differentiation, before the developing organ is transplanted back into the patient [35]. Despite some positive results, ethical feasibility of these techniques is challenging and hampers their progression into clinically relevant research phases.

2.1.4. Decellularized Kidneys

In 2008, Ott *et al.* succeeded in removing all cellular materials from a murine heart to obtain a native scaffold with preserved macro- and micro-architecture that could be repopulated with another cell source [36]. Over the last decade, detergent-based decellularization techniques have been further developed and extended to other organs, including the kidney [37]. Decellularized rat kidneys could be recellularized with renal progenitor and endothelial cells, and were reported to produce rudimentary urine *in vitro* as well as *in vivo* after orthotopic transplantation [38]. However, 'decell-recell technology' is currently limited to a few cell types and is dependent on perfusion. Precise and complete scaffold repopulation with this technique has not yet been achieved. Up-scaling for human purposes will pose a challenge and advanced bioreactors will be needed. Therefore, this method is not expected to enter clinical trials in the near future.

Nevertheless, decellularized kidney scaffolds create a niche for stem cell differentiation, analogous to the embryogenic environments in aforementioned whole kidney engineering approaches. In fact, tissue architecture is not only a consequence, but also a cause for tissue development, differentiation, homeostasis and remodeling [39]. The extracellular matrix (ECM) is a complex and highly charged network composed of collagen, elastin, laminin and glycoproteins that provide a 3D structure for spatial organization of cells. Moreover, it acts as growth factor reservoir and signal transduction pathway regulator. Thereby, it modulates cell shape, gene expression, and protein levels and distribution, and it actively contributes

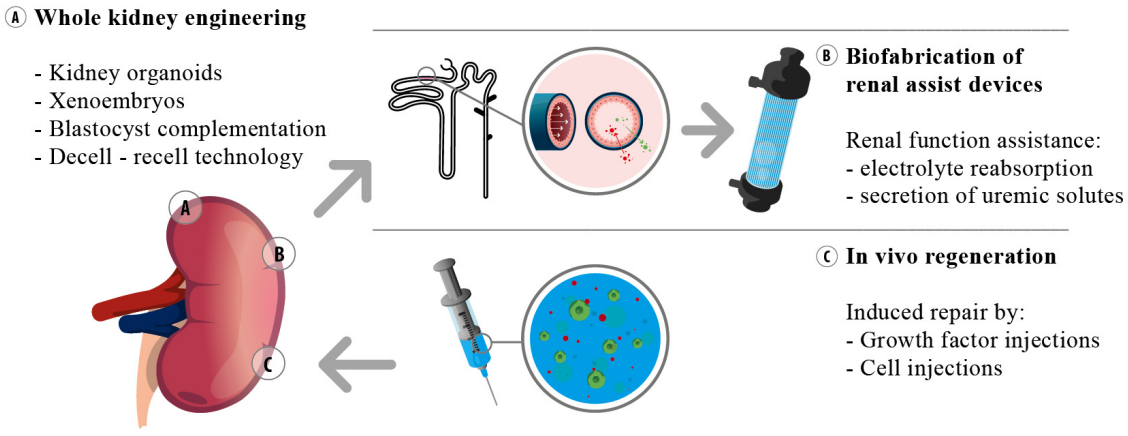

Fig. (1). Schematic overview of current strategies for renal replacement therapies development. (**A**) Whole kidney engineering aims for a lab-grown replication of the organ as transplant. (**B**) Renal assist devices are biotechnological approaches to complement conventional dialysis, with extracorporeal and implantable applications. (**C**) Biological injections of therapeutics promote *in vivo* regeneration *via* direct growth factor delivery and/or paracrine cellular effects.

to cellular survival, proliferation, migration, adhesion and differentiation behavior. Decellularized kidneys preserve the architecture of the ECM, including cell–ECM binding domains, which are tissue-specific and critical in promoting cell attachment, migration and proliferation [37]. Therefore, native ECM provides an ideal platform for kidney engineering. This topic will be discussed further in 3.2.1.

2.2. Biofabrication of Renal Assist Devices

In contrast to engineered whole kidneys, so called renal assist devices (RADs) are currently much closer to the bedside. In 2004, the RAD developed by Humes *et al*. was the first and to date only one to enter a clinical trial [40-42]. Jansen *et al.* gave a historical overview on the development of RADs that represent a more biotechnological approach to replace essential renal functions [6].The concept of RADs has not essentially changed since conceptualization by Aebischer *et al.* in 1987 [43]. Synthetic hemofiltration cartridges are supported in series by a bioreactor unit containing renal cells to excrete uremic toxins while reabsorbing water and salts, and exerting other favorable metabolic functions, such as glutathione metabolism and vitamin D activation [44]. The clinical phase I/II trial with RADs in an *ex vivo* setting in patients with acute tubular necrosis indicated cell viability and metabolic performance, but also reported events of hypoglycemia, thrombocytopenia and hypotension [40-42]. In 2008, a phase II multicenter, randomized, controlled trial suggested a 50% reduction in mortality risk [45]. However, this study has been criticized among others for being underpowered and was terminated prematurely because a significant decrease in mortality was also observed in the sham group [46]. However, the concept of RADs is still promising and therefore implemented correspondingly in several research groups. More diverse is the quest for a bioactive membrane with optimal ECM coating for tubular cell attachment, and formation and maintenance of differentiated epithelia. Zhang *et al.* systematically tested coatings with different ECM components and demonstrated that laminin and collagen IV are most suitable for renal cell culture in RADs [47]. This method could be improved further by double-coating with L-DOPA, and led to upgraded RADs with proven active toxin uptake and secretion *in vitro* [48, 49].

RADs mimic the relevant functions of proximal tubules to complement hemodialysis, mostly by using primary renal cells, cell lines like HK-2 and ciPTEC, or differentiated renal progenitor cells with expression of kidney-specific membrane transporters [6]. Other renal structures, such as glomeruli, microvasculature and the collecting duct system, will rather make progress as part of whole organ engineering or in *in vivo* regeneration. Noteworthy, the presence of other cell types, such as distal tubule cells, is suggested to enhance proximal tubule integrity and function [50]. Furthermore, in noncontact co-culture, endothelial cells altered expression of 99 genes in proximal tubule cells and significantly improved monolayer integrity [51]. Thus, communication between different cell types could improve RAD performance. In addition, conventional hemodialysis is being improved by advanced filter membrane technologies enabling implanted or wearable therapy; examples are the silicon nanopore membrane developed by Kim *et al.*, the wearable dialysis device by Gura *et al.* that has recently entered a clinical phase I trial, and the wearable bioartificial renal epithelial cell system (BRECS) by the Humes research group [41, 52, 53].

2.3. *In vivo* Regeneration

Although limited, the kidney has an inherent regeneration capacity after mild and acute renal damage. Growth factors like epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin-like growth factor 1 and transforming growth factor beta (TGF-β) are up-regulated in injured kidneys and are suggested to stimulate tubular cell proliferation and, thereby, tissue repair [54]. Moreover, injected mesenchymal stem cells have been shown to exert regenerative effects, mainly through paracrine stimulation of antioxidative, antiapoptotic, proliferative, differentiative, immunomodulatory and antifibrotic mechanisms [55]. Therapeutic growth factor or stem cell delivery to the injured site could potentiate cell repair and *in vivo* renal regeneration. However, systemic off-target effects of soluble factors hamper their injection, and injected cells quickly vanish from the site of injection [56, 57]. Hence, the development of novel therapies should focus on effective delivery systems, *e.g.* hydrogel carrier systems, which will be further discussed in 3.1.3.

2.4. Future Perspectives and the Possible Role of Hydrogels

Whole organ engineering, biofabrication of RADs and stem cell- or growth factor-mediated *in vivo* regeneration are three completely different strategies to replace or regenerate kidney function. However, all approaches require scaffold or carrier systems that promote and maintain cellular organization, differentiation and function. The most promising results have been obtained with physiological or mimetic micro- and macro-environments, such as embryonic signaling environments, decellularized kidneys, tubular structures with ECM coatings, or paracrine signaling *in situ*. In this regard, hydrogels are interesting biomaterials capable of supporting all three strategies. In the next paragraph, we introduce the concept of hydrogels and the perspectives within the fields of RRT and *in vivo* kidney regeneration. Although all abovementioned approaches merit scientific investigation, we consider biofabrication of RADs and *in vivo* regeneration most relevant for imminent translation from bench to bedside and therefore focus on hydrogel applications within these two strategies.

3. HYDROGELS AS POTENTIAL MATERIALS IN RENAL REPLACEMENT THERAPIES

3.1. Hydrogels

3.1.1. Historic Perspective and Classifications

Hydrogels are highly hydrophilic polymeric materials that swell in contact with water [58]. They have been studied extensively over the past 50 years as biomaterials in a multitude of applications, *e.g.* as contact lenses or injectable drug release depots [59, 60]. Usually, hydrogels are classified into three categories: synthetic, natural and hybrid, depending on the source of the polymers. Within the natural category, a subdivision between human endogenous (*e.g.* heparin) and non-human endogenous biopolymers (*e.g.* chitosan or dextran) can be made. Some of these biopolymers can form hydrogels as such, but most need to be chemically modified in order to facilitate hydrogel formation. A myriad of approaches to form hydrogels from a wide variety of biopolymers have been reported [61]. Hydrogels are formed by crosslinks between polymer chains based on either non-covalent (*i.e.* physical) or covalent (*i.e.* chemical) binding, or a combination of both types [62-65].

3.1.2. Hydrogels as Scaffolds for Tissue Engineering

In recent decades, hydrogels are being investigated as scaffold biomaterials in tissue engineering for several reasons. First and foremost, the materials resemble natural soft tissue due to their high water content contributing to biocompatible properties [66]. An important caveat here is the possibility of complement activation by some hydrogels containing hydroxyl groups [67, 68]. The high porosity of the polymer network is beneficial for a continuous exchange of nutrients, gasses, waste products and signaling molecules with cells embedded in or grown on the hydrogel. Many biopolymers are enzymatically degradable *in vivo,* with most retaining these properties even when chemically altered and crosslinked to produce a hydrogel network [61, 69]. Synthetic hydrogels can be modified to slowly hydrolyze under physiological conditions. Thereby, cells can actively reshape their surroundings, while low molecular weight waste products are safely removed from the body [70]. The nature of the targeted cell environment can be mimicked

further through utilizing ECM-based hydrogels or by incorporating biologically relevant cues, *e.g.* tissue-specific growth factors [71, 72].

Due to their polymeric nature, hydrogels are highly tunable in terms of mechanical properties. By changing system parameters like crosslinking density or polymer concentration, different mechanical moduli, *e.g.* stiffness, can be adjusted to better represent a specific type of tissue [73, 74]. The strategy of crosslinking reactive hydrophilic polymer solutions into hydrogels allows formation into different geometries, *e.g.* microspheres, tubes or nanofibers. Moreover, composites with other hydrogels or non-hydrogel materials can be used either as *de novo* scaffolds or to reinforce existing scaffolds.

3.1.3. Hydrogels as Cell Carrier Systems for In Vivo Regeneration

Due to the versatility of hydrogels, they can be used as carrier systems for controlled delivery of drugs, microparticles, exosomes or growth factor producing cells [72]. Release rate of these bioactive molecules is predominantly determined by the hydrodynamic size of the encapsulated molecule, pore size, degradation rate, and electrostatic interactions [71, 75]. For encapsulation into a hydrogel, cells and/or growth factors are suspended in the preferred medium together with a known concentration of hydrogel precursors. Crosslinking can be induced through a myriad of cell-friendly methods, including temperature triggered hydrogen bonding, initiation of covalent crosslinking through free radicals produced by (photo-)initiator molecules, or complexation with divalent salt ions like calcium [76]. With a crosslinked network established around the cells, the fabricated scaffold can be implanted or otherwise utilized. In general, the polymer network will be slowly degraded mainly *via* hydrolytic or enzymatic degradation, while the cells can divide, differentiate and produce a microenvironment that corresponds to their native tissue. Eventually, the initial hydrogel network will be completely degraded and replaced by native ECM (Fig. **2**).

4. DESIGN CRITERIA FOR RENAL REPLACEMENT THERAPIES

4.1. Extracellular Matrix-Mimetic Scaffold Composition

The high heterogeneity of the kidney is reflected in varying ECM compositions within the organ [77, 78]. O'Neill *et al.* showed that kidney-derived stem cells recognize organ-specific ECM, and that ECM from renal cortex, medulla or papilla modulate cell proliferation and metabolic activity in a region-specific manner. Disruption of the ECM ultra-structure did not diminish these effects, suggesting that cells process these ECM cues based on ECM composition rather than structure [79]. Moreover, a recent study indicated that adhesive ligand density plays an important role in maintenance of an epithelial phenotype with epithelial-to-mesenchymal-like transition at lower densities [80]. Even along the nephron, varying basement membrane compositions and selective ECM receptor distribution have been described. For instance, laminin expression is approximately 50% higher and integrin α-subunits are differently expressed in proximal tubules compared to distal tubules [81, 82]. However, it remains elusive to what extent these differences contribute to cell state and disease development.

In addition to biochemical cues from specific ECM compositions, also biophysical ECM properties play a key role in controlling cell fate. Collagen and elastin concentrations determine stiffness and elasticity of a tissue, which can range from soft brain tissue (Young's Modulus E ≈ 0.1-1 kPa) to rigid tissues like bone (E ≈ 1 GPa) [83]. Young's modulus in the kidney approximates 4.5 kPa, although Bensamoun *et al*. observed a spatial distribution of stiffness that was slightly increased in the renal sinus (E ≈ 6.8 kPa) compared to the cortex (E ≈ 4.3 kPa) [84]. Several cell functions are influenced by ECM stiffness, such as cell migration and stem cell lineage specification. Cells feel tissue rigidity by applying traction forces through integrin-based focal adhesions, which guide a migration process called 'durotaxis' [85]. Furthermore, mesenchymal stem cells have been shown to commit to a lineage based on matrix elasticity in long-term culture, which is only in the initial culture phase reversible by growth factors [83]. Also, maintenance of a differentiated phenotype is dictated by amongst others ECM elasticity. For instance, valve interstitial cells cultured on hydrogels with physiological elasticity preserved a quiescent phenotype, whereas stiff materials induced pro-fibrogenic gene expression through PI3K/AKT signaling [86]. Notably, the addition of growth factors to the hydrogel, in this case TGFβ1, could also activate the quiescent phenotype [87].

Since ECM-cell interactions play a key role in tissue development and maintenance, hydrogels with ECM-mimetic and bioactive properties are generally favorable, such as collagen or hyaluronic

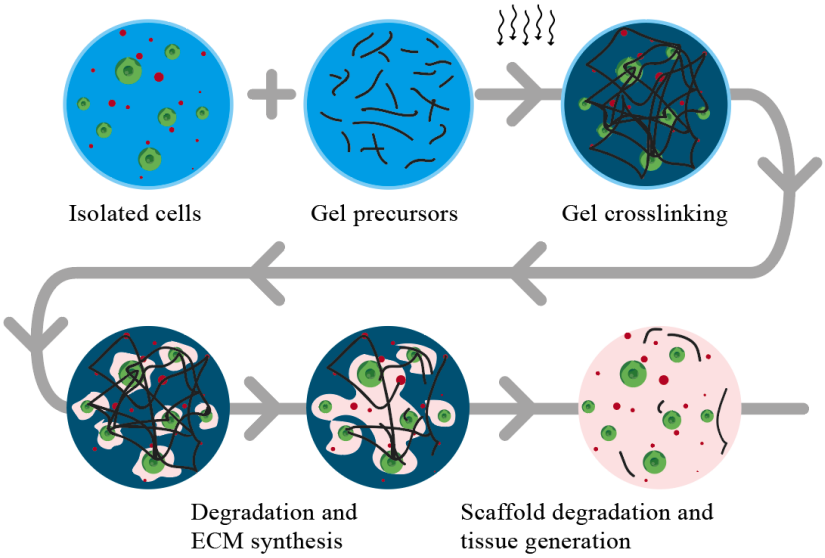

Fig. (2). Degradable hydrogels for biomedical applications. Cell-laden hydrogels can be prepared from isolated cells and/or growth factors plus gel precursors in the preferred medium. By crosslinking the reactive hydrophilic polymer (gel precursors), a certain 3D structure can be obtained. This scaffold can slowly degrade *via* hydrolysis or enzymatic degradation, while cells produce the surrounding ECM until a native-like tissue is generated.

acid-based hydrogels, which are made from an endogenous ECM components and, therefore, inherently bioactive and biocompatible with cells and host. However, considering the unique nature and immense complexity of tissue-specific ECM, establishing and prioritizing design criteria are crucial steps to success, which depend on the specific research objective. For accurate mimicry of a physiological setting, hydrogel mixtures with solubilized tissue-specific ECM components appear to hold the greatest potential [79]. It should be noted, however, that the use of biologically derived materials is accompanied by the risk of contamination with residual cellular debris or endotoxins, quick degradation or contraction, and batch-to-batch variabilities [88]. Moreover, hydrogels will have to meet application-specific requirements, such as suitable viscosity, overall charge, biodegradability and mechanical properties.

4.2. Design Criteria for Biofabrication of Renal Assist Devices

RADs make use of proximal tubule cells seeded onto porous membranes for active metabolite removal from the blood into a separate compartment. The latest RAD from the Humes research group comprises porous carbon disks, while others make use of hollow fiber membranes similar to those currently used in dialysis cartridges [41, 44, 89]. For the purpose of blood clearance, tight monolayer formation, well-differentiated cells with clear polarization and expression of various transport proteins, and resistance to fluid shear stress are essential properties of an RAD.

4.2.1. Tight Monolayer Formation and Polarization for Transepithelial Transport

Tube structures confer dual benefits as not only two separate compartments can be formed, but cells are also grown on a curved surface. This is an important parameter for cell attachment and function; a clear barrier formation and transport activity have been obtained with ciPTEC grown on polyether sulfone fibers with an inner diameter of 300 µm [89].

Hydrogels could be advantageous to RADs in two ways. Firstly, a thin ECM-mimetic hydrogel layer on a curved membrane could provide additional mechanical and biochemical cues as described earlier, possibly leading to enhanced cell differentiation and further improved cell organization and functionality (Fig. **3**). The hydrogel could eventually be degraded by the cells and replaced by newly synthesized basement membrane. However, permeance of the hydrogel must be fine-tuned to enable an unimpeded membrane separation performance, *e.g.* for transport of middle molecules like β-microglobulin and phosphate [90]. Moreover, diffusion of albumin must be possible for close delivery of protein-bound uremic solutes to the basolateral site of the cells [89]. Diffusion of macromolecules can be problematic if the size of the molecule is larger than the average mesh size of the hydrogel network [91]. The properties of the hydrogel have to be chosen carefully to allow for sufficient albumin transport in RADs. Kaemmerer *et al.* reported a diffusion retention with a factor of 2.3 compared to water for 70 kDa fluorescein isothiocyanate (FITC)-labelled dextran in 7% gelatin methacrylate [92]; these materials correspond to the size of albumin and the stiffness of kidney tissue, respectively.

The second advantage of hydrogels for RADs is their potential for implantation. Current RADs are designed for extracorporeal use, *i.e.* in series with conventional hemodialysis. Ultimately, we strive for implantable constructs that allow for continuous blood clearance and more independence for the patient. A breakthrough would be a wearable or implantable artificial kidney with an integrated RAD that supersedes conventional dialysis treatment. Fissell and Roy are pioneers in developing such a device, but several technical challenges must be met before entering clinical trials, which are sizing *versus* upscaling of mass transport and dialysate regeneration [93]. Meanwhile, progress in tissue engineering and stem cell biology could lead to kidney tubes consisting of differentiated autologous or human leukocyte antigen (HLA)-matching cells and biocompatible hydrogels; implantable and biocompatible constructs to complement conventional dialysis treatment. Emergent technologies, such as micromolding and microfluidic spinning, enable the production of biological, cell-containing fibers [94-96]. These techniques are still in early stages and do not yet apply to RRTs, but their expected value is discussed in paragraph 5.

4.2.2. Fluid Shear Stress

When we aim for kidney tubes made of hydrogel, the biomaterial itself must be strong enough to create a lumen and to withstand fluid flow. Mechanical strength could be enhanced *via* hydrogel reinforcement with for example micro- or nanofibers [97,98]. Moreover, cell attachment can be promoted through hydrogel components with Arg-Gly-Asp (RGD) motifs such as fibronectin, which serve as recognition site for integrins [99]. Of note, fluid shear stress (FSS) is not an unwanted technical challenge, but can be considered a beneficial factor for tube generation and function. Apical perfusion of cells causes axial FSS, which is sensed by microvilli, glycocalix and primary cilia. Moreover, perfusion of the lumen causes radial stretching of the tubule, which is transduced by integrins, stretch-activated channels and cell-cell contacts. Raghavan *et al.* reviewed the role of these mechanosensors in upholding glomerulotubular balance [31]. FSS around 0.02-0.1 Pa/cm^2 is considered physiologically relevant, with 0.1 Pa/cm^2 resulting in significantly increased membrane transport proteins expression, albumin endocytosis and cytoskeletal reorganization *in vitro* [99-104]

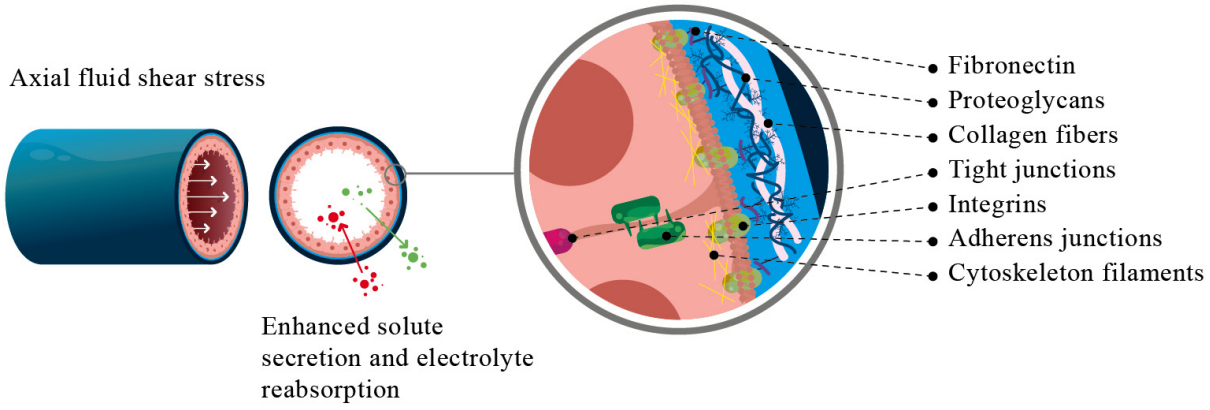

Fig. (3). Hydrogel-based RADs could improve cell function. Mechanical and biochemical cues by various ECM COMPOUNDS are shown to improve cell organization and function; an ECM-mimicking hydrogel layer (depicted in blue) could, therefore, enhance proximal tubule cell characteristics like solute secretion and electrolyte reabsorption. (The color version of the figure is available in the electronic copy of the article).

Increased ion transport and endocytic capacity counteract increasing glomerular filtration rates to maintain a constant reabsorption fraction of approximately 70% of filtered solutes and water [31]. Moreover, FSS-induced cytoskeletal reorganization enables the formation of tight junctions and adherens junctions, indicating enhanced polarization [104-107]. Therefore, hydrogel parameters should be attuned to resist physiological shear stress of around 1 Pa/cm^2 and allow for radial stretching to promote epithelial monolayer formation. Dankers *et al.* emphasized the synergistic effect of bioactive scaffolding and FSS, showing that primary renal tubule cells have improved epithelial-specific gene expression profiles when cultured in a perfusion system on supramolecular electrospun polycaprolactone (PCL) nanofiber meshes with intercalated ECM-peptides [107].

4.3. Design Criteria for *In Vivo* Regeneration

Injections of hydrogel-encapsulated cells into the kidney do not primarily aim for stem cell differentiation into proximal tubule cells in order to replace damaged cells in the affected nephron. Instead, injected stem cells can trigger signaling pathways through paracrine effects to facilitate endogenous repair processes [56]. Similar effects can be achieved with injections of exosomes or specific growth factors like EGF, HGF, insulin-like growth factor 1 and TGF-β, but stem cells potentiate renoprotective effects by generating signaling molecules on a broader range and on a longer term [108]. However, without any carrier system, retention of injected cells in the target tissues is smaller than 10% [109, 110]. Retention can be significantly prolonged *via* formulation of hydrogels as carrier systems: cell-laden microgels of 50-200 µm have already been applied in heart, bone and cartilage regeneration, and a ten-fold increased retention period was observed for cardiac progenitor cells in microcarrier injections in infarcted mouse hearts [111, 112]. Also in RRT, hydrogels could function as a depot to retain stem cells or renal progenitor cells, exosomes and/or growth factors. The encapsulated molecules will be gradually released *via* diffusion or hydrogel degradation, which can be tuned *via* chemical modification of the hydrogel [63]. Meanwhile, stem cells can produce their own micro-environment.

Hydrogels could be introduced as nano- or micro-sized injectables, or as *in situ* gelling materials, injected into the renal artery, the parenchyma or under the renal capsule [113-115]. Of note, renal tubules have a diameter of around 50 µm *in vivo*; obstruction with microgels of the same size should be avoided. To positively modulate kidney regeneration *in vivo*, design criteria for the preferred spatiotemporal drug release should be prioritized. Drug release mainly depends on degradation rate, electrostatic interactions between the predominantly negatively charged ECM materials and cationic growth factors, and the polymer mesh size, which sterically hinders the molecules in the hydrogel. ECM-mimetic scaffolding could also be beneficial for *in vivo* applications, *e.g.* by stimulating the release of the desired cocktail of signaling molecules. An example of such work has been published by Feng *et al.,* who covalently linked C-domain peptides of IGF-1C (a known renal prosurvival factor) to a chitosan based hydrogel matrix in order to create a more favorable environment for adipose derived stem cells. It was shown that this formulation showed higher cell retention and promoted cell survival and angiogenesis when injected intrarenally in a mouse AKI model, leading to improved renal function when compared to injections of cell suspension [116].

5. EMERGENT BIOFABRICATION TECHNOLOGIES AND THEIR POTENTIAL IN RENAL REPLACEMENT THERAPIES

5.1. Current State of the Art and Challenges in Biofabrication

To date, biofabrication has focused mostly on *de novo* creation of 'hard' tissues like bone and cartilage, whereas the kidney is a relatively new subject in this field [117, 118]. Consequently, only

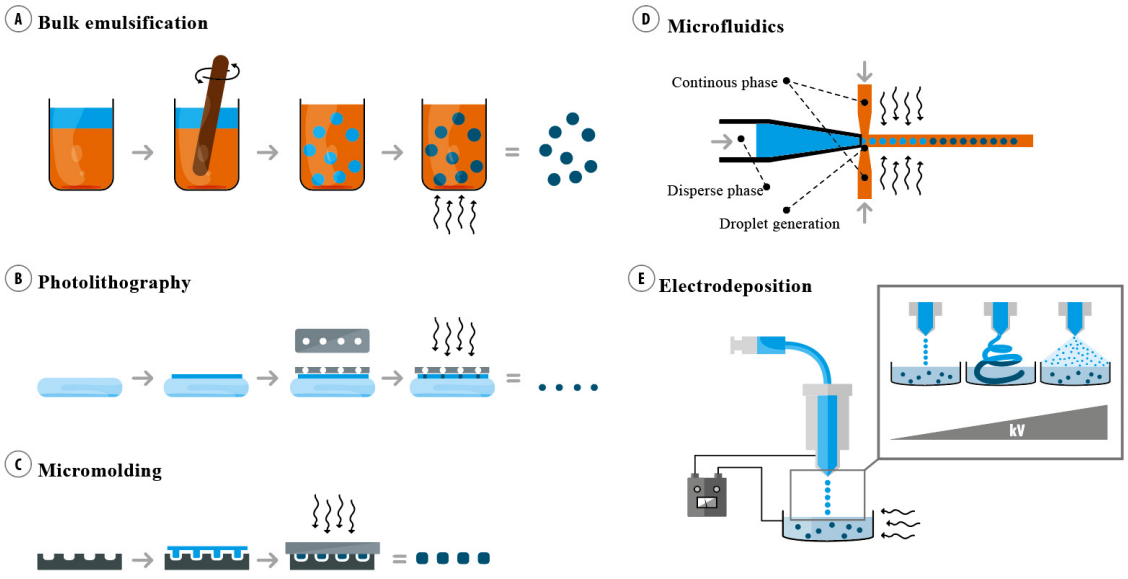

Fig. (4). Schematic overview of biofabrication techniques relevant to RRTs. (**A**) Bulk emulsification: the disperse phase of aqueous reactive polymer (depicted in blue) is stirred into the continuous oil phase (depicted in orange). After crosslinking, the oil phase is washed away with organic solvents. (**B**) Photolithography: the aqueous reactive polymer is spread onto a plate, and a pre-cut photolithographic mask is placed above. After crosslinking, the mask can be removed and the hydrogel particles can be collected. (**C**) Micromolding: a pre-fabricated mold is filled with aqueous reactive polymer. After crosslinking, the mold can be removed and the patterned hydrogels can be collected. (**D**) Microfluidics: a micron-sized channel is used to drip or jet the disperse phase of aqueous reactive polymer into the continuous phase (depicted in orange). After crosslinking, specific hydrogels are formed, *e.g.* micron-sized spheres (depicted in dark blue). (**E**) Electrodeposition: pressure-driven flow of aqueous reactive polymer is pumped through an electrically charged conductive nozzle towards an oppositely charged or grounded collector plate. Depending on the voltage and other factors, the polymer stream can drip, spin or spray from the nozzle to form several types of hydrogel geometries. A bath filled with a continuous phase can be used to collect the polymer for subsequent crosslinking. (The color version of the figure is available in the electronic copy of the article).

Table 1. Overview of current biofabrication techniques for hydrogel-based in renal replacement therapies.

Technology	Advantages	Disadvantages	Possible Geometries of Formed Hydrogels	Typical Dimensions
Bulk emulsification	- fast - low cost - easy to set up - easy to make large amounts of droplets	- deleterious oil phases hamper cell encapsulation - large polydispersity of created droplets	- hollow, solid and multicompartmental hydrogel spheres	~ 50 nm to 1 mm in diameter
Photolithography	- spatial design freedom in both X- and Y-directions - high throughput - low polydispersity - high reproducibility	- no shape control in Z-direction - costly equipment	- any solid shape with at least one straight dimension (*e.g.* cylinders or cubes)	~ 10 nm to several cm in any dimension
Micromolding	- design freedom in any dimension (depending on mold fabrication) - high monodispersity - high reproducibility	- precise mold fabrication in nano- and lower micrometer ranges is expensive	- any	~ 10nm to several cm in any dimension
Microfluidics	- relatively low cost to set up - high monodispersity - high reproducibility	- low throughput unless devices are parallelized - most methods rely on oil as a continuous phase, hampering cell encapsulation	- droplet-based: solid, hollow, multicompartmental and hybrid spheres - coaxial stream-based: solid, tubular, porous, flat, hybrid and grooved fibers	- droplet-based: 2 µm to 800 µm in diameter - coaxial stream-based: 20 µm to 800 µm in diameter and up to meters in length
Electrodeposition:dripping spinning spraying	- relatively easy set-up - large diversity of dimensions possible with a similar set-up - high monodispersity - high reproducibility	- high voltage power (laboratory safety) - low throughput (single nozzle) unless parallelized	- dripping: spheres - spinning: fibers - spraying: spheres	- dripping: 50 to 500 µm in diameter - spinning: 10 to 500 nm in diameter and 10 to 1000 µm in length - spraying: 10 to 500 nm in diameter

few biofabrication studies have been published in the field of RRT. Therefore, we extended this paragraph to studies pertaining to other organs and to articles with a more technical orientation. We discuss bulk emulsification, photolithography, micromolding, biofabrication through microfluidics and several forms of material deposition facilitated by applied electrical fields, *e.g.* electrospinning (Fig. **5**). We have chosen to focus on these techniques because of their direct applicability in RRT. Table **1** provides an overview of these techniques with their technical advantages and disadvantages, as well as the attainable dimensions and possible geometries.

5.2. Bulk Emulsification

Emulsification is certainly the simplest method of obtaining spherically shaped hydrogels. Emulsification requires two liquid phases that are immiscible, *e.g.* aqueous and organic phases. These phases are stirred to form droplets of one phase in the other, the so called disperse phase and continuous phase, respectively (Fig. **4A**). Often, a surfactant is added to stabilize the droplets, *i.e.* to suppress coalescence of droplets and to retard demixing of the two-phase system. These stable droplets can then be utilized as miniature reaction vessels. Reactive hydrophilic polymers can be dissolved in the aqueous phase and crosslinked through a myriad of different chemical reactions or physical linking strategies, such as thermal initiation [119-122], photoinitiation and complexation with divalent ions. Examples of bulk emulsification are found ubiquitously in literature [123]. Protocols have been described for the formation of nano-sized droplets, *e.g.* through sonication or repeated passage through a microfluidizer, and micron-sized droplets, usually through high speed mixing [124-126]. Disadvantages of the bulk emulsification method are the rather broad size distribution when compared to more sophisticated techniques (*e.g.* microfluidic fabrication) and the limitation to spherical shapes. Furthermore, the use of oil and surfactants necessitate the use of organic solvents or additional purification steps that are not biocompatible [127, 128]. This purification step makes the encapsulation of biological molecules or cells problematic due to the risk of denaturation and cell death. Therefore, the therapeutic use of spherical gels obtained from bulk emulsification is generally limited to cell adhesion on the surface of purified microgels, *i.e.* as a microcarrier system [112, 129, 130]. An exception in terms of biocompatibility are microgels that gelate through cell-friendly complexation reactions, *e.g.* alginate, which allow the encapsulation of living cells [131]. A downside to the use of alginate is its limited degradability *in vivo* [122]. In short, applicability of emulsification is currently hampered by the relatively high polydispersity and the limited biocompatibility of oils and organic solvents used in purification.

5.3. Photolithography

Photolithography was originally developed as a means of scaling down the size of transistors on microchips [132]. In the past decade, it has found direct use in the formation of sub-micron- to millimeter-sized hydrogels. Photolithography employs a light impenetrable mask that is pre-cut into the desired shapes of the hydrogel. This mask is then suspended above a solution of reactive polymer and photoinitiator (Fig. **4B**). Next, UV light is applied from a lamp above the mask, resulting in hydrogels with a precisely controlled shape. Photolithography has been shown to form hydrogels with a great variety of shapes, *e.g.* stars, rods and discs [133]. Working with cell-laden hydrogels is possible because the technique only utilizes a single aqueous phase, and could potentially be used in bioartificial membranes for RADs. However, a large caveat is the lack of control over shape in the z-direction, as it is only possible to alter the height of the formed construct due to the 2D mask. Application of photolithographically-based scaffolds has been investigated for use in regeneration of various tissues as single units of cell-filled hydrogels [134-137].

5.4 (Micro)molding

Micron-scale molding is based on injecting pre-polymer solutions into a mold and subsequently initiating crosslinking or formation of a physical network. This technique is very versatile in terms of shape, size (nanometers to centimeters) and scale [138-141]. The limiting factor in design is the fabrication of the mold. Most recent studies utilize polydimethylsiloxane (PDMS) 'stamp' like molds, which are made with a master mold made on a silica wafer using conventional photolithography equipment from the field of microelectronics Fig. (**4C**). For an in depth review of soft photolithography we refer to article by Xia *et al.* [141]. The initial stamps and eventual hydrogels made in this fashion still retain the same drawback as the hydrogels that are directly made by photolithography, as control of shape in the Z-direction is limited. Other mold fabrication methods involve embedding of solid inert materials with a desired shape into hydrogels and removing these objects after gelation. A representative example is given by the Khademhosseini group, who used a commercially available steel needle embedded into a gelatin methacrylate (GelMA) scaffold, which was removed after gelation to form a tunnel in the hydrogel with a diameter equal to the outer diameter of the needle [142]. The surface of these tunnels was used for cell seeding *via* perfusion with a cell suspension. This method has recently been applied in kidney-on-chip developments. The Lewis group used micromolding in combination with 3D printing for the formation of a model system of proximal convoluted tubules inside bulk hydrogels [143]. Unfortunately, these tubules are not suitable for RADs as there is no outer compartment for active solute exchange. Schumacher and co-workers used the retracted needle technique referenced earlier to create tunnels of around 200 micrometers in an ECM hydrogel; these tunnels were subsequently seeded with Madin-Darby Canine Kidney (MDCK) cells (Fig. (**5**), adapted from [95]). Eventually, through perfusion over time, the core cells of these tubular structures became necrotic and were washed out, while the cells near the interface with the hydrogel attached to the gel, thereby forming tubular structures. After approximately 10 days, the ECM gel was removed to yield freestanding cell-based tubules. Although promising, it remains to be seen whether this technique can be used to form tubular structures from human-derived cell types.

5.5. Microfluidic Fabrication

Microfluidic fabrication is a powerful technology for the generation of spatially controlled hydrogels, and it is rapidly gaining significance in tissue engineering applications [144, 145]. By being able to precisely create channels with micron-sized diameters, it is possible to control minute fluid flows. The small scale of these channels results in laminar flow conditions, which allow more control over the processes in these flows. This can then be leveraged to form hydrogels of a wide range of shapes using the principle of phase immiscibility. A simple representation of a microfluidic device is given in Figure 4D, where a disperse phase of aqueous reactive polymer is slowly dripped into a perpendicularly flowing immiscible continuous oil phase. Given the right flow speeds, the nozzle at the interface of the two phases drips single droplets of disperse phase into the continuous phase. These droplets can be processed into hydrogels further downstream. Most microfluidic platforms are either based on soft lithography using PDMS, etched glass, injection-molded polymer, pulled glass microcapillaries or formats using conventional well plate-based designs [141,146-149]. There is a large diversity in publications on novel microfluidic methods and products. Advances in kidney-on-a-chip technologies like their use as diagnostic systems have been discussed elsewhere [150]. Here, we focus on two types that are considered especially interesting for the purposes of RRT because of the specific geometries of hydrogel they can produce: droplet generation and fiber production. In essence, both microfluidic techniques rely on the same immiscibility between liquid phases as the bulk emulsification technique. The added value of a microfluidic system over bulk

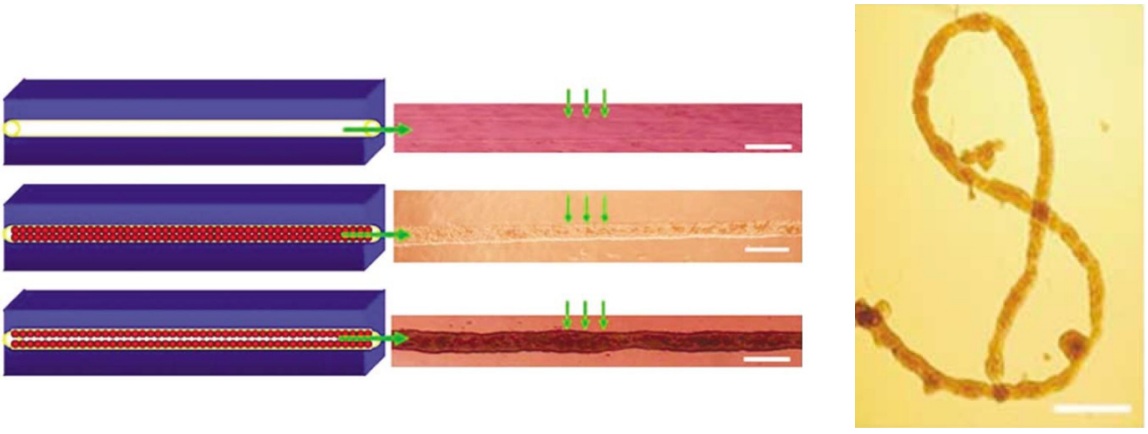

Fig. (5). Free-standing hollow tubule of MDCK cells. Channels were formed in an ECM gel using a retractable needle, and filled with MDCK cells; after 5-10 days, cells without contact to the ECM underwent apoptosis, leading to a tubule-like system (left and middle panel, scale bar = 200 μm). After 10 days, MDCK-based tubules could be released from the ECM mold, leading to a free-standing hollow tubule (right panel, scale bar = 500 μm). Adapted with permission from [92].

emulsification lies in controlling the formation of individual micron-sized droplets or stream. This allows the formation of highly monodisperse and reproducible gel products. The multitude of newly available devices and different strategies is also steadily increasing the number of possible geometries that can be made. These developments are leading to more freedom of design when fabricating hydrogel scaffolds, a good example is the combination of microfluidic flow and photolithography (*i.e.* stop flow lithography), allowing for high throughput generation of many different 2D shape controlled hydrogel particles [151, 152].

Controlled droplet generation using microfluidic methods has flourished as one of the initial innovations coming from this field [153]. Recently, droplet generation techniques have become increasingly proficient in encapsulating individual cells and cell clusters in droplets and hydrogels [127, 154]. For example, Choi *et al*. recently published an article on cell-laden microgels based on GelMA, where MDCK cells were encapsulated *via* a novel droplet formation technique utilizing a minimized amount of oil as a sacrificial layer that detaches quickly after polymerization. This in order to easily form the microgel whilst keeping encapsulated cells viable. These MDCK cell-filled gels were then cultured *in vitro* until the MDCK cells started self-assembling into cyst-like morphologies, showing the typical characteristics of this cell line (Fig. **6**).

Besides microgels which are most suitable for *in vivo* regeneration therapies, microfluidic spinning also enables the production of hollow hydrogel fibers filled with cells. Devices similar to the droplet generators are used at different, usually higher, fluid flow rates to form co-flowing streams of dissolved reactive polymer within another (continuous) phase. Due to the laminar nature of flows in the micron range, the two flows do not mix apart from transverse diffusion of molecules [155]. With this technology, it becomes possible to quickly fabricate a large variety of microfibers made from different gelling materials. As example in this field related to RRT, we refer to Hammer *et al.* who encapsulated Human Embryonic Kidney (HEK) cells into alginate fibers of around 200 µm in diameter [156]. These cell-laden microfibers where used as a cell delivery system for colonization and formation of hollow tubes within an empty bulk hydrogel. This interesting principle was also implemented in the work of Onoe *et al.*, who presented a relatively facile way of spinning hollow alginate microfibers [157]. Another example of cell-laden hydrogel tubules is shown in Fig. (**7**), taken from Hu *et al.*, who used an enzymatically crosslinkable gelatin-based hydrogel to encapsulate MDCK cells [158,159]. The cells were able to form a rudimentary epithelium on the inner surface of the tube. For further reading on microfluidic spinning of hydrogels, we would like to refer to the review by Jun *et al.* [94].

5.6. Electrodeposition

Electrospinning is rapidly evolving for tissue engineering applications, allowing the creation of a wide range of fibers, nano- and microspheres [160]. The principle is relatively simple: a syringe is filled with a polymer solution. While the plunger is slowly driven down the syringe to establish a flow from the needle, an electrical (usually DC) current is applied at a conductive part of the syringe and a plate resting at a certain distance below the needle tip Fig.

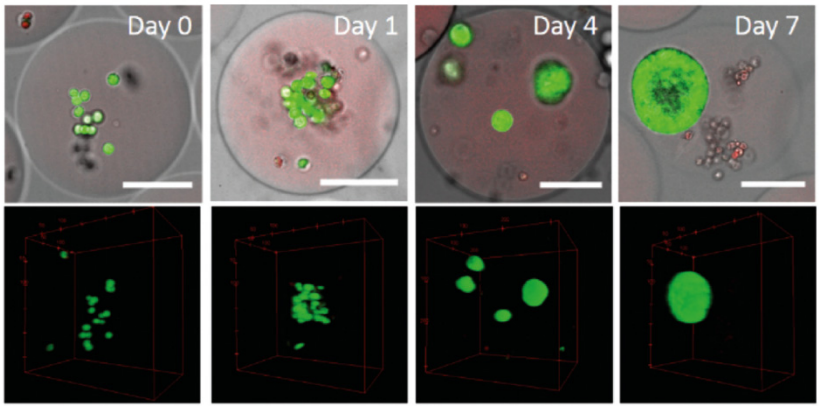

Fig. (6). Fluorescence microscopy images of GelMA microgels (light red) with encapsulated MDCK cells (green) and corresponding 3D spheroid reconstructions below. *In vitro* cultured cell-laden microgels show cell growth and cyst formation over time. This suggests potential of these gels as injectables for *in vivo* regeneration. Scale bar = 100 µm. Adapted with permission from [127]. (The color version of the figure is available in the electronic copy of the article).

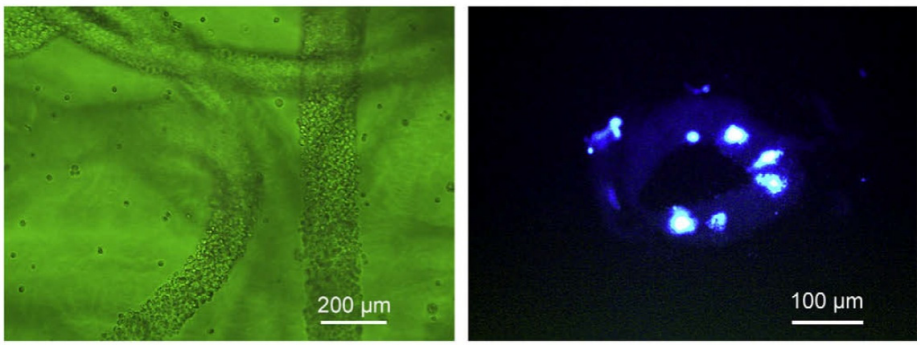

Fig. (7). Hollow gelatin–hydroxyphenylpropionic acid hydrogel fibers seeded with MDCK cells. Optical micrograph (left) and cryosectional image (right) of fibers formed through coaxial stream-based microfluidics using an inner H_2O_2 and an outer PBS flow with a middle flow of hydrogel precursor and MDCK cells. Adapted with permission from [158].

(**4E**). The static electric field produced by this current exerts a pulling force on the fluid emerging from the nozzle, a phenomenon called 'Maxwell stress' [161]. This force, in unison with the gravitational pull, overcomes the surface tension of the fluid, causing the pendant droplet to elongate and/or detach. Depending on the applied voltage that can range from two to several tens of kilovolts, the fluid flow takes on different appearances ranging from micron-sized droplets at low voltages (dripping), to nanofibers at medium to high voltages (spinning) and nano-sized droplets at high voltages (spraying) [118, 160, 162, 163]. Other important parameters that influence charged fluid behavior are electrical conductivity of the solute and solvent, viscosity of the fluid being sprayed, diameter of the needle, and distance of the needle tip to the ground plate or collector ring [118]. In general, electrospinning of biopolymers is challenging due to their great viscosities and high charge densities [164]. A novel approach is based on dripping an aqueous mixture of alginate and dextran methacrylate (DexMA) with HEK cells into a collector bath filled with polyethylene glycol (PEG) dissolved in water [158]. Due to the different hydrophilicities of DexMA and PEG, it is possible to form water in water emulsions, of which the disperse phase can be crosslinked to form hydrogel spheres [165]. This technique enables the formation of cell-laden microgels without any oil phase or purification steps, thereby enabling encapsulation of viable cells.

Electrodeposition of biocompatible non-hydrogel materials can also be interesting for the formation of scaffold meshes. For example, the recent works of Dankers and co-workers presented electrospun supramolecular membranes of modified PCL for use in enhanced monolayer formation of human primary tubule epithelial cells. Tubular electrospun meshes could also be applied for hydrogel reinforcement in RADs [107, 166].

CONCLUSION

While still in its infancy, advances in stem cell biology, sophisticated culture systems and processing technologies promise great advances in regenerative medicine. Available tools and technologies make steady progress and ultimately, some of these techniques could revolutionize the field, individually or in combination. The kidney, with its intricate architecture and function, remains one of the biggest challenges, while the clinical need for causative treatment is still unmet. Although whole kidney engineering approaches are worth to mention, RRT development for clinical application focusses on bioengineered functional units like RADs or induction of *in vivo* repair. For all three strategic lines, hydrogels represent an overarching tool as they mimic physiological cellular surroundings to promote cell organization and function; a basic concept that has emerged as most fruitful in tissue engineering. Hydrogels also interlink several research fields with each other, including cell biology, biomaterials, chemistry, physics and engineering. An interdisciplinary approach could shift the paradigm of future RRT, but therefore it is crucial to retain an overview of current developments in all fields. This review summarized current trends in kidney regeneration and biofabrication technologies, and highlighted the multifaceted potential of hydrogels.

LIST OF ABBREVIATIONS

CKD	=	Chronic kidney disease
ECM	=	Extracellular matrix
ESRD	=	End-stage renal disease
FSS	=	Fluid shear stress
GelMA	=	Gelatin methacrylate
HEK	=	Human Embryonic Kidney
iPSCs	=	Induced pluripotent stem cells
MDCK	=	Madin-Darby Canine Kidney
PDMS	=	Polydimethylsiloxane
PEG	=	Polyethylene glycol
PCL	=	Polycaprolactone
RADs	=	Renal assist devices
RRTs	=	Renal replacement therapies

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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