Development of an upscaled bio-artificial kidney

Natalia Vladimirovna Chevtchik

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Front page: confocal microscopy image of conditionnaly imrtalized proximal tubule renal epithelial cells (ciPTEC) cultured on polymeric hollow fiber membranes (HFM) with DAPI staining of the nuclei and immunostaining for the tight junction protein ZO-1.

DEVELOPMENT OF AN UPSCALED BIOARTIFICIAL KIDNEY

DISSERTATION

to obtain
the degree of doctor at the University of Twente,
on the authority of the rector magnificus,
Prof. Dr. T.T.M. Palstra,
on account of the decision of the graduation committee,
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The dissertation has been approved by the promotor:

Prof. Dr. D. Stamatialis

Chapter 1

Introduction

1. General Introduction

1.1. The kidneys

The kidneys are responsible for several physiological and regulatory functions, including the production of hormones, the regulation of blood pressure by controlling fluid volume in the body and keeping physiological pH by maintaining appropriate acid-base homeostasis. Kidneys are also responsible for nutrient reabsorption and most importantly for blood purification thanks to their excretory capabilities. Drugs, metabolic bi-products, endogenous wastes and environmental toxins, together named xenobiotics, are among the many compounds that are removed from systemic circulation by the kidneys, via urine production [1-3].

The kidneys are composed of hundreds of thousands of filtration units called nephrons (see Figure 1). In the nephron, blood initially passes through the glomerulus where small and middle-size solutes and excess fluids are removed out of the blood by convection. This glomerular filtrate is then transferred to the proximal tubules, which are responsible for reabsorbing essential components of the pre-urine but also for additional removal of a great variety of solutes and wastes from the blood stream, among which, the protein-bound toxins. More details about kidney physiology are explained in chapter 2 of this thesis.

1.2. Kidney disease

More than 10% of the worldwide population is estimated to present a more or less severe form of kidney disease [4-6]. Population ageing and the combination of various factors such as genetic predisposition, diabetes or cardiovascular diseases lead to the deterioration of kidney function and the development or progression of chronic kidney disease (CKD). The kidney function in CKD patients progressively and irreversibly declines until total loss, or end stage renal disease (ESRD). There, the patients require a permanent

renal replacement therapy (RRT). In addition, the ingestion of nephrotoxic agents, such as recreational or medicinal drugs, and intravascular contrast phase agents induces further progression of the disease, or can cause a brutal loss of kidney function, termed acute kidney injury (AKI) [7, 8]. AKI, which is sometimes irreversible, can occasionally lead to patient's death and frequently require the urgent application of a renal replacement therapy.

Figure 1: Schematic kidney and nephron morphology. Cross-section of the human kidney (left) which consists of approximately one million nephrons (center). The nephron is composed of several parts among which the proximal tubule, where proximal tubule cells (right) are playing an active role in the excretion of protein-bound toxins. Reproduced from [9] with the permission of Elsevier.

1.3. Renal replacement therapies

The most common treatment for CKD, ESRD and AKI patients is artificial kidney or dialysis, applied for 2.2 million patients worldwide [10]. This treatment only covers a fraction of the physiological renal function - mostly that of the glomerulus in the normal kidney - and its efficiency in waste removal is incomplete [11, 12]. Indeed, only small water-soluble molecules, inferior to 40 kDa, present in free fraction in the blood, can be eliminated [13] whereas most of the big size and protein bound toxins cannot be removed. Their accumulation is strongly linked to the fatal outcome of the hemodialysed population [14-16]. These toxins are in large part handled by the proximal tubules in the healthy kidneys [13, 17]. Besides, dialysis is removing a part of the toxin population but is not replacing the kidney endocrine and metabolic physiological functions.

1.4. The need for a more complete renal replacement therapy

There is a strong need for a device, extracorporeal or implantable, which could fully replace the kidney function. Such a device could be a hybrid combination of polymeric membranes and renal proximal tubule epithelial cells (PTEC), called the bioartificial kidney (BAK). The BAK is conceived to be used in combination with a classical hemofilter [18, 19]. In this way, there is a direct similitude with the natural kidney. First, the glomerular function is replaced by the classical hemodialysis for removal of small size water-soluble molecules. Second, the glomerular filtrate, which comes out of the hemodialysis module, can be processed by the PTEC of the BAK. The principle of application of the BAK is presented in Figure 2.

The first BAK prototype was presented by Aebischer *et al.* in 1987 [21]. Since then, several other prototypes have been proposed by the groups of Humes, Zink and Saito [18, 22-29]. The first prototypes made use of animal cells, which was not acceptable for a clinical application. The later versions made use of human cells mostly from primary sources and thus characterized by a high variability, low availability and a rapid senescence.

Figure 2: Combination of hemodialysis and of the bioartificial kidney for achieving a complete removal of uremic solutes from blood. Reproduced from [20] with the permission of Wichtig.

The PTEC should be from human origin, and should form a tight monolayer to be functional and act as a barrier against the loss of components. They should present a high availability, a limited replicative senescence and should not evoke an immune response in the host. One potential candidate could be conditionally immortalized PTEC line (ciPTEC) [30, 31]. They present high replicative capability with limited senescence and preserved organic ionic transporters function. This cell line is used in this thesis.

The cells should be supported by a permeable membrane that is cytocompatible on one side and haemocompatible on the other. Since these two surface properties are antagonist, a functionalization step is most of the time necessary to modify one of the membrane surfaces. The membrane should also have a high flux to allow on the one hand the passage of nutrients and toxins to the cells from the blood compartment, and on the other hand the release of hormones, vitamins and other beneficial solutes into the patient's body fluid. It should also not evoke an immune response. A review of the materials and coatings used in the development of a BAK are also presented in chapter 2 of this thesis.

2. Aim and outline of this thesis

The principal aim of this thesis is the development of an upscaled bioartificial kidney using human conditionally immortalized PTEC (ciPTEC) and presenting functional organic ionic transporters to allow a more complete removal of uremic wastes. Additionally, this thesis aims at investigating the ability of several materials and coatings to support the adhesion and the function of the ciPTEC.

Chapter 2 introduces the background of the kidney anatomy and function. It gives an overview of the current renal replacement therapies (RRT), their advantages and limitations. The need and requirements for a BAK are identified and presented. Finally, a short history of the BAK is presented, as well as new developments and needs in this research field [32].

Chapter 3 presents the upscaling of a “living membrane” for BAK device, on the external surface of commercially available hollow fiber membranes (HFM) mounted in modules. The development of a double L-Dopa and collagen IV coating is reported and the HFM transport properties are studied. We report the seeding of organic cationic transporter (OCT)-expressing ciPTEC and investigate the homogeneity of the monolayer and its barrier function. Furthermore, the uptake of a fluorescent OCT substrate is evaluated to assess the active function of the OCT [33].

Chapter 4 investigates strategies for achieving a good quality OCT2-expressing ciPTEC monolayer on the inside surface of the polymeric HFM. This configuration could be preferred for the development of a clinically relevant BAK. We first optimize the functionalization of the internal surface of the polymeric fiber to achieve high transport of metabolites. Secondly, we investigate several cell seeding parameters in order to achieve a tight ciPTEC monolayer on the inside surface of the HFM.

Chapter 5 investigates the ability of alternative flat surfaces to support the formation of a “living membrane”. Following our hypothesis, not only collagen

IV but also other elements of the natural kidney epithelial extra cellular matrix can be used to coat membranes to support ciPTEC. Moreover, we investigate the ability of positively charged polymer membrane to support the adhesion of the negatively charged cells. We use transport experiments to evaluate cell function as well as a novel setup to assess cell attachment strength.

Chapter 6 presents the upscaling of a BAK device capable of actively removing anionic uremic wastes. We study the quality of the ciPTEC monolayer by confocal microscopy and paracellular inulin-FITC leakage. The transport of indoxyl sulfate, an anionic uremic toxin, is studied to prove the function of, among others, the organic anionic transporter 1 (OAT1). Furthermore, the polarization of the secretion of immune response mediators is assessed by measuring the production of relevant cytokines both in the extraluminal and in the intraluminal spaces.

Finally **chapter 7** presents the general conclusions and reflections on the future directions in the development of a clinically relevant BAK.

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Chapter 2

Membranes for Bioartificial Kidney Devices

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1. Introduction

The kidneys play a fundamental role in maintaining whole body homeostasis and are responsible for several physiological processes, including the production of hormones, the regulation of blood pressure, by controlling fluid volume in the body and keeping physiological pH by maintaining appropriate acid-base homeostasis. Kidneys are also responsible for nutrient reabsorption. At the heart of renal function are their excretory capabilities that account for the kidneys blood purification function. Drugs, metabolic bi-products, endogenous wastes and environmental toxins, together named xenobiotics, are among the many compounds that are removed from systemic circulation by the kidneys, via urine production [1-3]. In severe renal diseases, either chronic kidney disease (CKD) or end stage renal disease (ESRD) a break-down in renal function leads to the accumulation of xenobiotics in the body, which subsequently results in disease progression. Moreover, sudden break-down in renal function, termed acute kidney injury (AKI), is sometimes irreversible and can lead to patient's death.

The current treatment for AKI, CKD and ESRD patients is mainly hemodialysis. Hemodialysis only covers a fraction of the physiological renal function and its efficiency in waste removal is incomplete [4, 5]. Indeed, most of the large solutes and protein-bound toxins cannot be removed. Their accumulation is strongly linked to the fatal outcome of the patients [6, 7]. There is therefore a strong need for a device, extracorporeal or implantable, which could mimic and/or replace fully the kidney function.

In recent years, the research around bioartificial kidneys and bioengineered renal replacement therapies has brought together different disciplines, combining technical expertise with cellular and molecular biology. Different from organ regeneration, this research is focused on the creation of devices that can mimic (partially) the function of a healthy kidney. Such device could be a hybrid combination of polymeric membranes and renal proximal tubule cells, called either the bioartificial kidney (BAK), or renal assist device (RAD), or

bioartificial renal tubule device (BTD). This chapter focusses on the role of artificial polymeric membranes for the development of these devices.

Figure 1. Renal physiology. A cross-section of the human kidney (left) which approximately consists of 1 million nephrons (right), the functional components of this organ. (right) Unfiltered blood will enter the glomerulus (G) and small solutes and H_2O will be excreted via ultrafiltration into Bowman's space, which is contiguous with the lumen of the proximal tubule. Subsequently, PTEC (P) mediate reabsorption of H_2O and compounds such as amino acids, glucose and albumin from the filtered fraction, next to the active excretion of endo- and xenobiotics into the pro-urine. In addition, 65% of the total amount of electrolytes will be reabsorbed via paracellular pathways. Downstream the proximal tubule segment the loop of Henle (L), the distal convoluted tubule (D) and collecting tubule and duct cells (C) are localized. In brief, these cell types are equipped with specific water and ion channels involved in the homeostasis of water and electrolyte balance, finally contributing to homeostasis. Reproduced and adapted from [11] with the permission of Elsevier.

2. Renal function

2.1. Renal tubular function

The kidney is composed of filtration units called nephrons (Figure 1). In the nephron, blood initially passes through the glomerulus where its capillary network significantly increases pressure, causing filtration of small and middle-size solutes and removing excess fluids out of the blood by convection. This glomerular filtrate is then transferred to the proximal tubules, which are responsible for reabsorbing essential components of the pre-urine but also for additional removal of a great variety of solutes and wastes from the blood stream, among which, the protein-bound toxins. To this end, proximal tubule epithelial cells (PTEC) are equipped with highly specialized molecular machinery (Figure 2). These polarized cells act as a barrier that compounds have to cross from the basolateral (capillary) side to the apical (pre-urine) side. The functional characteristics of PTEC are to a great extent derived from the presence of multiple energy dependent membrane transporters (carrier proteins) that mediate the transport of ions, small molecules, nucleotides, xenobiotics and other substances. These transporters can move solutes against steep concentration gradients, and provide the cells their barrier-specific selectivity and high excretion capacity. The transporters of PTEC can be unidirectional efflux pumps, co-transporters, facilitated diffusion carriers and exchangers, belonging to either the adenosine triphosphate-ATP binding cassette (ABC) [8] superfamily or the solute carrier family (SLC; www.slc.bioparadigms.org) of proteins [9]. Function of ABC transporters requires ATP hydrolysis, a feature that has enabled PTEC to develop an increased mitochondrial activity to meet the energy demand.

Activity of SLC transporters involves co-transport driven by the membrane potential, established by the basolaterally expressed Na,K-ATPase. Basolateral and apical transporters are complementary in substrate specificity to enable compounds that are taken up by the cells to be excreted subsequently [10].

Figure 2. Schematic representation the major basolateral and apical membrane transport systems in renal proximal tubule cells. Reabsorption mechanisms are presented in grey and drug transporters are presented in dark blue.

A comprehensive depiction of all major PTEC transporters is presented in Figure 2. The most prominent uptake transporters are the organic anion transporter 1 (OAT1; *SLC22A6*), organic anion transporter 3 (OAT3; *SLC22A8*) [12], and organic cation transporter 2 (OCT2; *SLC22A2*) [13]. The predominant efflux transporters are the breast cancer resistance protein (BCRP; *ABCG2*) [14], P-glycoprotein (P-gp; *ABCB1*) and the multidrug resistance proteins 2 and 4 (MRP2/4; *ABCC2/4*) [15]. Other carriers, such as the transporter organic anion transporter polypeptide (OATP4C1; *SLCO4C1*), and the multidrug and toxin extrusion 1 and 2 transporters (MATE1 and 2K; *SLC47A1-2K*) are also present at PTEC. These transporters share common regulatory pathways and their functional expression can be influenced by drugs and external factors. After uptake, PTEC can also metabolize drugs and compounds via phase I and II enzymes, in a process that can increase the excretory efficacy of certain compounds [16]. An elegant example of the relevance of this process is the

fact that glucuronidation augments substrate affinity for multidrug resistance proteins (MRP's) and incidentally MRP4 is highly expressed in the apical membrane of PTEC [17, 18]. Active membrane transport at the basolateral side also facilitates the removal of compounds that are bound to plasma proteins in blood. In fact, a great variety of toxins and drugs are transported in the blood stream coupled to plasma proteins, like albumin. Those protein-bound molecules can only be removed from the circulation via active membrane transport.

In addition to xenobiotic excretion, PTEC also play an extensive role in the reabsorption of nutrients and ions back into the blood stream, a process initiated from the apical (luminal) side (Figure 2). An important re-uptake mechanism is receptor mediated endocytosis. In this mechanism, proteins with different sizes, that initially pass through the glomerulus, can be shuttled back from the filtrate to circulation, mediated by three receptors expressed at the apical membrane, megalin, cubilin and amnionless [19]. A number of key ions is also reabsorbed, although this process is not exclusive to PTEC, but can also take place along other segments of the nephron and occurs passively with the high amount of water that diffuses back to the circulation or via a number of membrane carriers that handle *eg.* potassium (K^+), magnesium (Mg^{2+}), calcium (Ca^{2+}), phosphate (PO_4^{3-}) [20, 21]. In addition, glucose is reabsorbed exclusively by PTEC via Na^{2+} -dependent co-transporters (*SLC5A* family members) [22]. Furthermore, the lumen of the proximal tubules is convoluted where the apical side of PTEC is organized into dense microvilli that form a brush border, a feature that provides an increased surface area of the membrane to enhance the reabsorption processes. Throughout the nephron, continuous water reabsorption is facilitated by selective water channels known as aquaporins (AQP), with different channels expressed in the proximal tubules, loop of Henle and collecting duct [23]. Though renal function derives from the actions of the different nephron segments, PTEC are key, since their activity account for a significant part of xenobiotic excretion.

2.2. Renal dysfunction

More than 10% of the worldwide population is estimated to present a more or less severe form of kidney disease [24-26]. Population ageing and the combination of various factors such as genetic predisposition, diabetes or cardiovascular diseases lead to the deterioration of kidney function and the development or progression of chronic kidney disease (CKD). In addition, the ingestion of nephrotoxic agents, such as recreational or medicinal drugs, and intravascular contrast phase agents induces further progression of the disease, or can cause a brutal loss of kidney function [27, 28]. Table 1 presents a classification of the stages of kidney disease based on KDIGO guidelines [29].

Table 1: classification of chronic kidney disease (CKD) progression, according to KDOQI CKD guidelines [30]; GFR: glomerular filtration rate.

Stages of Chronic Kidney disease of all types		
Stage	Qualitative description	Renal function (mL/min/1,73 m ²)
1	Kidney damage – normal GFR	≥ 90
2	Kidney damage, mild ↓ GFR	60-89
3	Moderate ↓ GFR	30-59
4	Severe ↓ GFR	15-29
5	End-stage renal disease	< 15 (or dialysis)

The stage of kidney disease is expressed based on the glomerular filtration rate (GFR). The GFR is calculated from the concentration of serum creatinine and several other markers in urine and/or plasma. CKD or chronic renal failure (CRF) is diagnosed in case of a low GFR for a prolonged period of time. The kidney function in CKD patients progressively and irreversibly declines until total loss, or ESRD. There, the patients require a permanent renal replacement therapy (RRT). According to Fresenius Medical Care report [31]: “The number of patients being treated for ESRD globally was estimated to be 3,2 million at the end of 2013 and, with a ~6% growth rate, continues to increase at higher rate than the world population.” According to Hedgeman et al. [32] “population prevalence estimates of CKD stages 3–5 in adults ranged from approximately

1 to 9%". For these patients a palliative RRT is frequently proposed in developed countries.

A brutal loss of the kidney function is the AKI or acute renal failure (ARF). In this case an immediate renal therapy is urgently required [28]. AKI remains a major unmet medical need and a global public health concern impacting ~13.3 million patients per year [24]. AKI occurs in half of the intensive care patients. Although it is most of the time reversible, the patients have worse renal function at the time of hospital discharge and 42% of them may develop CKD [27]. Moreover, although direct causality between AKI and death has been controversial, increasing AKI severity - classified based on the serum creatinine and the urine output [28] - is associated with morbidity, increased costs, and mortality - about 1.7 million deaths per year.

2.3. Existing renal replacement therapies (RRT) and their limitations

Figure 3 presents schemes of the three current RRT. The most popular therapy is kidney transplantation from deceased or living donors. Indeed, in case of a successful transplantation, the kidney function is fully replaced and the quality of life of the patient is almost back to normal. More than 75000 patients worldwide receive a kidney transplant yearly [33]. However, not all of the kidney patients are eligible, for example in case of comorbidities a transplantation is not possible. Moreover, the success of the therapy is not total: after 10 years, about 50% of the transplanted kidneys are still functional [34]. Besides, even after transplantation patients have the medical need for immunosuppressive therapy, which coincides with a number of side effects.

The major limitation of transplantation remains the availability of organs. The waiting list varies per country and region but is about 3 to 5 years. In 2016, the number of patients registered on the waiting list for receiving a kidney transplant was 10900 in Europe [36] versus more than 100000 in the US [37].

Patients who are not eligible for transplantations or registered on a waiting list for a transplant whose residual renal function (RRF) is insufficient - as well as

AKI and ESRD patients – require other therapies such as peritoneal dialysis (PD) and/or hemodialysis (HD).

In PD, the peritoneum of the patient is used as filtration membrane. This therapy can be used as ambulatory or home dialysis, which presents the advantage of a continuous filtration (in case of home-dialysis). Despite this, only 8.5% of the ESRD patients are treated with PD. HD is widely preferred [31] due to the high prevalence of catheter access problems and peritonitis episodes for the PD therapy. These complications often lead to ultrafiltration failure and volume overload of the patients. In addition, even in the absence of complications, the use of glucose as osmotic agent in PD solutions damages the peritoneum quickly, after one to two years. For these reasons, more than 35% of the PD patients switch to HD within 2 years after the beginning of the treatment [38]. More importantly, due to the intrinsic property of the peritoneum, only small size solutes are being cleared.

HD remains the most common therapy, applied for 2.2 million patients worldwide [31]. They undergo HD 3 to 4 times a week, for 3 to 4 hours session in a hospital. Shorter dialysis times, chosen for budgetary and logistics reason, have been associated with higher mortality among patients. In fact, dialysis sessions inferior to 4 hours have been associated with a 42% increase in mortality [39-41]. To palliate this problem, home-HD is being developed for longer treatment sessions, preferably at night. However, the risk of infections and the fragility of the vascular access remain major issues, as well as the logistics for pure water production and storage of disposables [42].

For in center-HD as well as for home-HD, the size and range of toxins being removed is limited. Indeed, only small water-soluble molecules, inferior to 40 kDa, present in free fraction in the blood, can be eliminated [43]. Research is ongoing to further improve existing techniques by varying parameters such as flow rates, membrane permeability and surface area, and combining diffusion, convection and/or adsorption mechanisms. [44]. Molecules up to 200 kDa can be removed via adsorption, which is more solute-specific rather than size-specific. Protein-bound toxins can also be targeted via adsorption. For

example, multilayered mixed matrix membranes (MMM) using activated carbon for the removal of protein-bound toxins have been developed in the recent years [45-48].

Figure 3. schematic presentation of the three available renal replacement therapies (RRT) (A) Kidney transplant. The transplanted donor organ is implemented in the patient while both affected kidneys will remain in the body. A donor organ is the preferred treatment option for patients suffering from ESRD. (B). Peritoneal dialysis (PD). During PD, a permanent tube will be connected to the patients' abdomen and the peritoneum will be used as a membrane which allow for the removal of small waste products from the blood into the dialysis waste fluid. Peritoneal home-dialysis is possible as long as infections are suppressed and the quality of the peritoneum is maintained. (C). Hemodialysis (HD). For hemodialysis, vascular access can be accomplished via three ways i) a central venous catheter ii) an arteriovenous fistula which is a surgical connection between an artery and a vein to achieve a suitable access point or iii) an arteriovenous graft which is a biocompatible tube surgically connected between an artery and a vein. The patients' blood will pass the dialyzer, which consists of numerous hollow fiber membranes, and ultrafiltration process occur. During dialysis, small waste products will be removed from the blood into the dialysis waste fluid and the filtered blood will return to the patient. In general hemodialysis is performed three times a week and four hours per session, often in a dialysis center or hospital. Reprinted and adapted from Jansen [35] with the permission of the author.

2.4. The need for a more complete RRT – the BAK

Several studies have established a direct link between the concentration of protein-bound toxins - namely indoxyl sulfate and paracresol sulfate - and cardiovascular events and/or mortality in ESRD patients [7, 49]. These toxins are in large part handled by the proximal tubules [50] and, although traditional dialysis is able to remove small water-soluble toxins, the protein-bound solutes can only be removed through the biological processes inherent to PTEC [43, 51]. Besides, dialysis is removing a part of the toxin population but is not replacing the kidney endocrine and metabolic physiological functions. Therefore, the BAK, thanks to the use of PTEC, appears as a possible solution to bring a more complete kidney replacement therapy.

The requirements for a BAK should be the following:

- (1) The cells used should be functional, from human origin, with a high availability, and stability in time. These cells should form a tight monolayer to be functional and act as a barrier against the loss of components. The production of pro-inflammatory cytokines should be minimal and preferably oriented towards the waste compartment.
- (2) The cells should be supported by a permeable membrane that is cytocompatible on one side and haemocompatible on the other. The membrane should allow on the one hand the passage of nutrients and toxins to the cells from the blood compartment, and on the other hand release of hormones, vitamins and other beneficial solutes into the patient's body fluid. It should also not evoke an immune response.
- (3) The whole device has to be adequately designed to support cell growth and function. The device should allow gas exchange, and pH, pressure and temperature control. Moreover, it should remain stable in practice, including during transport and storage, as well as cost efficiently.

The BAK is conceived to be used in combination with a classical hemofilter [52, 53]. In this way, there is a direct similitude with the natural kidney. First, the glomerular function is replaced by the classical hemodialysis for removal of small size water-soluble molecules. Second, the glomerular filtrate, which comes out of the hemodialysis module, can be processed by the proximal tubules of the BAK. As explained in the paragraph 2.1, the BAK should replace not only the excretory function to eliminate the protein-bound and larger size toxins, but also the essential endocrine and metabolic functions of the kidney.

Figure 4. BAK composition and mechanism. Separated in- and outlets for the patient's blood and the dialysate are incorporated in a BAK. The device will consist of numerous [hemocompatible HFM]. The inner surface of the HFM will be modified in order to induce cytocompatibility to stimulate monolayer integrity. A homogeneous and polarized cell monolayer will stimulate excretion of endo- and xenobiotics (e.g. protein-bound uremic toxins) and reabsorption of solutes (e.g. phosphate). Importantly, host albumin and IgG components will be retained due to appropriate molecular cut-off values of the membrane. Furthermore, potential metabolic and endocrine functions of the cells can contribute to an improved homeostasis of the patient. Preprinted from [11] with the permission of Elsevier.

Figure 4 shows the composition of a BAK. It commonly has the configuration of a classical module for hemodialysis. The HFM bioreactor presents the advantage of a three dimensional configuration, close to the natural PTEC configuration within the kidney. This model has therefore the advantage of a simple extracorporeal circuit, but due to the presence of cells, its fabrication under conditions compliant to good manufacturing practice (GMP) conditions, storage and transport should be planned and organized carefully in case of clinical applications. The action of the system entirely relies on the integrity and function of the cell monolayer. It is, therefore, important to develop non-destructive testing for these devices and obviously, to choose the appropriate cells (see paragraph 5.3).

3. Cells for bioartificial kidney

Renal cells can perform and regulate, with extreme efficiency, highly complex and specific chemical and physical processes simultaneously. Harnessing and exploiting cells to study renal physiology and bioengineered kidneys is central in this line of investigation. Nowadays a variety of renal-derived cell types are available, from different sources and used in fundamental bioengineered kidney research. A fundamental aspect of culturing cells *in vitro* is assuring that the cells retain a phenotype that closely resembles the *in vivo* situation.

Cells can drastically change their properties while being in culture, due to the artificial environment. Cellular plasticity allows cells to change their gene and protein expressions as well as their metabolic activity when confronted with an artificial environment. *In vitro*, cells are grown on flat plastic surfaces, fed with a cocktail of nutrients and factors (culture medium) and maintained in a humidified environment at physiological temperature. *In vivo*, cells are arranged in three-dimensional structures often containing multiple cell types that cross-talk and are nourished from the blood stream. Culture medium composition can be tailored to maintain tissue specific phenotypes, however, the addition of growth factor and serum, often required to maintain proliferation, can influence chromosome stability and can lead to gene mutations, affecting the phenotype of the cells [54]. To monitor that cells maintain their phenotype, an array of assays and techniques has to be performed.

When growing PTEC *in vitro* it is key to determine whether the specific molecular machinery of this cell type is expressed at the gene and protein levels. It is also important to determine the proper morphology, capability of the cells to polarize, tight monolayer formation, generation of the appropriate membrane potential and selective barrier function. Subsequently, it is crucial to evaluate the functional activity of the cells, ensuring recapitulation of the activities of native PTEC.

3.1. Primary cells

Primary cells are directly derived from renal tissue or urine, collected from healthy donors (either through a biopsy or from a discarded kidney transplant). The renal tissue is then disaggregated into a heterogeneous cell suspension that is purified further via flow cytometry or magnetic beads, making use of membrane markers in order to isolate PTEC from other cell types. Afterwards, cells can be cultured and characterized to confirm the cell phenotype. These primary cells retain only temporarily the PTEC phenotype, losing their epithelial characteristics with each population doubling in culture, and their use is limited to the availability of donors [55]. Due to these limitations, primary cells are not a preferred source for long-term applications and are mostly used for cellular and molecular research into the inner works of PTEC, as well as for drug efficacy and safety testing. An alternative source of PTEC cells also explored is urine [56, 57]. Being easily accessible, urine is an abundant source of cells and incidentally PTEC are shed in reasonable numbers.

3.2. Stem cells

Another primary cell source are stem or progenitor cells for which cells can be derived also from other tissues than the kidney. Stem cells are undifferentiated cells that can, in one hand, self-regenerate and, on the other, give rise to various terminally differentiated cell types [58]. These cells are found in developing embryos, being pluripotent (able to generate any lineage) at earlier stages. As embryonic development progresses, stem cells differentiate into particular tissue lineages and gradually occupy specific niches. Stem cells can be isolated using specific membrane markers and cultured under defined conditions, they can be expanded without losing their properties, or differentiate upon specific inducers [59]. As with primary cells, stem cells are also limited by donor availability. They can be collected from embryos, which is directly associated with the additional challenge of being a highly controversial ethical issue and quite limited source. Alternatively, stem cells can be derived from adults, mainly from blood, bone marrow and adipose tissue. The latter source is the least invasive and relatively abundant. However,

the biggest bottleneck in the use of these cells is the differentiation *in vitro*, which is a time and resource consuming process for which adequate PTEC phenotype still needs to be demonstrated. Nonetheless, stem cells have the promise of providing an autologous cell source for biomedical research applications and can potentially be expanded in large quantities.

3.3. Induced pluripotent stem cells

A cell type that was introduced less than a decade ago are induced pluripotent stem cells (iPS), that now make their way to the spotlight of cellular research. The technique to produce this new type of cells, bypasses the issues with limited sources since they can be derived from somatic cells [60]. Furthermore, the cells can differentiate into virtually any cell type in the body, hence subscribing their pluripotency. These iPS cells are generated by introducing a specific factor in adult cells (terminally differentiated) that will trigger the cells to re-arrange their genetic program and change their phenotype into undifferentiated stem-like cells, in a process labeled trans-differentiation. Several factors, namely pluripotency encoding genes, have been identified and novel delivery vectors have been explored to prevent the use of viral transfection.

Human iPS cells have been obtained from fibroblasts and other sources, and kidney organoids grown from such cells formed functional PTEC [61]. iPS cells are a potential source of autologous cells, however their use and generation are still a laborious process. Cells trans-differentiated using viral vectors may not be appropriate for clinical use. The use of ectopic transcription factors can be potentially tumorigenic and an incomplete re-programming compromises the cells pluripotency [62, 63]. As renal derived iPS cells become a reality, comprehensive validation is needed to confirm the cells function and determine the correct phenotype [60].

3.4. Cell lines

Cell lines are also a prominent source of renal cells that usually originate from a primary cell culture that is transformed to enable prolonged culturing while maintaining the cell-type specific phenotypical properties. These cells are widely used in research and can be obtained commercially or generated in an adequate facility, both for research or commercial uses. Drug screening and fundamental research into kidney pharmacology and physiology are important applications for kidney-derived cell lines. Two commonly used kidney cell lines, and examples of early developments in this area, are the Human embryonic kidney 293 (HEK293) and the Human kidney 2 (HK-2) cells.

HEK293 cells are derived from primary cultures of human embryonic kidney cells and transduced by adenovirus particles to achieve sustainable cell growth *in vitro* [64]. Although originally derived from human renal tissue, these cells show abnormal chromosomes and lack a defined phenotype (namely the transport machinery characteristic of PTEC), while culture conditions for optimal proliferation are well established [65]. HEK293 cells are easy to transfect, stimulating their use in cellular research study protein expression on a molecular level.

HK-2 are derived from human primary PTEC cultures transfected with the human papilloma virus 16 E6/E7 genes (HPV) in order to obtain a stable cell line [66]. These cells express several enzymes present in primary PTEC along with certain functional aspects, such as glucose uptake. However, the HK-2 cells do not entirely resemble a PTEC phenotype and show, at most, residual active transport activity of xenobiotics [65, 67]. These early generations of renal cell lines underline the problems with generating a cell that is well-defined in terms of phenotype and that retains key features of differentiated cells.

Non-human cells lines, isolated from mammals are also widely applied. The Madin-Darby canine kidney (MDCK) cells retain a strong epithelial phenotype and are simple to culture [68]. Pig derived LLC-PK1 cells also possess a well characterized PTEC phenotype [69]. Nevertheless, these cells have contributed

to elucidate the cellular and molecular mechanisms involved in renal physiology and pathophysiology and also paved the way to more advanced and complex *in vitro* models that are becoming of increasing importance.

3.5. Conditionally immortalized cell lines

In recent years, the amount of cell lines generated has increased as a consequence of improved molecular techniques, the need for more representative and well-defined cells and also in an attempt for refining or replacing the use of animals in research. A hand-full of cell lines has been developed relying on immortalization tools that reduce genetic variability and thereby improve the stability of the cells, largely for purposes of renal *in vitro* pathophysiology and drug safety testing.

The renal PTEC line (RPTEC) and NKi-2 cell line were generated by overexpressing the human telomerase reverse transcriptase (hTERT) via viral transfection. This transformation allows the cells to maintain their intact chromosomes after every doubling, resulting in stable lines [70-72]. Functionally, the cells express metabolic enzymes, including esterase and glucuronidase [73], and are used as an *in vitro* model for kidney toxicity studies with emphasis on drug screening [74]. These cells were non-invasively harvested, and can be cultured *in vitro*. Subsequently, the cells are transformed to grow in a sustained way and characterized to confirm a PTEC phenotype. Conditionally immortalized human proximal tubule cells (ciPTEC) are a type of PTEC cells generated by overexpressing the simian virus 40 large T tsA58 antigen (SV40T) together with hTERT [56]. These transformations enable the cells to proliferate at a temperature of 33 °C and subsequently mature at 37 °C, inactivating the large T antigen and acquiring a differentiated PTEC phenotype. CiPTEC can be derived both from urine or adult renal tissue [56, 57] and functionally express OCT2, BCRP, P-gp and MRP4, key drug transporters that are native to PTEC. Consequently, the cells are sensitive to nephrotoxic drugs and can extrude protein-bound uremic toxins [50, 75]. These cells can be grown abundantly and are functional after high population doublings. Arguably, cells derived from urine are different from cells derived from tissue; the fact they

were shed from the proximal tubule epithelium can indicate a loss in functionality. Nonetheless, ciPTEC derived from both urine and kidney biopsies, and immortalized according to the same procedure, show similar gene expressions, membrane transport functions and enzyme activities, supporting the validity of urine derived PTEC [57].

3.6. Cell models - challenges and perspectives

The concept of a bioartificial device that combines the properties of cells and membranes was pioneered by growing Madin Darby canine kidney (MDCK) cells and pig kidney epithelial cells (LCC-PK1) on permeable membranes impregnated with matrigel (extracellular matrix extracted from mouse sarcoma) [76]. This strategy proved that cells can confer selectivity and maintain transport function when grown on an artificial membrane. Further studies used human or porcine kidney cells seeded into modified hemofiltration cartridges to recover kidney function in uremic animals [77]. These early approaches revealed that cellular properties can be harnessed to improve dialysis, however, the use of animal cells and/or animal-derived substrates hampers clinical applications. Therefore, human-derived cell sources have to be perused. Table 2 summarizes the human PTEC proposed for use in the development of bioartificial kidney devices.

Table 2: Sources of human PTEC used or proposed for use in bioartificial kidney

Cell	Type	PTEC phenotype	Availability	References
HK-2	Cell line	Intermediate	High	[65-67]
Primary	Primary	Strong	Low	[77-81]
RPTEC	Cell line	Strong	Reasonable	[70, 81]
ciPTEC	Cell line	Strong	Reasonable	[82-84]
Stem cells	Primary	Intermediate	Low	[59, 85, 86]
iPS	Primary	Intermediate	Reasonable	[60, 87]

Despite all innovations in developing advanced cell models that recapitulate renal PTEC function, there are still considerable challenges on the road towards a bioartificial kidney that incorporates living cells [11]. A potential problem can arise from using PTEC to directly or indirectly remove solutes from the blood of patients with kidney disease due to differences in genetic background which can exert immune compatibility issues. The cells can excrete factors and express major histocompatibility complex (MHC) surface proteins, that can be recognized as foreign and trigger an immune response. Although this response may not affect the PTEC themselves, if MHC peptides are shed and end up in the patients systemic circulation, immune cells can be activated leading to unwanted inflammatory events. Immunogenic responses can be avoided if the cells used are derived from stem cells or iPS cells, providing that the recipient is also the donor. In addition, the use of surfaces/materials that absorb such soluble factors and peptides can circumvent the issue as well [11].

Immortalized PTEC lines form a tight monolayer, however implementation raises issues about monolayer stability and integrity. Cells can breakaway and compromise the barrier function and also being shed from any compartment that is not self-contained. Finally, the cell characteristics such as proliferation or function are strongly dependent on the culturing environment, and especially the two-dimensional (2D) or three-dimensional (3D) configuration [88-90]. Therefore, many PTEC cell models which were first studied in 2D may show a very different behavior when cultured on hollow fiber membranes for BAK applications. A recent work with ciPTEC grown on the outside of hollow fiber membranes has proven that these cells can form a tight monolayer around the fibers and actively take up and secrete substrates when perfused in dose dependent manner [84, 91, 92].

4. Artificial membranes for the bioartificial kidney

The requirements for artificial membranes for a BAK should be the following. First, one side of the membrane has to be in prolonged contact with blood or body fluid and has, therefore, to be haemocompatible has low fouling and thus avoid cell adhesion. The other side of the membrane should be highly cytocompatible and favor PTEC adhesion and function. The membrane should also provide high solute fluxes to allow the exchange of solutes between the PTEC and the patient fluid. Finally, it should act as an immunoprotective barrier to preserve the PTEC from an eventual immune attack. The following paragraphs will summarize the membrane properties reported in literature which played an important role in the development of the BAK. Moreover, since the development of BAK began with commercially available membranes (presented more in detail in the paragraph 5), surface modification was necessary in order to improve their cytocompatibility. These modifications will be discussed, as well.

4.1. Membrane materials

The first BAK prototypes were build using the existing ultrafiltration or hemodialysis HFM, mostly from polysulfone (PSU) [52, 77, 93]. The reasons for this choice were the improved haemocompatibility, the high filtration rates and the high reproducibility and availability of the HFM. More recent work reported newer generations of HFM based on polyarylethersulfone (PAES) from Gambro [94], ethylene vinyl alcohol (EVAL) from Kasei Kuraray Medical [78, 80], polyethersulfone (PES) from 3M-Membrana [92]. Since the commercial membranes were available in various materials, several groups even compared them in terms of their ability to support cell adhesion. For example Saito and co-workers reported a comparison between polyimide, PSU and EVAL [95], and their results supported the use of EVAL membranes. In addition to better cell adhesion, EVAL presented there a high mechanical strength and therefore HFM had thin walls (25 μm). Table 3 (at the end of the chapter, page 38) summarizes the major HFM materials reported in literature as components of BAK in combination with cells from human origin.

4.2. Membrane permeability and selectivity

As mentioned earlier, mostly commercially available hemodialysis membranes were used for BAK. They usually present high permeability, in the ultrafiltration range. This property is important allow the transport of nutrients to the cells during the proliferation and maturation phases. During the function of the BAK, the high permeability should also allow:

- (1) the easy access of the toxins from the blood to the cells,
- (2) the excreted toxins from the cells to the waste,
- (3) the reabsorbed metabolites from the cells to the waste/blood

The high fluxes are achieved thanks to the relatively thin HFM wall and high porosity. The wall thickness reported in the literature varies between 25 μm and 145 μm . This parameter is strongly dependent on the material used, since the HFM needs to have a sufficient mechanical strength to allow handling. Most of the hemodialysis membranes applied are “asymmetric”, porous and open on the one side (dialysate) and have a “skin layer” with reduced pore size in contact with the blood. This skin layer really determines the membrane selectivity or MWCO. The hemodialysis membranes presents MWCO in the range 40-65 kDa, in order to prevent albumin leakage of the patients. Interestingly, the ideal MWCO for the BAK application is not very well defined in literature. According to most authors, the HFM should prevent albumin leakage. However, if the HFM are covered by a tight monolayer of ciPTEC, the cells should be able to act as a barrier against albumin loss. Moreover, in order to optimize the removal of protein bound toxins, albumin, the carrier, should be brought in close proximity to the cells. The MWCO of membranes used for BAK are presented in Table 3.

4.3. HFM diameter size and curvature

It has been shown that the increase of substrate curvature could up-regulate the PTEC functions without altering the confluent cell morphology [96]. Researchers cultured either canine MDCK cells or human HK-2 on HFM from PSf and PSf-PEG, with inner diameters of 0.4 mm, 0.8 mm and 1.2 mm. Although the cell monolayer morphology was always good, the activity of the brush border enzyme, of the glucose transporter (GLUT) and of the multidrug resistance- associated protein 2 (MRP2) was higher on HFM with a smaller inner diameter. In the literature, the range of internal diameters of the HFM is rather broad. In reality, since most researchers used commercially available membranes, inner diameters in the range 175-250 μm are used. Larger diameter membranes (490 μm) were reported too ([79]. Finally, it is interesting to consider the natural size of the proximal tubule. The diameters for human proximal tubule are ranging from 30 μm 60 μm [97]. Therefore, one could expect that a further decrease in the diameter of the HFM would be beneficial to the BAK function.

4.4. Membranes surface modifications for cell adhesion

All HFM for dialysis therapies, are developed to have a low cell adhesion and fouling during blood filtration. The chemical groups present at the membrane surface may explain its ability to support or not cell adhesion. The presence of apolar groups, such as methyl, has been shown to inhibit cellular attachment, whereas polar or charged functional groups, such as amino or carboxylic groups has been identified as promoting cell attachment [98]. Therefore, the cytocompatibility of the materials can be tailored by the incorporation of the desired groups to their surface, by synthesis of the copolymers or by surface modification. Most of the time, the easier and more commonly reported solution remains a HFM surface modification.

- Chemical surface modifications

The group of Zink [99] reported an extensive list of surface modifications applied to HFM membranes, among others, poly(maleic anhydride-alt-1-octadecene), oxygen plasma treatment and hydrogen peroxide. These techniques mostly aimed at increasing the presence of carboxylic acid groups on the surface of the membranes. The best cell attachment was achieved with a coating of L-3,4-dihydroxyphenylalanine (L-Dopa) (Figure 5). The same coating is used for many materials [100, 101], and various cell lines (human pluripotent stem cells for example [102], HK-2, HPTC and ciPTEC). This favorable cell attachment to L-Dopa coated substrates may be explained by the presence of additional groups such as amines. The group of Zink [99] also reported greater cell attachment while using a natural ECM coating, applied in addition to a surface treatment. These coatings will be described in the next sub-section.

Recent research makes use of the charge properties of the materials. The adhesion of the negatively charged cells is promoted to slightly positive surfaces. The group of Thomas Groth reported the use of Poly(ethylene imine) and / or poly(ether imide) as having good haemocompatibility, and as promoting cell attachment, proliferation and/or differentiation. [103-105]. In the case of membranes for BAK, the patent from Gambro reports coatings of poly(ethylene imine) to improve cell adhesion, too [106].

Figure 5. Scheme of cross-linking of L-Dopa during coating. Reprinted from [101] with the permission of Elsevier.

- Biologically derived extra cellular matrices

It has been shown that cell adhesion is linked to the presence and conformation of specific attachment proteins on material surfaces. The ECM, which surrounds cells in tissues (Figure 6), is composed of structural proteins, like collagens, adhesive proteins, and glycosaminoglycans [107]. The understanding of the composition of the ECM can help designing the membrane surface properties.

In the past few years, researchers tested various ECM compounds as coatings for adhesion of cell lines (HK-2) or HPTC. ECM coating was applied to the membranes for several hours [92, 108] and at a fixed concentration, prior to seeding the renal epithelial cells. The ECM coating stimulates cell adhesion and differentiation; successful cell differentiation causes ECM production by the epithelium (Jansen et al., 2014).

Collagen IV from human sources appears to be one of the best ECM coatings in both PES/PVP and PET membranes to support the adhesion and function of human PTEC, (HK-2, HPTC, ciPTEC) [82, 91, 92, 99, 108]. Interestingly, optimal results were obtained when collagen IV was coated after a first layer of L-dopa, which is shown to be involved in the formation of mussel's adhesive proteins [100]. L-dopa is negatively charged, and the combination with collagen IV (positively charged) can create optimal conditions for cell attachment and differentiation. One other successful ECM is Attachin from Bio999, reported by [81], in combination with EVAL HFM and lifespan extended PTEC. Attachin is reported to improve the adhesion of many cell-lines, however its formulation is not known and the availability of the product is limited to the Asian countries. Finally, Humes et al used pronectin-L and murin laminin to coat PSU HFM to support primary PTEC, from *in vivo* studies to clinical trials [109].

Figure 6. Essential extracellular matrix components. The native ECM is a key factor in inter- and intracellular signaling, regeneration, support and is a depot for growth factors, indicating its high relevance in cell maintenance. The cell-ECM adhesion and signaling is mediated by integrins, which are transmembrane receptors located in the PTEC plasma membrane. The ECM composition can be divided into two major components: the basement membrane (BM) and the stromal matrix (SM). The BM is a sheet-like scaffold mainly characterized by fibronectin, proteoglycans, laminin and collagen IV. The SM is made up of larger, fibrous structures, which provide the major structural support of the ECM, mainly Collagen I, proteoglycans and GAGs. Reprinted from [11] with the permission of Elsevier.

4.5. HFM challenges and perspectives

The combination of parameters – such as surface chemistry and topography – can have a significant impact on the cell attachment. Hulshof et al [110] reported a striking difference between the response of ciPTEC on PS and PES membranes (coated with L-dopa). While for PS the large topographic features did not adversely affect ciPTEC cell numbers and monolayer formation, the same features fabricated on PES disrupted the cell monolayer.

As discussed earlier, many HFM characteristics have shown to play a crucial role in cells attachment, growth, morphology and function, such as material, selectivity and curvature. The research to study the impact of those parameters was often performed by modifying one parameter at a time, while keeping the other parameters constant. A more systematic research, including design of experiments or “high throughput screening” [110, 111] could allow a better understanding of the impact of several parameters and their combination on the cell attachment and function. In depth knowledge of the effects of the above cited parameters could avoid the use of additional surface modification or ECM coatings to favor cell attachment. Finally, the surface properties can be optimized for a given cell line, but be detrimental for another one [112].

5. An improved replacement of the renal function – the BAK - history and perspectives

Table 3 highlights the major BAK prototypes - using human PTEC - and their characteristics.

Table 3: BAK systems using human kidney cell sources, main characteristics.

System - Name - Cell type - Cell seeding / culturing	Membrane characteristics - Material - Coating - MWCO	Testing and key output parameters	References
RAD primary – isolated renal tubule progenitor cells internal seeding culture under perfusion	PSU coated with murine laminin or bovine collagen IV 45 kDa / 50 kDa	1/ <i>in vitro</i> and preclinical: increased excretion of ammonia, glutathione metabolism, and production of 1,25- dihydroxyvitamin D3 2/ clinical- phase I and phase II a: more rapid recovery of kidney function, RAD well tolerated	1/ [77] 2/ [113]
RAD HK-2 cell-line transfected with pcDNA3.1-hEpo, internal seeding static culture	PSF coated with Laminin 50 kDa	<i>in vitro</i> : Gene expression and secretion of Erythropoietin (Epo)	[67]
BAK primary HPTC internal seeding culture under perfusion	PES/PVP/ NMP coated with L-Dopa and human collagen IV < 65 kDa	<i>in vitro</i> : immunostainings and qPCR gene expression	[79]

BAK primary: HPTC external seeding culture under perfusion	PAES (Gambro) no coating PSU (Fresenius) no coating PES/PVP – self- made, coated with L- Dopa and human collagen IV < 65 kDa	<i>in vitro</i> : OAT transport (Lucifer yellow) and uptake of urea and creatinine, high levels of IL-6 and IL-8	[94, 115]
BTB primary RPTEC with siRNA - mediated lifespan extension internal seeding culture under perfusion	EVAL - (Asahi Kasei Kuraray Medical) Attachin < 65 kDa	1/ <i>in vitro</i> : reabsorption of water, sodium and glucose, metabolisation of β 2- microglobulin and pentosidine 2/ <i>in vivo</i> : (AKI goats) expended life span; clearance of small solutes; decreased inflammatory cytokines 3/ <i>in vivo</i> : (AKI goats) culture in serum free media with a similar performance	1/ [81] 2/ [78] 3/ [80]
BAK ciPTEC cell line external seeding static culture	PES - MicroPES (3M - Membrana) L-Dopa and human collagen IV 150 kDa	<i>in vitro</i> : immunostainings and active uptake of organic cations	[92]

The first BAK, composed of proximal tubule epithelial cells grown inside ultrafiltration HFM, was proposed by Aebischer et al. in 1987 [93]. They achieved a continuous ultrafiltration for relatively long periods of time when using non-human derived (canine or porcine) cells. Since then, the group of David Humes developed a RAD system based on PSU HFM seeded first with porcine renal proximal tubule cells (LLC-PK1) and then with human PTEC. They first treated uremic animals [52, 77], showing active vectorial transport of sodium, bicarbonate, glucose and organic anions, enabling functional maintenance. Moreover, endocrine activity with conversion of 25-hydroxy(OH)-vitaminD3 to 1,25-(OH)₂ vitD3 was demonstrated in the RAD. Subsequently, the system passed the Phase IIa clinical trials successfully in 2005 for the treatment of

patients with AKI and CRF [113]. The Phase IIb clinical trials however were suspended for safety reasons: platelet count levels reached a lower limit of 35,000 per mm³ [109]. Moreover practical drawbacks such as cell expansion, differentiation, storage and transport issues were reported.

To overcome the previous issues, the Humes group recently developed a bioartificial renal epithelial cell system (BRECS) [114] composed of porous, niobium-coated carbon disks, retaining a dense population of allogenic renal epithelial cells (REC). After the cells reach an optimal density, the BRECS can be cryopreserved at -80 °C or -140 °C, transported, and stored. This unique design allows for long-term storage and should permit on-demand use for acute clinical applications. It could also be incorporated to a PD circuit and provide an improved PD wearable dialysis. This device has been recently tested *in vivo* on nephrectomized sheep for 24 hours and was demonstrated a stable uraemic state and endocrine support in the form of 1,25 vitamin D3.

Ni et al. used PES/PVP, PSU/PVP and PSU HFM in combination with a double coating and human PTEC [94, 99]. The first trials were however performed with MDCK cells, which adhered perfectly without coating on PES/PVP HFM [112]. The human PTEC reacted differently and required an additional coating or a different membrane material.

The same group presented a new model of BAK with PTEC seeded on the extraluminal side of the HFM in 2013 [94], [115] and showed improved PTEC performance without using coatings.

The group of Akira Saito has firstly worked with LLC-PK1 cells (porcine kidney) and MDCK cells (canine kidney) seeded inside coated polysulfone or cellulose acetate HFM. Later, they switched to human cells and had to readapt materials and coatings previously optimized for animal cells. They further developed a RAD using lifespan-extended human PTEC cultured in a newly developed serum-free medium. They compared its performance with BAK prepared with PTEC cultured in serum-containing conventional medium in AKI goats with positive results [80, 81]. Moreover, the group also considered developing a bioartificial glomerulus using CD133+ progenitor cells to replace the

conventional hemofilter which precedes the BAK [116]. Gambro [106, 117] proposed combination of a distal tubule part with a proximal tubule part. Their patent is based on internal, confidential reports and no published research articles are available. As a comparison, the group of Zink patented their BAK system [115] based on their publication [94].

Finally, the previously cited BAK prototypes showed function of the PTEC in terms of albumin uptake, transport of various ionic solutes, as well as the expression of several markers. However, none of them has shown the removal of protein-bound toxins. The groups of Stamatialis and Masereeuw have collaborated since 2009 to propose a “living membrane”. It is supported by PES based HFM with a double coating and ciPTEC [57], seeded on the extraluminal HFM side. The first *in vitro* tests of the small scale living membrane shown a healthy cell monolayer with function of several transporters [91], and active removal of several protein bound toxins [84]. Their living membrane has been also successfully upscaled [92], see Figure 7.

In order to ameliorate patients' quality of life and facilitate logistics, several research projects are focused on developing a wearable BAK – WEBAK - or even an implantable BAK - IBAK [118-121]. For this system, all of the elements of the “conventional” BAK extracorporeal circuit have to be miniaturized. An adequate source of energy has to be developed, as well. More importantly, the filtration system has to be stable on the long term when in contact with body fluids. The membranes have to be durable with excellent antifouling and anticoagulating properties [120].

The configuration proposed for WEBAK so far made use of PD and sorbent technology to regenerate PD fluid, in combination with a BRECS system, described in the previous section [122]. Currently, an IBAK which should be connected to the blood and bladder, fully replacing a kidney transplant is being developed in the University of California, San Francisco. In order to allow prolonged contact with blood and tissues, the prototype is making use of nanoporous silicone membranes for the blood filtration step [118, 119, 123, 124].

Figure 7. Functional upscaled "living membrane". (A) Picture of one module used for upscaled "living membrane" model. Three MicroPES hollow fiber membranes (HFM) within a housing composed of PE, PP and silicone parts, LuerLock fittings and caps. (B) SEM image of three MicroPES HFM. (C) Scheme of a transversal cut of one "living membrane". Not at proportional scale. (D) Representative confocal microscopy images of ciPTEC cultured on HFM with in blue the DAPI staining of nuclei and in green the immunostaining for ZO-1. (E) quantification of ASP+ uptake (10 mM) in the absence or presence of specific inhibitors (cationic uremic toxin mix (UTmix), cimetidine (cim, 100 mM)) in matured ciPTEC cultured on upscaled HFM. Reprinted from [92] with the permission of Elsevier.

6. Conclusion, challenges and perspectives

Since the first BAK has been presented 30 years ago [93], much progress happened in the field. The results clearly indicated the need of a reliable and consistent cell line as well as device related logistics such as cost-effective manufacturing, storage, and distribution process.

New renal cells, from human origin have been developed and fully characterized. Still many challenges exist, including the generation of cells with defined PTEC activity. Currently available cell lines yield promising results, nonetheless they are mostly highly valuable research tools. Such cell types face several questions when it comes to its use in an actual biomedical device, namely the immunological considerations. Since these cells are genetically modified, viruses were used to promote transfection of the vectors that enable immortalization and stability, which might provoke safety issues and require systematic testing as set by regulation authorities. For any cell type, it is important to design the BAK with the right fluidics to maintain cell viability and functionality. Indeed, high sheer stress may damage the cell monolayer. The study of the maximal toxins concentrations to which the device can be exposed is also of crucial importance, high toxin concentrations are leading to high cell mortality [125].

The BAK comes with many questions concerning logistics and costs. It should be first produced in a GMP environment, stored, transported, and used by qualified personal. Precise estimates for the number of expected AKI patients per day per participating medical center would be required to adjust the production rate and limit the storage time. The BRECS could be a solution to overcome the storage problems of the HFM based BAK since there it is possible to store the system using commercially available and FDA-approved cryopreservation media [114]. The BRECS is also tested to be part of a WEBAK. This device is now on the fast track process of the FDA registration, showing the importance of a treatment for kidney injury.

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8. List of abbreviations

AKI -	Acute kidney injury;
ARF –	Acute renal failure;
BAK –	Bioartificial kidney device;
BRECS –	Bioartificial renal epithelial cell system;
BTD –	Bioartificial renal tubule device;
CKD –	Chronic kidney disease;
CRF –	Chronic renal failure;
ESRD –	End stage kidney disease;
GFR –	Glomerular filtration rate;
HD –	Hemodialysis;
HF –	Hollow fiber;
HFM –	Hollow fiber membrane;
IBAK –	Implantable bioartificial kidney;
iPS -	induced pluripotent stem cells;
L-Dopa –	3,4-dihydroxy-L-phenylalanine;
LCC-PK1 -	Pig kidney epithelial cells;
MDCK –	Madin Darby canine kidney cells;
MHC -	Major histocompatibility complex;
MWCO –	Mass weight cut-off;

OAT -	Organic anion transporter;
OCT -	Organic cation transporter;
PD -	Peritoneal dialysis;
PSU -	PolySulfone;
PTEC -	Proximal tubule epithelial cell;
RAD -	Renal assist device;
RPTEC -	Renal PTEC;
RRF -	Residual renal function;
RRT -	Renal replacement therapy;
SLC -	Solute carrier family;
WEBAK -	Wearable bioartificial kidney.

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Chapter 3

Upscaling of a living membrane for bioartificial kidney device

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1. Abstract

The limited removal of metabolic waste products in dialyzed kidney patients leads to high morbidity and mortality. One powerful solution for a more complete removal of those metabolites might be the application of the bioartificial kidney device (BAK), which contains a hybrid “living membrane” with functional proximal tubule epithelial cells (PTEC). These cells are supported by an artificial functionalized hollow fiber membrane (HFM) and are able to actively remove the waste products. In our earlier studies, conditionally immortalized human PTEC (ciPTEC) have been shown to express functional organic cationic transporter 2 (OCT2) when seeded on small size flat or hollow fiber polyethersulfone (PES) membranes. Here, an upscaled “living membrane” is presented. We developed and assessed the functionality of modules containing three commercially available MicroPES HFM supporting ciPTEC. The HFM were optimally coated with L-Dopa and collagen IV to support a uniform and tight monolayer formation of matured ciPTEC under static culturing conditions. Both abundant expression of zonula occludens-1 (ZO-1) protein and limited diffusion of FITC-inulin confirm a clear barrier function of the monolayer. Furthermore, the uptake of 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP⁺), a fluorescent OCT substrate was studied in absence and presence of known OCT inhibitors, such as cimetidine and a cationic uremic mixture. The ASP⁺ uptake by the living upscaled membrane was decreased by 60% in the presence of either inhibitor, proving the active function of the OCT2. In conclusion, this study presents a successful upscaling of a living membrane with active organic cation transport as a support for BAK device.

2. Introduction

Within the growing worldwide population of kidney patients undergoing dialysis treatment, mortality (15–20% per year) and morbidity remain high [1]. One of the reasons could be the limited removal of protein-bound retention solutes [2, 3]. Their accumulation is strongly associated with the fatal outcome in the patients [4]. In the functional kidney, proximal tubule epithelial cells (PTEC), equipped with a broad range of transporters, mediate the excretion of those solutes. One of the PTEC transporters involved in the excretion of cationic uremic metabolites and drugs is the basolateral organic cation transporter – 2 (OCT2; SLC22A2).

The development of a PTEC-based bioartificial kidney (BAK) device could improve existing dialysis therapies for the removal of protein-bound uremic retention solutes [1, 5]. A key requirement for the BAK is the formation of a “living membrane” consisting of a tight monolayer of renal cells with preserved functional organic ion transporters, grown on an artificial porous hollow fiber membrane (HFM). One side of these HFM need to be highly haemocompatible since it would be in contact with blood, whereas the other side should be bioactive to support the formation of a cell monolayer.

Several groups have presented their achievements in upscaled BAK systems in recent years [6-8]. In terms of materials for the HFM, Polyethersulfone (PES), polysulfone (PSF), polyacrylonitrile (PAN) and cellulose acetate membranes have been mostly evaluated [9]. Those materials were chosen for their good haemocompatibility properties and limited fouling, and therefore had to be functionalized by various extracellular matrix (ECM) components, such as laminin, polylysine, pronectin, gelatin, and collagen IV [9-11]. As for the renal cell lines, the ones originating from humans are preferred to those from other species [12], [13]. Several groups showed the presence of various markers, indicating that the cells preserved their phenotype [14] without characterizing their function [10, 14-17]. Moreover, the most important drawbacks of primary cells are limited availability, low proliferative capacity and donor-to-donor variation.

To overcome these drawbacks, the recently developed and well-characterized conditionally immortalized human PTEC line (ciPTEC) is a suitable candidate to develop an efficient BAK system [18, 19]. The ciPTEC were transduced with human telomerase (hTERT) that limits replicative senescence by telomere length maintenance. In addition, their proliferation is controlled by the temperature sensitive vector SV40tsA58, allowing proliferation at 33 °C and differentiation in mature PTEC at 37 °C. Two of our recent studies proved the concept of using the PTECs for BAK application. In fact, when the cells were cultured on bioactive Polyethersulfone (PES)-based flat sheet membranes [11] or small size hollow fibers [20], they presented functional OCT2.

In this work, we investigated the upscaling of this concept and developed modules containing three “living membranes”. The transport properties and the quality and function of the grown ciPTEC monolayer were systematically investigated, including the expression of zonula occludens-1 (ZO-1) protein and the diffusion of fluorescein isothiocyanate (FITC)-labelled-inulin (inulin-FITC). Moreover, we also studied the uptake of a specific fluorescent OCT substrate in the presence or absence of the OCT inhibitors [21].

3. Materials and Methods

3.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) unless stated otherwise. MicroPES TF10 hollow fiber capillary membranes (HFM) (wall thickness 100 µm, inner diameter 300 µm, max pore size 0.5 µm) were purchased from 3M - Membrana GmbH (Wuppertal, Germany). Conditionally immortalized human renal proximal tubule epithelial cells (ciPTEC) were cultured as described previously [18].

3.2. Module preparation

MicroPES HFM were mounted into mini modules composed of Kartell PP T-shaped connectors (Fisher Scientific, Landsmeer, the Netherlands) and PE rigid semi-transparent tubing diameter 6-8 mm (VWR International B.V, Amsterdam, the Netherlands), see Figure 1. The modules were potted with Polyurethane bi-component resin (Intercol B.V, Ede, the Netherlands), allowed to dry for at least 24 h, and cut open. To ensure further ease of use of the modules, we added Luer Lock fittings and caps (Cole Palmer, Metrohm Applikon BV, Schiedam, the Netherlands) to the inlets and outlets, and connected them together using silicon tubing (VWR International B.V, Amsterdam, the Netherlands). Three fibers were mounted in every module, with an effective length of 8.5 ± 0.5 cm and a surface of 4.01 ± 0.25 cm² available for cell seeding. After cell seeding, extra luminary inlets were supplemented with gas exchange Sartorius Minisart sterile filters (Fisher Scientific, Landsmeer, the Netherlands).

3.3. Membrane sterilization and coating

The bioactive coating was performed on the extraluminal side of the HFM, with slight modifications to the previously established methods [9, 20]. The modules were sterilized using 70% (v/v) EtOH incubation for one h, washed and incubated using sterile 10 mM Tris buffer (pH 8.5) for 1 h. The primary coating component L-Dopa (L-3,4-dihydroxyphenylalanine, 2 mg·ml⁻¹) was dissolved in Tris buffer at 37 °C for 45 min. The L-Dopa solution was sterile filtered and injected in the extraluminal space of the module, completely filling it. The primary coating was performed at 37° C, on a shaking device, for 20 h. Afterwards, the L-Dopa solution was removed; the modules were washed with Hank's balanced salt solution (HBSS) (Fisher Scientific, Landsmeer, the Netherlands) buffer and filled with the second coating component - human collagen IV (25 µg·ml⁻¹ in HBSS). The coating with collagen IV was performed at 37 °C, on a shaking device, for 2 h. Finally, the collagen IV solution was removed and the modules were washed with HBSS.

Figure 1. **Upscaled "living membrane"**. (A) Picture of one module used for upscaled "living membrane" model. Three MicroPES hollow fiber membranes (HFM) within a housing composed of PE, PP and silicone parts, Luer Lock fittings and caps. (B) SEM image of three MicroPES HFM. (C) Scheme of a transversal cut of one "living membrane". Not at proportional scale.

3.4. Membrane characterization

The membrane topography of both uncoated and coated HFM was visualized using scanning electron microscopy (SEM), as described previously [11, 20]. We used an OSMO Inspector automated setup (Convergence B.V, Enschede, The Netherlands) to quantify the transport of pure water (Merck Millipore, Billerica, MA) and PBS solutions with $1\text{ mg}\cdot\text{ml}^{-1}$ bovine serum albumin (BSA) and $0.02\text{ mg}\cdot\text{ml}^{-1}$ immunoglobulin G (IgG) solutions through the uncoated and

coated cell-free HFMs. The flux through the membranes (J , in $\text{l}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$) was plotted as a function of the Transmembrane Pressure (ΔP , in bar). The permeance (L , in $\text{l}\cdot\text{m}^{-2}\cdot\text{h}^{-1}\cdot\text{bar}^{-1}$) was calculated from the slope of this curve. Every pressure step was maintained for 30 min. The sieving coefficients (SC) for BSA and IgG were determined by dividing the concentration of these proteins in the permeate by their concentration in the feed protein solution. BSA and IgG concentrations were measured by spectrophotometric analysis (Agilent Technologies, Cary 300 UV-Vis system) using quartz cuvettes at 280 nm.

3.5. Cell culture

The ciPTEC cell line [18] was cultured at a 33°C proliferating temperature in a ciPTEC complete medium prepared as follows: phenol red free Dulbecco's modified Eagle medium DMEM-HAM's F12 (Lonza; Basel, Switzerland) was supplemented with 10% v/v foetal calf serum (FCS; Greiner Bio-One; Alphen a/d Rijn, The Netherlands), insulin, transferrin, selenium (ITS I = $5\mu\text{g}\cdot\text{ml}^{-1}$; T = $5\mu\text{g}\cdot\text{ml}^{-1}$; S = $5\text{ng}\cdot\text{ml}^{-1}$), $36\text{ ng}\cdot\text{ml}^{-1}$ hydrocortisone, $10\text{ ng}\cdot\text{ml}^{-1}$ epidermal growth factor and $40\text{ pg}\cdot\text{ml}^{-1}$ tri-iodothyronine. Antibiotics were added to the medium only in the proliferating culture. During maturation at 37°C, the culture media were antibiotic free. CiPTEC were cultured up to a maximum of 40 passages, during which the proximal tubular characteristics remain unaltered [18].

3.6. HFM Modules handling

Prior to cell seeding, modules were incubated for 1 h in ciPTEC complete medium. Proliferating 90% confluent ciPTEC were detached using Accutase (StemPro® Accutase®, Life Technologies Europe BV, Bleiswijk, the Netherlands), centrifuged and suspended at $2.5\text{ million cells}\cdot\text{ml}^{-1}$ density in the ciPTEC complete medium. The modules' extraluminal space was completely filled with the cell suspension. To promote initial cell attachment, the modules were placed at 33°C, 5% CO_2 for 8 h, with a rotation of 90 degrees every 2 h. Afterwards, modules were washed with the ciPTEC complete medium, provided

with gas exchange filters and cellular proliferation was allowed for additional 64 h. Finally, the temperature was changed to 37°C for 7 days to promote the formation of a differentiated monolayer. During the whole culture period, ciPTEC were supplemented with fresh culture media every day.

3.7. Immunochemistry

The expression of OCT2, zonula occludens-1 (ZO-1) in ciPTEC monolayers on hollow fibers were investigated using the immunocytochemistry methodology as previously described [19] and examined under the Nikon confocal A1/ super resolution N-STORM microscope (Nikon Instruments Europe B.V, Amsterdam, The Netherlands). Images were captured using the NIS-elements analysis software, version 4.40.000.

3.8. Transepithelial barrier function

Paracellular permeability was quantified in the living membranes following the previously described method [20]. Shortly: after washing the modules with Krebs-Henseleit buffer supplemented with HEPES (10 mM; KHH buffer), inulin-FITC ($0.1 \text{ mg}\cdot\text{ml}^{-1}$ in KHH buffer) was perfused at $18 \text{ ml}\cdot\text{h}^{-1}$ at 37°C for 15 min. The fluorescence of the samples and of a standard range of concentrations was measured using a Tecan infinite M200PRO plate reader (Tecan Austria GmbH) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Coated and uncoated HFM without cells were used as negative controls. The flux of inulin-FITC (J) was calculated in $\text{pM}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$, using an average molar mass of $4500 \text{ mg}\cdot\text{mM}^{-1}$ for inulin-FITC.

3.9. Functional organic cation transport

The activity of OCT2 was evaluated by perfusing the modules with the fluorescent OCT substrate 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP⁺, 10 μM) in KHH buffer, at $18 \text{ ml}\cdot\text{h}^{-1}$ at 37°C for 30 min. The assay was performed in the presence or absence of a cationic uremic toxin mix (UT mix; 10 times the uremic plasma concentrations reported in the literature [21]) or cimetidine (100 μM). After perfusion, the modules were washed with KHH

buffer and fixed for 30 min in a fixing solution (4% Sucrose, 2% Paraformaldehyde in HBSS).

The modules were carefully cut open and the extracted fibers were mounted on microscopy slides using Dako fluorescent mounting media (Dako Netherlands B.V, Heverlee, Belgium). Slides were directly analysed at a constant laser intensity using the Nikon confocal A1/ super resolution N-STORM microscope. Images were captured using the NIS-elements analysis software (version 4.40.000) and quantification of the data was performed using ImageJ software (ImageJ 1.40g, NIH, USA). The average pixel intensities of four cells in the focal plan were extracted for the various conditions.

3.10. Data analysis

Every experiment was performed at least in duplicate. The number of samples (n) measured is indicated in each case and presented in each figure legend. The results are presented as mean $\pm$ standard deviation. Statistical analysis was performed in the SPSS software (IBPM SPSS Statistics version 23.0) using a one-way analysis of variance (ANOVA) or Student's t-test, where appropriate. A P-value of < 0.05 was considered significantly different.

4. Results

4.1. Membrane characterization

The surface of MicroPES HFM was functionalized via a double coating to allow the formation of a tight homogeneous cell monolayer. This coating was successfully used on a flat PES membrane [11] and then on a single small (1 to 2 cm long) HFM [20]. Here, we performed the coating on the upscaled system of three HFM with a total surface area of $4 \pm 0.25 \text{ cm}^2$. Some previous studies [22] and preliminary experiments with shorter coating time (data not shown) suggested that a prolonged coating of membranes is necessary to generate a reliable and reproducible cell monolayer. Therefore, the previously optimized L-

Dopa coating time was extended until 20 h instead of 5 h (on small HFM) [20] or a few min (on flat membranes) [11]. The collagen IV coating time was also extended to 2 h instead of 1 h as was done previously [20].

Figure 2. **SEM images of MicroPES HFM.** (A) uncoated and (B) with L-Dopa and collagen IV double coating.

Fig. 2 compares the SEM images of the surface and cross-sections of uncoated and coated HFM in the absence of cells. Although we applied a longer coating time for L-Dopa, the pores remain open. The slight colour difference between uncoated and coated HFM is due to the charge of the sample during SEM analysis and does not reflect the coating state.

Figure 3. **HFM transport properties.** (A) Clean water fluxes (CWF) and (B) sieving coefficients of bovine serum albumin (BSA) and immunoglobulinG (IgG) for uncoated and coated HFM. (A) The slope of the CWF as a function of the pressure gives the membrane permeance. Data are shown as mean $\pm$ standard deviation of three samples per case. * $P < 0.001$ using an unpaired t-test.

The effect of the coating on the membrane transport properties was measured by clean water and proteins transport experiments. Figure 3A presents the clean water flux (CWF) through the uncoated or coated HFM at different pressures and Figure 3B presents the SC of BSA and IgG at the same pressure range. The clean water permeance of the coated membranes ($(10.6 \pm 1.7) \cdot 10^3 \text{ l} \cdot \text{m}^{-1} \cdot \text{h}^{-1} \cdot \text{bar}^{-1}$) is lower ($P < 0.001$) than the permeance of the uncoated membranes, $((18.2 \pm 1.9) \cdot 10^3 \text{ l} \cdot \text{m}^{-1} \cdot \text{h}^{-1} \cdot \text{bar}^{-1})$ but is still very high. These results are consistent with the SEM observation that the pores of the coated membranes are open (Figure 2B). Besides, BSA with a molecular weight ~ 66 kDa still passes freely through the coated membrane, as demonstrated by the SC (0.99 ± 0.00 versus 0.98 ± 0.01), Figure 3B. The passage of a larger protein molecule, IgG (molecular weight ~ 166 kDa), is partially affected (SC 0.79 ± 0.04 versus 0.97 ± 0.02 for the uncoated HFM, $P < 0.001$). These results indicate a slight decrease of the pore size caused by the prolonged coating.

4.2. Cell monolayer integrity

Figure 4 shows representative images of ciPTEC cultured on MicroPES HFM. Only a few cells can be found on the uncoated HFM (Figure 4A). The L-Dopa (5 h) and collagen IV (1 h) double coating strongly improves cellular adhesion of the ciPTEC (Figure 4B), but does not lead to the formation of a complete cell monolayer. The prolonged L-Dopa (20 h) and collagen IV (2 h) coating allows the formation of a uniform ciPTEC monolayer (Figure 4C and D). In this condition, the abundant expression of the Zonula Occludens 1 (ZO-1) protein along the cell boundaries confirms the presence of tight junctions between the cells. In addition to a polarized epithelial barrier, the tight junction proteins contribute to fluid and ion homeostasis mediated by paracellular transport [23]. This tight cell monolayer is present on the whole length of the HFM within a module and is achieved for all three HFM (data not shown).

Figure 4. Monolayer quality of ciPTEC cultured on HFM. (A, B, C, D) Representative confocal microscopy images of ciPTEC cultured on HFM with in blue the DAPI staining of nuclei and in green the immunostaining for ZO-1 (A, B, C, D). A – Uncoated HFM. B – HFM with short coating time. C and D – HFM with optimal coating time.

Figure 5 presents the paracellular leakage of inulin-FITC as mean $\pm$ standard deviation of three experiments performed at least in triplicate. Since no active transcellular transport has been reported for inulin-FITC, its paracellular leakage was used as an indicator of passive diffusion and thus monolayer tightness [11]. When compared to the uncoated or coated HFM without cells, the coated HFM with renal cell monolayer presents a low inulin-FITC leakage

($973 \pm 70 \text{ pM} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ for the coated HFM and $1016 \pm 2 \text{ pM} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ for the uncoated vs $301 \pm 103 \text{ pM} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$, respectively ($P < 0.001$ using an unpaired t-test)). Consistent with the previous microscopy data, the low inulin-FITC paracellular leakage demonstrates formation of a tight ciPTEC monolayer.

Figure 5. **Monolayer integrity of ciPTEC cultured on HFM.** Inulin-FITC paracellular leakage. Data are presented as the mean $\pm$ standard deviation of at least 4 samples. * $P < 0.001$ using an unpaired t-test.

4.3. Cell function – ASP⁺ uptake

To further examine the functionality of the matured ciPTEC monolayers grown on functionalized HFM modules, we assessed the activity of OCT2, which is expressed along the basolateral membrane of PTEC. This transporter is responsible for the uptake of cationic uremic metabolites [21] and may be crucial for their renal elimination. Here, we perfused the HFM modules intraluminally with ASP⁺, an OCT2 fluorescent substrate, in the absence or presence of competitive inhibitors of OCT-2 mediated uptake: either Uremic Cationic toxin mix (UT mix) or cimetidine [21].

Figure 6. **Functional organic cation transporter - 2 transport.** (A) Representative confocal microscopy images and (B) quantification of ASP⁺ uptake (10 μ M) in the absence or presence of specific inhibitors (cationic uremic toxin mix (UT mix), cimetidine (cim, 100 μ M)) in matured ciPTEC cultured on upscaled HFM. The perfusion lasted for 30 min. Data are normalized against ASP⁺ uptake in the absence of inhibitors and presented as mean $\pm$ standard deviation of four measurements of the intensity of four cells in the projection plan, from two HFM containing modules. * $P < 0.001$ using ANOVA.

Figure 6A shows representative confocal images of the three conditions. The uptake of the ASP⁺ alone gives a strong fluorescent signal, which is significantly reduced in presence of either uremic toxin mix or cimetidine (Figure 6B). The intensity of the fluorescent signal in presence of inhibitor is normalized to the signal of the uptake of the ASP⁺ alone, after 30 min of perfusion. The ASP⁺ uptake is inhibited by ~60% by both UT mix and cimetidine (39% and 36% of the original intensity, respectively), confirming a functional monolayer is created on double-coated HFM in the modules.

5. Discussion

In this study, we developed upscaled modules of “living membranes” for BAK with a functional ciPTEC monolayer grown on coated MicroPES HFM. The double coating of L-Dopa and collagen IV was thin and preserved the membrane transport properties. The cells formed homogeneous monolayers with well-expressed ZO-1 protein and low paracellular leakage. As proof of concept, the obtained ciPTEC monolayers demonstrated active uptake a specific OCT substrate, demonstrative of the successful upscaling of a “living membrane” suitable for a BAK device.

The MicroPES membrane is designed for plasma fractionation and, therefore, has antifouling properties. Hence, it does not promote cell adhesion and requires a functionalization on the extraluminal side. The first coating component - L-Dopa - is enriched in mussel-like adhesive proteins and can adhere to a wide range of surfaces [24]. The second coating component - collagen IV - is an essential positively charged component of the extracellular matrix of renal tubular epithelial cells and has been successfully used previously, in combination with the negatively charged L-Dopa, to establish a proximal tubule cell monolayer both for primary cells [14] and ciPTEC [11]. To support the growth of ciPTEC on the outer surface of the HFM (4 cm²), this double coating, initially applied for a few min for PES flat membranes [11], has been later applied for several h (5 h L-Dopa + 1 h collagen IV) for single fiber

HFM (0.13 cm^2) [20]. However, when such coatings were applied in our module system, no reproducible monolayer could be obtained (Fig. 4B). We had to prolong the L-Dopa coating to 20 h, and therefore came closer to the coating conditions previously applied for primary renal cells [14]. As to the collagen IV, the coating was also extended to obtain the desired deposition pattern to promote the initial cell adhesion. Moreover, a low speed dynamic regime during the L-Dopa coating (shaking or low flow) was applied to ensure a good homogeneity of the coating on the whole membrane length and within all the HFM of the same module.

The double coating seems to decrease the membrane pore size as shown by the clean water flux and the sieving of BSA and IgG experiments. However, the transport through the membrane is still high, allowing transport of nutrients and toxins. Compared to the previous study with the small HFM [20], both clean water permeance and IgG sieving coefficients are decreased ($(10.6 \pm 1.7) \cdot 10^3 \text{ l} \cdot \text{m}^{-1} \cdot \text{h}^{-1} \cdot \text{bar}^{-1}$ versus $(16.4 \pm 0.7) \cdot 10^3 \text{ l} \cdot \text{m}^{-1} \cdot \text{h}^{-1} \cdot \text{bar}^{-1}$ and 0.79 ± 0.04 versus 0.90 ± 0.01 respectively). The fact that both BSA and IgG can pass through the HFM and reach the ciPTEC monolayer should not be detrimental for a BAK application, since the ciPTEC tight monolayer is intended to form the functional barrier against the loss of essential proteins by the patient. Besides, the transport of albumin through the membrane towards the ciPTEC monolayer may be advantageous for the elimination protein bound compounds [1], since those need to be in close proximity to the cell transporters.

The ciPTEC monolayer was characterized after 7 days of maturation at 37°C in the upscaled modules. The confocal microscopy analysis shows regular nuclei, homogeneous cell shapes and abundant expression of the tight junction protein ZO-1. This underlines the epithelial character of the ciPTEC monolayer, which is confirmed further by low inulin-FITC diffusion. When compared to the results obtained for the single HFM [20], the inulin transport through the HFM both with and without cells is similar (without cells: $973 \pm 70 \text{ pM} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ now compared to $1200 \pm 193 \text{ pM} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ previously; with cells: $303 \pm 129 \text{ pM} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ now compared to $373 \pm 42 \text{ pM} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ previously). A similar range of inulin-FITC permeability was found earlier *in vitro*, too [25]. Apparently,

the longer coating time in this study (versus the small scale HFM) does not affect the inulin diffusion ($973 \pm 70 \text{ pM} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ for the coated HFM versus $1016 \pm 2 \text{ pM} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ for the uncoated HFM). This result was to be expected since the inulin-FITC is much smaller than BSA (inulin has a molecular weight of $\sim 4.5 \text{ kDa}$ and BSA has one of $\sim 66 \text{ kDa}$), which passes freely through the membrane. Finally, the high standard deviation ($303 \pm 129 \text{ pM} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ Fig. 4) obtained for the HFM with cell monolayer may suggest some local irregularities in the epithelial barrier due to the more complex handling and characterizing procedures. In other studies, intraluminal seeding of the PTEC was preferred [6, 26, 27] and required an inverted design of the HFM, with a selective layer on the extraluminal wall to avoid pore clogging [14]. Though the extraluminal cell seeding has been reported less frequently [15], it allows the use of commercial HFM designed for plasma fractionation, having the selective layer on the intraluminal wall. This, together with the compatibility with our imaging techniques, justifies the configuration used here.

Finally, our upscaled system can preserve the functionality of the OCT-2 of the ciPTEC monolayer, which is crucial to ensure the clearance of cationic uremic solutes [28, 29] in a BAK application. We analysed the uptake of the specific fluorescent substrate ASP^+ and its inhibition in the presence of a polyamine and guanidino cationic uremic toxin mixture (UT mix, [21]), as well as in the presence of cimetidine [30-32]. The intensity of the intracellular fluorescence signal corresponding to the ASP^+ uptake alone is very strong (Fig. 5A). This intensity decreases dramatically in presence of either inhibitor. The inhibitory effect of the UT mix observed here, is slightly stronger than that for a small fiber [20], probably due to either the prolonged uptake time (30 min instead of 13 min), or the system upscaling (4.01 cm^2 against 0.13 cm^2 previously). Indeed, in the upscaled system, 4.01 cm^2 of cells received daily 2.5 ml of medium. The small HFM were cultured in 6 well plates, and thus 0.13 cm^2 of cells were receiving 2.5 ml of medium every 2 or 3 days. In order to compare more systematically both systems, systematic analysis should be performed at several time points.

6. Conclusion & outlook

This work presents the upscaling of a “Living Membrane” comprising of functionalized MicroPES HFM supporting conditionally immortalized PTEC. This Living Membrane, developed under static culturing conditions, exhibits a uniform, reproducible and tight ciPTECs monolayer. Our work shows that PTEC cultured in this upscaled system feature active organic cationic transport, crucial for the removal of uremic cationic metabolites.

The next step towards a functional BAK device is to culture the cells while exposing them to a unidirectional flow with relative shear stress to mimic the natural kidney proximal tubule physiology. There is evidence [33] the ciPTECs brush-border barrier height and therefore surface available for uremic metabolites uptake increases significantly when culturing cells under shaking conditions in a 2D environment. This result, supported by similar evidence for other cell lines [34-37], suggests better toxin removal under dynamic conditions. Finally, a ciPTEC-based BAK device should also ensure a sufficient toxin clearance for a prolonged session. Here, the upscaled “living membrane” has been tested for 30 min. Therefore future work should evaluate longer clearance periods.

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8. List of abbreviations

ASP ⁺ -	4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide
BAK -	bioartificial kidney device
BSA -	bovine serum albumin
ciPTEC -	conditionally immortalized human proximal tubule epithelial cells
CWF -	clean water flux
ECM -	extracellular matrix
HBSS -	Hank's balanced salt solution
HFM -	hollow fiber membrane
IgG -	immunoglobulin G
Inulin-FITC -	fluorescein isothiocyanate (FITC)-labelled-inulin
KHH -	Krebs-Henseleit buffer supplemented with HEPES (10 mM)
L-Dopa -	3,4-dihydroxy-L-phenylalanine
PES -	polyethersulfone
PTEC -	proximal tubule epithelial cell
SEM -	scanning electron microscopy
SC -	sieving coefficient
UT mix -	uremic toxin mix
ZO-1 -	zonula occludens-1

9. References

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Chapter 4

Strategies to achieve good quality kidney cell monolayer on the inside of a hollow fiber polymeric membrane

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Abstract

The bioartificial kidney (BAK) might be a powerful solution for the active removal of protein bound toxins and the replacement of endocrine and metabolic functions for patients with end stage renal disease. In chapter 3 we developed an upscaled BAK prototype containing human conditionally immortalized proximal tubule epithelial cells (ciPTEC) monolayer. The ciPTEC, grown on the external surface of functionalized polyethersulfone (PES) hollow fiber membranes (HFM), expressed functional organic cationic transporter 2 (OCT2). In order to further upscale the system, more HFM, and therefore a tight fiber packing in the module, are necessary which could damage the cell monolayer. Since the monolayer integrity is essential to the BAK function and longevity, cell culture on the internal surface of the HFM would be highly beneficial. Besides, a monolayer of PTEC grown on the inner surface of the HFM presents greater similarity with the real nephron, and it appears to be a necessary requirement for a functional BAK.

Here, we investigate strategies for achieving a good quality CiPTEC monolayer on the inside of the polymeric HFM. We first optimize the functionalization of the internal surface of the polymeric fiber to achieve high transport of metabolites. Secondly, we investigate several cell seeding parameters in order to achieve a tight ciPTEC monolayer on the inside of the HFM.

1. Introduction

Currently, about two million people worldwide having end stage renal disease (ESRD) are treated with hemodialysis [1]. This therapy can only remove small waste compounds, leaving larger molecules and protein-bound toxins to accumulate in a patient. This limited clearance capacity, as well as, the lack of metabolic and endocrine kidney functions, contributes to disease progression, leading to high morbidity and mortality rates [2-4].

A solution to circumvent those problems and to replace essential renal functions could be the application of a bioartificial kidney (BAK). The BAK uses proximal tubule epithelial cells (PTEC) to actively remove protein-bound toxins. By formation of a tight monolayer on a hollow fiber membrane (HFM), toxins could be removed from the blood stream without loss of important blood components [5]. The BAK is conceived to be used in combination with a classical hemofilter [6, 7]. First, the glomerular function is replaced by the classical hemofiltration for removal of small size water-soluble molecules. Second, the glomerular filtrate, which comes out of the hemofilter, can be processed by the PTEC of the BAK [5].

In our recent work, we have developed an upscaled BAK where the human conditionally immortalized proximal tubule epithelial cells (ciPTEC) were cultured on the external surface of functionalized MicroPES HFM. The ciPTEC have limited senescence, high replication capability and they present several functional transporters [8, 9], such as organic anionic transporters (OCT). The cells, therefore, showed active uptake of organic cationic toxins [10, 11]. This BAK, with ciPTEC monolayer grown on the external surface of the HFM, presented the advantage of a straightforward characterization of the monolayer quality via immunostaining. However, in order to achieve a clinical-size BAK, more HFM, and therefore a tight fiber packing in the module, would be necessary. If the cells are on the outside of the fibers, the mechanical stress and friction between the fibers could damage the cell monolayer [12]. Since the monolayer integrity is essential to the BAK function for guaranteeing the selectivity of the excreted molecules, cell culture in the bore of the fiber would

be highly beneficial for the BAK function and longevity. Besides, a monolayer of PTEC grown on the inner surface of the HFM presents greater similarity to the natural biological organization of the real nephron and therefore we would expect an improved PTEC adhesion and function there.

It is obvious that development of a BAK with a ciPTEC monolayer on the internal surface of the HFM is an important step towards realization of the clinically relevant large size BAK. Therefore, in this work we focus on strategies for achieving this. In fact, we perform:

- (i) Optimal functionalization of the polymeric fiber to achieve good cell adhesion. In previous work [10, 11], we used a double L-Dopa and Collagen IV (CIV) coating on the external surface of the HFM. This coating was tailored to achieve cell adhesion while preserving the access of cells by the toxins. Here, we optimize the coating on the inside of the HFM to achieve the same important adhesion and mass transfer requirements.
- (ii) Optimal cell seeding on the fibers. Since it is important to achieve a homogeneous monolayer with a clear barrier function (see Table S1, page), several parameters were investigated, including the cell seeding concentration, the number of cell infusions (how many times the cells are inoculated in the module), the initial attachment time, and the number of days of proliferation.

We characterize the fibers transport properties before and after coating, as well as, the homogeneity of the collagen coating. The cell adhesion on the developed fibers is quantified via cell detachment, and the monolayer barrier function via Inulin-FITC leakage. The results are compared to our previous studies with the cell monolayer on the outside of the fibers, as well as, to the existing literature.

2. Materials and Methods

2.1. Chemicals – Hollow fibers

All chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) unless stated otherwise. MicroPES TF10 hollow fiber capillary membranes (HFM) (wall thickness 100 μm , inner diameter 300 μm , max pore size 0.5 μm) were used for the cell culture and were purchased from 3M - Membrana GmbH (Wuppertal, Germany).

2.2. Module preparation, membrane sterilization, and coating

The membrane modules were prepared following the protocol presented previously [11]. Briefly, MicroPES HFM were mounted into mini modules composed of Kartell PP T-shaped connectors (Fisher Scientific, Landsmeer, the Netherlands) and PE rigid transparent tubing diameter 6-8 mm (VWR International B.V, Amsterdam, the Netherlands). The modules were sterilized using 70% EtOH incubation for 1 h, washed and incubated using sterile 10 mM Tris buffer (pH 8.5) for 1 h. The L-Dopa and Collagen IV coatings were applied at concentrations established previously [11], in the lumen of the HFM, at 37 °C on a shaking device, for a duration of 20h and 2h respectively. During the incubation with the L-Dopa and Collagen IV coating solutions, the outer compartment was filled with Tris and Hank's balanced salt solution (HBSS) buffer solutions, respectively.

2.3. HFM characterization – permeability

The MicroPES HFM transport properties were measured before and after the double coating, in the absence of ciPTEC. We used an OSMO Inspector automated setup (Convergence B.V, Enschede, The Netherlands) to quantify the clean water (Merck Millipore, Billerica, MA) flux (CWF) through the HFM (J, in $\text{l}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$) as a function of the transmembrane pressure (TMP or ΔP , in bar). The permeance (L, in $\text{l}\cdot\text{m}^{-2}\cdot\text{h}^{-1}\cdot\text{bar}^{-1}$) was calculated from the slope of this curve.

Every pressure step was maintained for 30 min, the first pressure step was meant as a washing and it was performed at the highest of the pressure steps.

The transport of immunoglobulin G (IgG) solution ($0.02 \text{ mg}\cdot\text{ml}^{-1}$ in PBS) through the fiber was investigated similarly to the CWF experiment. The IgG concentration in the feed and the permeate was measured spectrophotometrically (Agilent Technologies, Cary 300 UV-Vis system) using quartz cuvettes at a wavelength of 280 nm. The sieving coefficient (SC) of IgG was then calculated by dividing the IgG concentration of the permeate by the concentration of the feed.

2.4. HFM characterization – immunostaining

Collagen coating quality and homogeneity was investigated via immunostaining. The membrane module containing three fibers was first washed with HBSS to remove any medium. The inner surface of the membrane was then flushed with a blocking solution (2% FCS + 2% BSA + 0.1% Tween20 in HBSS) for 30 min. The blocking solution prevents non selective attachment of the antibodies to the HFM. Afterwards rabbit anti-human collagen IV antibody (Invitrogen, Life Technologies, Carlsbad, USA) ($5\mu\text{g}\cdot\text{ml}^{-1}$ in blocking solution) was injected inside of the HFM and incubated for 90 min. The module was then washed three times with HBSS. Goat anti-rabbit antibody with Alexa Fluor® 488 (Invitrogen, Life Technologies, Carlsbad, USA) was then injected into the HFM at a concentration of $10 \mu\text{g}\cdot\text{ml}^{-1}$ in blocking solution and incubated for 30 min. Any residual antibody was washed away with HBSS. Images were taken with the inverted EVOS fluorescence microscope.

2.5. Cell culture, cell seeding techniques and modules handling

The ciPTEC cell line [8] was cultured at a 33°C proliferating temperature and at 37°C maturation temperature in a ciPTEC complete medium prepared as described previously [11]. The ciPTEC were cultured in the absence of antibiotics up to a maximum of 60 passages. The modules handling was performed as reported previously [11, 13]. Prior to cell seeding, modules were

incubated for 1 h at 33 °C in ciPTEC complete medium. When the proliferating cells reached 90% confluence, they were detached using Accutase (StemPro® Accutase®, Life Technologies Europe BV, Bleiswijk, the Netherlands), centrifuged and suspended at the chosen concentration in the ciPTEC complete medium. The HFMs' intraluminal space was completely filled with the cell suspension. We used a 1 ml syringe and injected the cells vertically, from the bottom of the module, taking care of filling the whole HFM without air bubbles.

We applied two different cell seeding densities: $1.0 \times 10^5 \text{ cells}\cdot\text{ml}^{-1}$ and $1.0\text{-}2.5 \times 10^6 \text{ cells}\cdot\text{ml}^{-1}$. Besides, two different cell infusions methods were performed:

- (i) the ciPTEC were seeded once at the desired concentration in the lumen of the HFM. The module inlets were closed and the cells were then left to attach for 4 h at 33 °C, with a 90° module rotation every 1 h to facilitate cell adhesion to the entire surface of the HFM.
- (ii) the ciPTEC were seeded 4 times at the desired concentration, at 1 h interval, inside the lumen of the HFM. We rotated the module 90° in between each infusion. The total attachment time was 4 h. In between each infusion the modules were kept closed at 33 °C.

After cell attachment, the modules were washed with the ciPTEC complete medium, provided with gas exchange filters and the cell proliferation was allowed for 2 to 5 days at 33 °C.

Most of the modules were sacrificed during the proliferation period in order to identify the cell proliferation time. For some of the modules, the cells were let to proliferate for the previously defined time and then kept for one additional week at 37 °C, to allow for their maturation. During the culture period, ciPTEC were supplemented with fresh culture media every day.

2.6. Characterization of the proliferating ciPTECs

The cell detachment experiment was performed on proliferating ciPTEC. The culture medium was first removed from the module, which was subsequently washed with HBSS. Afterwards, 1 ml of Accutase was injected inside of the HFM and incubated at 37 °C for seven minutes. The modules were hit to make sure that the cells would detach from the HFM. The module was rinsed with 1 ml of medium to collect the detached cells. The cell suspensions were collected & counted with a Neubauer counting chamber.

2.7. Transepithelial barrier function of the matured ciPTEC

Paracellular permeability was quantified in the HFM with mature ciPTEC via inulin-FITC transport experiments. This value was compared to the inulin-FITC permeability in the HFM without cells. The modules were first washed with Krebs-Henseleit buffer supplemented with HEPES (10 mM) (KHH) and connected to a pump at one of the inlets. In the outer compartment 2 ml of inulin-FITC, prepared at a concentration of 0.1 mg·ml⁻¹ in KHH, was injected. A flow of 0.3 ml·min⁻¹ KHH was applied through the lumen of the HFM. The KHH circulating through the module was collected for 15 min. The inulin-FITC concentration was measured with a fluorescence plate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

2.8. Data analysis

Every experiment was performed at least in duplicate. The number of samples (n) measured is indicated in each case and presented in each figure legend. The results are presented as mean ± standard deviation. Statistical analysis was performed in the SPSS software (IBPM SPSS Statistics version 23.0) using a one-way analysis of variance (ANOVA) or Student's t-test, where appropriate. A P-value of < 0.05 was considered significantly different.

3. Results

3.1. HFM functionalization

In order to achieve optimal membrane functionalization on the inside of the fibers, we coated them with L-Dopa and Collagen IV. Our goal was to achieve high membrane fluxes to allow solutes exchange between the fluids and the cells, as well as, to achieve a homogeneous collagen pattern to assure cell adhesion.

Figure 1. HFM functionalization – 1/2. (A) Clean water fluxes (CWF) coated MicroPES HFM on the inside surface (this work) and on the outside surface [11]. The slope of the CWF as a function of the pressure gives the membrane permeance. Data are shown as mean $\pm$ standard deviation of four samples. (B) Sieving Coefficient (SC) of ImmunoglobulinG (IgG) through uncoated and coated HFM. Data are shown as mean $\pm$ standard deviation of three samples.

The effect of the coating on the membrane transport properties was evaluated by clean water and IgG transport experiments. Figure 1A compares the CWF of the (i) membrane coated on the inside and (ii) outside of the HFM at different transmembrane pressures (TMP). For both, the clean water permeance is high - $(10.6 \pm 1.7) \cdot 10^3 \text{ l} \cdot \text{m}^{-2} \cdot \text{h}^{-1} \cdot \text{bar}^{-1}$ [11] on the outside surface versus $(11.4 \pm 0.2) \cdot 10^3 \text{ l} \cdot \text{m}^{-2} \cdot \text{h}^{-1} \cdot \text{bar}^{-1}$ on the inside surface. Besides, IgG with a molecular

weight ~ 166 kDa passes freely through both uncoated and coated membranes, (IgG SC = 1 for both, see Figure 1B). These results indicate that the HFM remains highly permeable after the double coating.

In order to investigate the integrity and homogeneity of the collagen IV coating, an additional test – immunostaining for collagen IV – was applied to the uncoated and coated HFM. Figure 2 shows the collagen pattern inside a MicroPES HFM. The presence of the collagen IV coating is confirmed through a homogeneous collagen IV pattern, previously shown to be sufficient for ciPTEC attachment [10]. Two different collagen coating times (2 h and 20 h) lead to a similar pattern (Figure 2), therefore, the shorter coating time of 2 h was selected for further studies. The cell adhesion on this coating is presented in the next section.

Figure 2. **HFM functionalization - 2/2.** Representative fluorescent microscopy images of HFM with (B, C) and without (A) collagen IV coating (in green). The collagen coating was applied for 2 h (B) and for 20 h (C).

3.2. Cell seeding

In this work we applied two different cell seeding concentrations in the lumen of the HFM: $1 \cdot 10^5$ cells·ml⁻¹ and $1\text{-}2.5 \cdot 10^6$ cells·ml⁻¹. The selection of these concentrations was based on our previous experiments where ciPTEC were seeded on the external surface of the HFM ([11], $1\text{-}2.5 \cdot 10^6$ cells·ml⁻¹) and on flat membranes ([10], $1 \cdot 10^5$ cells·ml⁻¹).

We also investigated two different cell infusion methods: (i) one infusion, as performed in our previous work on the outside of the HFM [11], and by Sun et al [14] with different membrane, coating and cell line (Table S1); (ii) four infusions, showed to be successful by several authors for other cell lines [15-17] (Table S1, page 98).

The proliferation time was set at minimum 2 days. In our previous work, we used 2 days proliferation time [11] but in the literature significantly longer proliferation time (Table S1), ranging from 4 days [14] to 10 days [15] are also applied. We therefore considered that a higher proliferation time may be necessary to reach the desired amount of cells.

To assess the presence of cells in the membrane, we initially attempted to slice the fiber open and stain the cell layer. This, however, was very challenging and often cell sheets were detached. Therefore we decided to instead, detach and count the cells. Figure 3A presents the cell amounts detached from the HFM after different days of proliferation, for a cell seeding in one time performed with two seeding concentrations. Based on the fiber surface area of 3.4 cm^2 and the cell size of $700 \pm 150 \mu\text{m}^2$ theoretically, $400\text{-}600 \cdot 10^3$ cells are needed to cover the inner surface of the HFM. The amount of cells attached directly following the 4 h attachment time was quantified (day 0). For both seeding concentrations, the amount of cells after 2 days of proliferation is similar or even lower to the cells present immediately at day 0. This is typical of a lag phase, which is a period of slow growth when the cells are adapting to the culture environment and preparing for fast growth [18]. When seeded with the lower cell seeding concentration of $1 \cdot 10^5 \text{ cells} \cdot \text{ml}^{-1}$, the modules did not contain sufficient amount of cells - $(30 \pm 7) \cdot 10^3$ cells - after 5 days of proliferation. However, the cell seeding concentration of $1\text{-}2.5 \cdot 10^6 \text{ cells} \cdot \text{ml}^{-1}$ led to $(600 \pm 13) \cdot 10^3$ cells after 4 days of proliferation. Therefore, this concentration, seeding technique and proliferation time were chosen as optimal seeding parameters for the investigation of the formation of a tight monolayer in the following experiments.

Figure 3: Cell numbers and barrier function (A) Proliferation of the ciPTEC in the modules. Cell seeding in one time, cell concentration of 1.0×10^5 cells·ml⁻¹ and 1.0×10^6 cells·ml⁻¹. (B): Inulin-FITC transport through HFM without and with cells, grown inside upscaled HFM (this work), outside small HFM [10] and outside upscaled HFM [11]. Data are presented as the mean $\pm$ standard deviation of at least 3 samples. * $P < 0.001$ using an unpaired t-test.

Figure 3D presents the transport of inulin-FITC through HFM without cells and with mature ciPTEC. Since no active transcellular transport has been reported for inulin-FITC, its paracellular leakage can be used as an indicator of passive diffusion and thus monolayer tightness [11, 19]. The HFM with cells has low inulin-FITC leakage compared to the uncoated or coated HFM without cells, ($295 \pm 117 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ vs $1030 \pm 80 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$, respectively ($P < 0.001$ using an unpaired t-test)). This low inulin paracellular leakage indicates the formation of a tight ciPTEC monolayer and it confirms that the applied seeding / culturing configurations are appropriate.

4. Discussion

In the context of the upscaling of a BAK, a PTEC monolayer grown on the inner side of the HFM would be highly beneficial for the longevity and function of the cells in the device. To achieve this, both optimal functionalization of the HFM and seeding parameters are of high importance.

In this study, we coated MicroPES HFM with L-Dopa and Collagen IV and characterized the coating quality. The double coating preserved the membrane transport properties. This is an important aspect of the BAK development since the membrane needs to support the cell monolayer and to allow high access of toxins and nutrients to the cells. We then compared several seeding techniques in order to achieve a ciPTEC monolayer on the inner side of the functionalized MicroPES HFM. One combination of parameters was selected: seeding in one time with a concentration of $1\text{-}2.5 \cdot 10^6 \text{ cells} \cdot \text{ml}^{-1}$, 4 h of initial attachment and 4 days of proliferation. Mature ciPTEC, seeded following these parameters, showed low FITC-inulin transport, demonstrative of a clear barrier function and therefore successful formation of a cell monolayer.

The BAK presented here contained three MicroPES HFM each and the surface available for cell seeding was $\sim 3.4 \text{ cm}^2$. The system was similar to our previous study [11]; however, the coating and the seeding here took place on the inner

side of the HFM. This adaptation was possible thanks to the absence of a “skin” layer on the MicroPES HFM. Indeed, both the inner and outer surface of the membrane present similar surface morphologies, see supplement, figure S1. At higher magnification, the inner surface even appears to be rougher and more open, which may facilitate cell attachment [20, 21]. We first performed the coating using the same protocol applied previously [11], L-Dopa for 20 h and collagen IV for 2 h. Our results show that the membrane is highly permeable after the applied coating. The CWF of the membranes was measured from the outside to the inside, opposite of what was performed previously [10, 11]. The permeability of both coatings, on the outside surface - $(10.6 \pm 1.7) \cdot 10^3 \text{ l} \cdot \text{m}^{-1} \cdot \text{h}^{-1} \cdot \text{bar}^{-1}$ [11] - versus on the inside surface - $(11.4 \pm 0.2) \cdot 10^3 \text{ l} \cdot \text{m}^{-2} \cdot \text{h}^{-1} \cdot \text{bar}^{-1}$ - are identical. We confirmed the presence of the collagen via immunostaining, which showed an irregular pattern, conform to what was reported previously [10].

Once the coating conditions were optimized, we evaluated several seeding methods. We had some difficulties to characterize the cells with the previously reported Dapi staining [13], by cryotome sample preparation, and by Alamar Blue viability assay (data not shown). Cell proliferation was therefore successfully characterized by detaching ciPTEC from the HFM using Accutase, suspending in known amount of medium and counting. This technique clearly indicated the presence of high amount of cells inside the HFM and allowed to identify the most favorable seeding conditions. The seeding concentration of $1\text{-}2.5 \cdot 10^6 \text{ cells} \cdot \text{ml}^{-1}$ led to enough cells (~ 600.000 cells) to cover the whole inner walls of three HFM after 4 days of proliferation, with the seeding in one infusion.

Cell seeding concentration of a few million $\text{cells} \cdot \text{ml}^{-1}$ for achieving good cell monolayer was also necessary in our previous study for cell culturing on the outside of the fibers [11] and it has been reported in many other studies (see Table S1, , page 98). Nevertheless, it is also important to note that due to the variety of diameters of HFM used, the surface to volume ratio are very different between literature studies. As a consequence, when the seeding concentration in $\text{cells} \cdot \text{ml}^{-1}$ is converted to a seeding density in $\text{cells} \cdot \text{cm}^{-2}$, one can identify more differences between the studies. In fact, the seeding densities, reported

in Table S1, vary from 10^4 to 10^5 cells·cm⁻² for the PTEC seeded on the inner side of the HFM, and even up to 10^6 cells·cm⁻² for cells seeded on the outer side of the HFM. The seeding concentration we applied ($1\text{--}2.5 \cdot 10^6$ cells·ml⁻¹) also corresponds to cell density in the order of 10^4 cells·cm⁻², which can probably explain our favorable result.

In general a single cell infusion is highly preferably compared to multiple ones, since for the latter the chance of infections in the culture increases. Besides, the seeding in one infusion is globally preferable for an upscaling procedure, since it has limited handling and requires lower amount of cells.

In this work, the proliferation time of 4 days was doubled compared to our earlier study where ciPTEC were cultured on the outside surface of the HFM [11]. This longer proliferation time was necessary due to the difference in surface to volume ratios. Although the seeding concentration was similar, the resulting seeding density in the present configuration is much lower. As a consequence, a lower quantity of initially attached cells may indeed require supplementary proliferation time. In addition, a shorter attachment time of 4 h here, instead of 8 h in the earlier study for seeding on the outside, may as well explain the need for a longer proliferation time to reach confluency. Interestingly, even longer proliferation times, between 7 and 10 days, are reported in most of the studies with internal cell layers (Table S1). The longer proliferation time may be inherent to the cell lines used. The ciPTEC used here require shorter proliferation time which is advantageous concerning costs.

After reiteration of the previously identified optimal seeding conditions and 7 days of maturation at 37 °C, the BAK were exposed to inulin-FITC leakage. The inulin-FITC flux was lower for the HFM with cells than without cells (295 ± 117 pmol·min⁻¹·cm⁻² vs 1030 ± 80 pmol·min⁻¹·cm⁻², respectively). When compared to the results obtained for both the single HFM [22] and the upscaled system [11], the inulin transport measured here is similar (without cells: 973 ± 70 pM·min⁻¹·cm⁻² and 1200 ± 193 pM·min⁻¹·cm⁻² previously; with cells: 303 ± 129 pM·min⁻¹·cm⁻² and 373 ± 42 pM·min⁻¹·cm⁻² previously. Humes et al [23] using renal assist devices (RAD) also reported inulin leak rates 4 times lower

compared to those without cells. We can therefore conclude that in this work we identified the optimal seeding conditions to achieve a cell monolayer inside the chosen upscaled BAK system.

5. Conclusion & outlook

In the present study, the internal surface of commercially available MicroPES HFM was functionalized and seeded with ciPTEC. Cell proliferation and monolayer formation for different seeding conditions was investigated. The successful seeding parameters for the formation of the tight barrier were: cell seeding in 1 time with cell concentration of $1\text{--}2.5 \cdot 10^6 \text{ cells}\cdot\text{ml}^{-1}$, 4 h attachment, 4 days of proliferation and 7 days of maturation. In comparison to other studies, the procedure developed here presents two advantages: a single cell infusion and short proliferation time. It is possible that longer initial attachment time of 6 or 8 h, instead of 4 h, could lead to even shorter proliferation time and is therefore recommended for further work. Furthermore, testing of the upscaled system in the future with uremic toxins to confirm the cell function is required.

6. Acknowledgements

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7. List of abbreviations

BAK -	bioartificial kidney device
CiPTEC -	conditionally immortalized human proximal tubule epithelial cells
CWF -	clean water flux
ECM -	extracellular matrix
HBSS -	Hank's balanced salt solution
HFM -	hollow fiber membrane
IgG -	immunoglobulin G
Inulin-FITC -	fluorescein isothiocyanate (FITC)-labelled-inulin
KHH -	Krebs-Henseleit buffer supplemented with HEPES (10 mM)
L-Dopa -	3,4-dihydroxy-L-phenylalanine
PES -	polyethersulfone
SC -	sieving coefficient
SEM -	scanning electron microscopy
TMP -	transmembrane pressure

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9. Supplement

9.1. BAK bibliographic table

Table S1: BAK systems using human kidney cell sources, main characteristics. Reproduced and adapted from [5]

System - Name - Cell type - Cell seeding / culturing	Membrane characteristics - Material - Coating - Dimensions : (SA (cm ²) / ID (μm) / OD (μm) / WT (μm))	Detailed cell seeding parameters: - C (cells·ml ⁻¹) / D (cells·cm ⁻²) - seeding technique, rotations, - AT/ PT	References
RAD primary – isolated renal tubule progenitor cells internal seeding	PSU - coated with either murine laminin or bovine collagen IV SA 400, 700 or 1000 (ID 200 / WT 40) and (ID 250 / WT 70)	C 3 × 10 ⁷ / D 3.5 × 10 ⁵ 4 cell infusions separated by 1.5 h and a 90 ° rotation AT 6 h / PT 7-10 days	[15, 23]
RAD HK-2 cell-line, transfected, internal seeding	PSF - coated with Laminin (source?) (ID 225 / WT 45)	C 6 × 10 ⁶ / D 3 × 10 ⁴ 1 cell infusion, rotation 90 ° every 1 h AT 4 h / PT 4 days	[14]
BAK primary HPTC internal seeding	PES/PVP/NMP - self- made – coated with L-Dopa and human collagen IV (ID 490 / WT 145)	C 3 -5 × 10 ⁶ 4 cell infusions separated by 1.5 h and a 90 ° rotation AT 6 h / PT 9 days	[16]
BAK primary HPTC. external seeding	1/ PAES or PSU – no coating 2/ PES/PVP – self- made, coated with L- Dopa and human collagen IV (OD 315 / WT 50)	C 3-5 × 10 ⁶ 1 cell infusion, rotation 90 ° every 2 h AT 8 h / PT 7 days	[12, 24]

BTD	EVAL – coated with	C $4 - 10 \times 10^6$ / D $4 - 9 \times 10^4$	[17, 25, 26]
primary RPTEC, lifespan extension	Attachin SA 65 and 8000 (ID 175 / WT 25)	4 cell infusions separated by 1 h and a 90 ° rotation	
internal seeding		AT 4 h / PT 7 days	
		Total 5×10^6 / $3-7 \times 10^8$ cells	
BAK	PES – coated with	C 2.5×10^6 / D 5×10^6	[11]
ciPTEC cell line, transfected	L-Dopa and human collagen IV SA 4.01 +/-0.25	1 cell infusion, rotation 90 ° every 2 h	
external seeding	(OD 500 / WT 100)	AT 8 h / PT 2.5 days	

Abbreviations: SA: surface area, ID: inner HFM diameter, OD: outer HFM diameter, WT: wall thickness, C: seeding concentration, D: seeding density, TA: total amount of cells, AT: attachment time, PT: proliferation time

9.2. SEM images of MicroPES HFM

Figure S1. **SEM images of MicroPES HFM.** (A, B, C) internal side, (D, E, F) external side and (G, H) cross-section. Magnification x500, x950 and x9000.

Chapter 5

Development of bioactive surfaces for optimal renal cell adhesion

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Abstract

The development of cell based bioartificial kidney device (BAK) could improve existing dialysis therapies for (i) the removal of a broad range of uremic toxins including protein-bound ones and (ii) the replacement of endocrine and metabolic functions. A key requirement for such device is the formation of a “living membrane” consisting of a tight monolayer of functional proximal tubule epithelial cells (PTEC), on suitable artificial membranes. Recently, some of the authors presented such a concept: a monolayer of conditionally immortalized human PTEC (ciPTEC) with functional organic cationic transporter 2 (OCT2), achieved on a polyethersulfone (PES)-based flat membrane, after application of L-dopamine and collagen IV (L-Dopa/CIV) double coating. The human CIV used in those studies is very costly, and presents batch-to-batch variability, which could affect the development of a clinical size BAK. Therefore, in this work, we investigate alternative surfaces for the formation of living membrane. We study (i) PES membranes coated with heparan sulfate (HS) since HS is widely present in the kidney epithelial tissue and (ii) polyetherimide membranes because their positive electrostatic charge might facilitate the adhesion of negatively charged cells. For both (i) PES coated with HS and (ii) PEI membranes, combined with an L-Dopamine coating, the expression of zonula occludens-1 (ZO-1) protein confirms the systematic formation of a tight monolayer. Moreover, our preliminary results on PES membranes coated with L-Dopa and HS show limited transport of inulin-FITC, characteristic of a barrier function of the monolayer, and an active transport of creatinine, indicating a functional OCT2. In case of the PEI membranes, due to the low membrane porosity no transport experiments could be performed, nevertheless, a novel setup is used to quantify the cell adhesion strength, which is equivalent to the one achieved on the positive control PES coated with L-Dopa/CIV.

1. Introduction

The development of cell based bioartificial kidney device (BAK) could improve existing dialysis therapies for (i) the removal of middle size, big size and protein-bound toxins and (ii) the replacement of endocrine and metabolic functions [1]. A key requirement for such device is the formation of a “living membrane” consisting of a tight renal cells monolayer with preserved functional organic ion transporters, on suitable artificial membrane surfaces. Recently, some of the authors presented such a concept: a monolayer of conditionally immortalized human renal proximal tubular epithelial cells (ciPTEC) [2, 3] with functional organic cationic transporter 2 (OCT2), achieved on a polyethersulfone (PES)-based flat membrane, after application of L-dopamine and collagen IV (L-Dopa/CIV) double coating [4]. The latter coating was adapted for the specific fibers and cells, following earlier studies [5, 6]. Furthermore, this double coating was adapted for three dimensional system: first on a single small size hollow fiber membrane (HFM) [7] and then in an upscaled BAK prototype [8].

The human collagen IV used in those studies is very costly and as all of the nature-derived products, it may have high batch-to-batch variability, which could affect the reproducibility of the coating quality. Therefore, in this work, we investigate the ability of alternative surfaces to support ciPTEC adhesion and achieve a living membrane. We focus on (i) PES membranes coated with the glycosaminoglycan GAG heparan sulfate (HS) and on (ii) polyetherimide membranes. The HS domains are specifically located in basement membranes and/or surfaces of renal cells and display a characteristic distribution over the nephron [9], HS coating may thus improve ciPTEC adhesion. Moreover, since the cells are negatively charged, they may adhere well on a positively charged polyetherimide (PEI).

First, the natural extra-cellular matrix (ECM) of the ciPTEC is composed not only of collagen IV, but also of many other components, such as laminin, integrins and proteoglycans (PG), see Figure 1. PGs are composed of a core protein and one or more glycosaminoglycan (GAG) chains. GAGs are produced in every cell

of the human body and are found not only in the ECM but also on the cell surface, as well as in intracellular granules [9, 10]. GAGs are linear, strongly negatively charged polysaccharides consisting of repeated disaccharide building blocks. Based on the disaccharide composition, GAGs are classified into four groups: (i) hyaluronan; (ii) keratan sulphate; (iii) chondroitin sulphate (including dermatan sulphate) and (iv) heparan sulphate (HS, presented Figure 2A) [11]. GAGs are involved in various biological processes, such as cell–cell and cell–matrix interactions, cell migration and proliferation, chemokine and cytokine activation [12], and therefore can affect cell activities. [10]. As a consequence, the presence of GAGs such as HS on the polymeric membrane surface may favorably influence renal cell adhesion, the formation of a cell monolayer and its function.

Figure 1. Essential extracellular matrix (ECM) components. The native ECM is a key factor in inter- and intracellular signaling, regeneration, support and is a depot for growth factors, indicating its high relevance in cell maintenance. The cell-ECM adhesion and signaling is mediated by integrins, which are transmembrane receptors located in the PTEC plasma membrane. The ECM composition can be divided into two major components: the basement membrane (BM) and the stromal matrix (SM). The BM is a sheet-like scaffold mainly characterized by fibronectin, proteoglycans, laminin and collagen IV. The SM is made up of larger, fibrous structures, which provide the major structural support of the ECM, mainly Collagen I, proteoglycans and GAGs. Reprinted from [13] with the permission of Elsevier.

In this study, we coated HS on PES membrane (PES 50 kDa) used in earlier studies [4] for the collagen IV coating. This membrane has one haemocompatible smooth side, and one side used to apply the coating and support the cells. In order to improve the wettability of the PES membrane prior to HS coating, the presence of an adhesive such as L-Dopa may be necessary. L-Dopa has been widely used in our recent publications with easy and successful application [4, 7, 8].

Figure 2. **Schemes of chosen surface molecules** (A) Repeat unit of heparan sulfate (HS). (B) Repeat unit of polyetherimide (PEI).

Here we also investigate the attachment of ciPTEC based on PEI positively charged membranes. The PEI, presented in Figure 2B, is an amorphous, hydrolysis-resistant, high-temperature polymer. Its high aromatic content makes it extremely stable: it can be sterilized by ethylene oxide and high-energy radiations like gamma and e-beam without losing its physical and mechanical properties [14]. Besides, it is non-toxic in cell cultures and in addition were found to be haemocompatible [15]. *In vitro* studies using PEI membranes showed similar activity and behavior to tissue culture polystyrene (TCPS) for osteoblast cell line [16] and human dermal fibroblasts and keratinocytes [17]. PEI has even been successfully used in bio-hybrid liver device application [18]. Thanks to its low cytotoxicity, mechanical strength and resistance to sterilization, PEI is already used for a variety of commercially available medical devices applications, such as staplers, tubing, and drug delivery components [14]. It is important not to confuse polyetherimide (PEI) used in this study with polyethylene imine - presented in the supplementary material figure S1 - often referred to in the literature as PEI, too. Polyethylene imines are widely used for

biomedical applications such as transfections and drug delivery [19]. They are also positively charged, which facilitates cell adhesion and generally interaction with cells. Nevertheless, they have been shown to exhibit a high cell toxicity [20] and induce both necrosis and apoptosis [21].

For both surfaces, PES coated with HS and PEI membranes, the quality of the ciPTEC monolayer was systematically investigated by the expression of zonula occludens-1 (ZO-1) protein. The function of the ciPTEC monolayer was assessed for the PES membranes by (i) the diffusion of fluorescein isothiocyanate (FITC)-labelled-inulin (inulin-FITC), (ii) the transport of a cationic uremic toxin in the presence or absence of the OCT inhibitors [22]. The PEI membranes used here were non-porous, therefore, instead of transport experiments, a novel setup was used to quantify the cell adhesion strength.

2. Materials and Methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) unless stated otherwise. PES (molecular weight cut-off 50 kDa) flat membranes (ref 1465047D) were purchased from Sartorius Stedim Biotech GmbH (Göttingen, Germany). Polyetherimide (PEI) ULTEM™ 1000 resin was a gift from SABIC (Bergen op Zoom, The Netherlands).

2.2. PEI membrane preparation

PEI membranes were prepared as follows. PEI was dissolved in NMP at concentrations of 10, 15, 20 and 25 weight to weight (w/w) percent. The dissolution happened in 3-4 days, on a rolling bench at room temperature and then the solution was let to degas for one day. Membranes were cast on a glass plate, using a metallic casting knife of 250 μm . Subsequently, they were immersed in water coagulation bath. They were afterwards rinsed several times under gentle water stream and were kept in water until use.

2.3. Membranes handling

The PES and PEI membranes were cut to size with a puncher, and sterilized overnight in 4% paracetic acid (PAA) solution. They were transferred into (i) sterile 24 wells culture plates for the immunostaining and the cell attachment strength experiments and (ii) Corning (Corning Costar, NY, USA) custom 12 wells Transwell® systems for the transepithelial transport measurements.

The PES membrane has an asymmetric structure: one membrane side is denser (it is the selective layer and determines the transport properties) and smooth, while the other membrane side is more porous and rough. The coating was performed on the later side (see paragraph 2.4). The PEI membranes were a lot less porous, without visible difference between glass and air side (SEM images not shown). The “air” side of the PEI membranes during the casting, was used to perform the coating.

- (i) The membranes were fixed at the bottom of the culture plates under fluoroelastomer (FKM) O-rings (Eriks, the Netherlands). Prior to be used, the O-rings were washed in HBSS overnight, dried and autoclaved. Placing a membrane under an O-ring allows an optimal flatness of the membrane surface for a homogeneous coating and cell seeding procedure.
- (ii) The original bottom membranes of the Transwell inserts were removed and the inserts were cleaned and sterilized in 4% PAA. The PES and PEI membranes were installed and fixed using custom PEEK rings. For the coating procedures, the Transwell® inserts were placed on a flat polycarbonate surface to avoid the permeation of the coating through the membranes. Figure 3 provides a schematic overview of a coated Transwell® membrane[4].

Figure 3. **Schematic overview of the Transwell® set-up**, as used for the examination of basolateral to apical inulin, creatinine and ASP^+ transmembrane transport across ciPTEC monolayers. Indicated on the left are the basolateral and apical compartments. On the right side the ciPTEC monolayer and the L-Dopa/CIV double coating have been schematically represented. An identical setup was used for the other coatings, as well as for the reference membranes without cells. Reproduced and adapted from [4] with the permission of Elsevier.

After placing the membranes and prior to any coating and/or cell seeding procedure, the membranes were sterilized one more time in 4% PAA, rinsed 3 times in HBSS buffer and kept in the last HBSS buffer bath overnight. The HBSS buffer was removed in the minutes preceding the coating procedure.

2.4. Membranes coating

PES membranes were coated with (i) L-Dopa (D9628, Sigma Aldrich, Zwijndrecht, The Netherlands), (ii) a double layer of L-Dopa and human collagen IV (referred to as CIV) (C6745, Sigma Aldrich, Zwijndrecht, The Netherlands), (iii) a solution of HS from bovine kidney (H7640 Sigma Sigma Aldrich, Zwijndrecht), (iv) a double layer of L-Dopa and HS. PEI membranes were coated with (i) L-Dopa solution only, (ii) a double layer of L-Dopa and HS.

- (i) L-Dopa ($2 \text{ mg}\cdot\text{ml}^{-1}$) was completely solubilized in a Tris 10mM buffer at pH 8.5 for 30 min at 37°C , under stirring. Subsequently the L-Dopa solution was filter-sterilized and used immediately for the coatings. The L-Dopa was applied for 4 minutes at 37°C on the

membranes surface. Next, the L-DOPA solution was aspirated, and the membranes were left to dry for 5 min at 37°C.

- (ii) A CIV solution ($25 \mu\text{g}\cdot\text{ml}^{-1}$ in HBSS) was used for the second step of the coating. The CIV solution was applied for 4 minutes at 37 °C on the membranes surface. After fluid aspiration and drying for 5 min at 37 °C, the membranes were washed three times in HBSS to remove any remaining solvent or unbound compound.
- (iii) The HS coating was performed directly on the PES membranes or after a first L-Dopa coating as described below. To achieve coating with HS, membranes were incubated overnight at 4°C with HS solution ($20 \mu\text{g}\cdot\text{ml}^{-1}$ in an optimized HS coating buffer). Afterwards the membranes were rinsed three times in HBSS.

2.5. Characterization of the PES membranes transport properties

The PES membranes transport properties were measured before and after the coating, in the absence of ciPTEC. Uncoated flat membranes were tested after immersing them in HBSS overnight to remove preservatives added during the manufacturing process. Coated membranes were immersed in HBSS buffer for 30 min prior to use. The membranes were mounted in a stirred, air-pressurized, dead-end “Amicon type” ultrafiltration cell (Millipore; Billerica, MA, USA), hosting 2.2 cm diameter samples. The selective layer of the membranes was placed facing the feed solution.

We used an OSMO Inspector automated setup (Convergence B.V, Enschede, The Netherlands) to quantify the flux of clean water (Merck Millipore, Billerica, MA) or BSA solution ($1 \text{ mg}\cdot\text{ml}^{-1}$ in phosphate buffered saline (PBS)) through the membrane (J , in $\text{l}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$) as a function of the transmembrane pressure (TMP or ΔP , in bar). The membrane permeance (L , in $\text{l}\cdot\text{m}^{-2}\cdot\text{h}^{-1}\cdot\text{bar}^{-1}$) was calculated from the slope of this curve. Every pressure step was maintained for 30 min. The sieving coefficient (SC) for BSA was determined by dividing the BSA concentration in the permeate by that in the feed solution. $\text{SC} = 1$ means that

the protein passes freely through the membrane, while $SC = 0$ means that the membrane rejects the protein completely. BSA concentrations were measured by spectrophotometric analysis (Agilent Technologies, Cary 300 UV-Vis system) using quartz cuvettes at 280 nm.

2.6. Cell culture

The ciPTEC OCT2-expressing urine-derived cell line [2, 7, 8] was cultured at 33 °C proliferating temperature and at 37 °C maturation temperature in a ciPTEC complete medium. The latter was prepared as described previously [8]. Antibiotics were only added to the medium in the proliferating culture. During maturation at 37 °C, the culture media were antibiotic free. Proliferating 90% confluent ciPTEC were detached using Accutase (StemPro® Accutase®, Life Technologies Europe BV, Bleiswijk, the Netherlands), centrifuged and suspended at a concentration of $150.000 \text{ cells}\cdot\text{ml}^{-1}$ in the ciPTEC complete medium. The cells were seeded at a concentration of $133.000 \text{ cells}\cdot\text{cm}^{-2}$ on the membranes and were allowed to attach and proliferate at 33°C, 5% CO₂ for 24 h or 48 h. Afterwards, the ciPTEC complete medium was refreshed and the temperature was changed to 37 °C for 7 days to allow ciPTEC maturation. During the culture period, the ciPTEC were supplemented with fresh culture media every second day.

2.7. Immunochemistry – microscopy

In order to investigate the morphological characteristics and the monolayer integrity of matured ciPTECs cultured on the PES and PEI uncoated and coated membranes, immunocytochemistry was performed to study the expression of the tight junction protein, zonula occludens 1 (ZO-1) following a protocol described previously [4]. Briefly, matured ciPTECs were fixed using 2% (w/v) paraformaldehyde in HBSS supplemented with 2% (w/v) sucrose for 5 min and permeabilized in 0.3% (v/v) Triton X-100 in HBSS for 10 min. To prevent non-specific binding of antibodies, cells were exposed to a blocking solution containing 2% (w/v) BSA and 0.1% (v/v) Tween-20 in HBSS for 30 min. Cells were incubated for 1 h with antibodies against the tight junction protein ZO-1

(1:50 dilution, Invitrogen, Carlsbad, CA) diluted in blocking solution, rinsed with HBSS and subsequently incubated with goat anti-rabbit Alexa 488 conjugate (1:200, Life Technologies Europe BV, Bleiswijk, The Netherlands) for 30 min. Finally, DAPI nuclei staining (300 nM, Life Technologies Europe BV) was performed for 5 min. The membranes were carefully dis-assembled from the culture setups and were mounted on microscopy slides using Dako fluorescent mounting media (Dako Netherlands B.V, Heverlee, Belgium). The slides were examined under the Nikon confocal A1/ super resolution N-STORM microscope (Nikon Instruments Europe B.V, Amsterdam, The Netherlands). Images were captured using the NIS-elements analysis software, version 4.40.000.

2.8. Transepithelial transport measurements

The same setup and the same PES membranes were used to analyze first (i) the monolayer barrier integrity via diffusion of inulin-FITC and second (ii) the transepithelial transport of creatinine in the absence or presence of inhibitors (see Figure 3). Mature cell monolayers were washed in modified Krebs–Henseleit buffer including 10mM Hepes (pH 7.4) (KHH). Subsequently, all the samples were pre-incubated for 2 h at 37 °C, 5% (v/v) CO₂ in KHH buffer (0.3 ml apically, 0.9 ml basolaterally) prior to testing. The basolateral compartment was emptied from KHH prior to the experiments. Transport was initiated by the basolateral addition of first (i) inulin-FITC solution (0.1 mg·ml⁻¹ in KHH buffer) and second (ii) creatinine (0.09 µMol·ml⁻¹ in KHH buffer), with or without cimetidine (100 µM) as competitor / inhibitor for the OCT2.

After 1 h of incubation with gentle agitation at 37 °C, two or three samples of 100 µl were removed from both the apical and basolateral chambers. The fluorescence of the inulin-FITC samples and of a standard range of concentrations was measured using a Tecan infinite M200PRO plate reader (Tecan Austria GmbH), at an excitation and emission wavelengths of 485-535 nm for inulin-FITC. The creatinine concentration was determined following the directions of the creatinine assay kit MAK080 [23] by colorimetry, measuring the absorbance at 570 nm of the samples and of a standard range of

concentrations. The membranes were incubated for 1 h in KHH buffer at 37 °C between the inulin-FITC and creatinine transport experiments.

2.9. Cell attachment strength

The transepithelial transport experiments presented above could not be applied to the PEI membranes due to their low porosity. We therefore implemented a novel technique to assess the cell attachment strength there. This set-up was adapted from previous work [24], where cells were grown on transparent surfaces and could be observed via transmission microscopy. Here we used reflection microscopy; the full set-up is presented in Figure 4. We used the PES membranes coated with L-Dopa/CIV as reference for which we expect an optimal attachment [4] strength.

The PEI and PES membranes with mature ciPTEC were rinsed with HBSS and incubated for 1 h in calcein-acetoxymethyl ester (calcein) (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The membranes were installed one by one in a small petri-dish half-filled with HBSS. Capillary tube with inner diameter of 250 μm was aligned perpendicularly to the monolayer. At least 2 experiments were performed on different areas of each membrane. The cell monolayer was exposed to a wall jet. The jetting liquid is HBSS, and was continuously jetted at a fixed flow rate using a syringe pump (LC-20AP, Shimadzu, Kyoto, Japan). To ensure laminar flow, a jet Reynolds number < 2300 was used. The theory of cell-jetting and shear stress calculations is explained in an earlier publication [24]. Automated image analysis was performed with Matlab (The MathWorks, Natick, MA). The cell detachment dynamics was monitored in real-time from the top using a camera (D5100, Nikon, Tokyo, Japan) operated at 25 frames per second and a microscope (Zeiss CFL40, Oberkochen, Germany). The magnification of the objective (5 $\times$, 10 $\times$ or 20 $\times$, Olympus, Tokyo, Japan) is chosen to match the cleared radius of the cell layer. The cell layer was co-axially illuminated from below using a white light source (LS-M352, Sumita, Saitama-City, Japan) and a filter set (Zeiss filter set 09) for fluorescent imaging of the calcein-stained cells.

Figure 4: **Cell attachment strength set-up.** (A) photograph of the setup and (C) detailed view of the membrane and jet, (B) Scheme of the setup. A submerged liquid jet impacts on a cell monolayer attached to a glass surface. The monolayer is illuminated and visualized using a fluorescent setup.

2.10. Data analysis

Every experiment was performed at least in duplicate. The number of samples (n) measured is indicated in each figure legend. The results are presented as mean $\pm$ standard deviation or standard error of the mean. Statistical analysis was performed in the SPSS software (IBPM SPSS Statistics version 23.0), using a one-way analysis of variance (ANOVA) or Student's t-test, where appropriate. A P-value of < 0.05 was considered as significantly different.

3. Results

3.1. PES membranes with HS coating

3.1.2. Membrane coating characterization

The PES membranes were coated with either a solution of HS from bovine kidney, or a double layer of L-Dopa and HS. In order to assess the reproducibility of the coating, the transport properties of PES membranes were evaluated before and after the coatings.

Figure 5. **Membrane transport properties.** (A) Clean water fluxes (CWF) for uncoated and coated membranes. The slope of the CWF as a function of the pressure gives the membrane permeance.

Figure 5 shows the Clean Water Flux (CWF) of the uncoated and coated PES membranes in function of the transmembrane pressure. The permeance estimated from the slope of the graphs are presented in in Table 1. Both HS coating alone and the double L-dopa/HS coating increase the permeability of the PES membrane. The SC of BSA is very low, which is consistent with our expectations since the PES membrane has a molecular weight cut-off (MWCO)

of 50 kDa. The coating reproducibility seems to be good, as indicated by the standard deviation range.

Table 1. Membrane transport properties. The permeance for clean water and BSA as well as the SC of BSA are summarized. Data are shown as mean $\pm$ standard deviation of three or four samples. As a comparison, the permeance values obtained by Schophuizen et al. for PES membranes and PES membranes coated with L-Dopa/CIV are presented in italic [4].

	PES uncoated	PES + L-Dopa + CIV [4]	PES + L- Dopa + HS	PES + HS
Clean water permeance (l·m⁻²·h⁻¹·bar⁻¹)	808 $\pm$ 126 <i>738 $\pm$ 110</i>	<i>146 $\pm$ 10</i>	927 $\pm$ 213	1036 $\pm$ 187
BSA permeance (l·m⁻²·h⁻¹·bar⁻¹)	237 $\pm$ 32 <i>57 $\pm$ 7</i>	<i>37 $\pm$ 27</i>	283 $\pm$ 56	283 $\pm$ 69
SC BSA	0.03 $\pm$ 0.01 <i>0.03 $\pm$ 0.01</i>	<i>0.02 $\pm$ 0.01</i>	0.15 $\pm$ 0.09	0.01 $\pm$ 0.01

3.1.3. Cell monolayer formation - immunochemistry

The ciPTEC monolayer formation was studied via the expression of the tight-junction protein Zonula Occludens 1 (ZO-1). In addition to a polarized epithelial barrier, the tight junction proteins contribute to fluid and ion homeostasis mediated by paracellular transport [25]. We performed two experiments containing each four membranes per type, supporting matured OCT-expressing ciPTEC. The results are presented in Table 2. The number of membranes (surface 0.92 cm²) with an intact cell monolayer is reported.

The reference PES membrane (C) with L-Dopa/CIV coating supports the formation of a uniform ciPTEC monolayer. The abundant expression of the ZO-1 (green color brightly expressed at the contact zone between the cells) is demonstrative for the presence of tight junctions between the cells. The use of L-Dopa only, without collagen IV, increases the cell adhesion and promotes the formation of a monolayer, but presents many defects.

Table 2. Summary of the monolayer formation on PES membranes with various coatings, as observed by confocal microscopy. Representative images of the ZO-1 tight junction protein (green) and nuclei (blue) in matured ciPTEC. The number of membranes with a visually intact monolayer is reported (example x/8). Red dots correspond to saturated pixels.

Membrane, coating; Zo-1 formation and description	Representative picture magnification x 20	Representative picture magnification x 60
PES uncoated; Zo-1 - 0/8 Few attached cells - no monolayer. Variable cell shapes.
PES + L-Dopa; Zo-1 - 0/8; 6/8 (partial ZO-1) Monolayers with many apparent defects and holes.
PES + L-Dopa + CIV; Zo-1 - 7/8 Clear Zo-1 pattern. Presence of little defects in the monolayer.

PES + HS;Zo-1 - 0/8 (partial
ZO-1 8/8)

A lot more cells
attached.
Sometimes
presence of small
areas with
expression of ZO-1,
with many defects.
Less ZO-1 areas
than on L-Dopa
alone (condition B).

PES + L-Dopa + HS;

Zo-1 - 4/4

Clear Zo-1 pattern.
Similar to condition
C (PES + L-Dopa +
CIV)

Compared to bare PES membrane where only a few cells are present, the HS coating improves cell adhesion. Several areas with monolayers are present, but many defects are present as well, indicating a possible coating inhomogeneity. The application of the double L-Dopa/HS coating results in an good monolayer. The shape of the nuclei and of the cells are regular for both L-Dopa/CIV and L-Dopa/HS double coatings. The little defects found during microscopy observation may be due to handling (tweezers, removal of O-Ring, microscopy sample preparation). Therefore, the paracellular transport experiment in the next section should indicate whether the defects are inherent to the cell monolayer itself or to the handling.

3.1.4. Paracellular transport

a. Transepithelial barrier function

In order to characterize the barrier-formation and the homogeneity of the observed monolayers, the transport of inulin-FITC through PES membranes coated with L-Dopa/HS and supporting mature ciPTEC was measured. The concentration of inulin-FITC in the permeate was measured and corrected for the exact volume of permeate. The amounts of transported inulin are presented in Figure 6A. Although the standard deviation is high, the inulin leakage decreases for about 30% in the presence of cells ($P < 0.05$ using one-way ANOVA). This may indicate the formation of a monolayer with a good barrier function but lacking of reproducibility and/or presenting local defects.

a. Functional organic cation transport

To further examine the functionality of the matured ciPTEC monolayers grown on PES membranes with the double L-Dopa/ HS coating, we assessed the activity of OCT2, which is expressed along the basolateral membrane of PTEC. Here, we exposed the apical side of the membranes to creatinine, a cationic uremic toxin, in the absence or in the presence of competitive inhibitor of OCT2 mediated uptake: cimetidine. Figure 6B shows the amount of creatinine transported through the membrane in the absence and in the presence of cells. The amount of creatinine transported without cells is higher than when cells are present. When the cells are present, there is a 26 ± 11 % decrease in the transport of creatinine in the presence of the inhibitor compared to the transport of creatinine alone ($P < 0.001$ using one-way ANOVA). This result is indicative of an activity of the OCT2 transporter in this configuration consistent with the Inulin-FITC transport results.

Figure 6: **Paracellular transport.** (A) Transport of inulin-FITC. Data are presented as the mean $\pm$ standard deviation of at least 5 samples for the membranes without cells and 15 samples for the membranes with cells. * $P < 0.05$ using a one-way ANOVA. (B) Transport of creatinine through the functionalized PES membranes without and with matured ciPTEC. Data are presented as the mean $\pm$ standard deviation of at least 2 samples. * $P < 0.001$ using a one-way ANOVA.

3.2. PEI surfaces

3.2.1. Cell monolayer formation

As explained in the paragraph 3.1.3, the monolayer formation was studied via expression of the tight-junction protein ZO-1. The results are presented in Table 3. Globally, qualitatively more cells adhere on PEI (Table 3) than on PES (Table 2), with a formation of monolayer presenting several defects. A good cell monolayer seems to be achieved when L-Dopa coating is applied.

Table 3. Summary of the monolayer formation on PEI membranes with various coatings, as observed by confocal microscopy. Representative images of the ZO-1 tight junction protein (green) and nuclei (blue) in matured ciPTEC. The number of membranes with a visually intact monolayer is reported (x/8). A total of 8 samples per case was used, from 2 different seeding experiments. The blue/green background color of the images is due to the auto-fluorescence of the PEI membrane. Red dots correspond to saturated pixels.

Membrane, coating; Zo-1 formation and description	Representative picture magnification x 20	Representative picture magnification x 60
PEI; Zo-1 - 0/8 Very high cell density. Several areas with expression of ZO- 1 but many defects.
PEI + L-Dopa ; Zo-1 - 6/8 Zo-1 expression on almost the whole surface of the samples. Presence of little

defects in the monolayer.

PEI + HS ;
Zo-1 - 0/8
Lower cell density than on PEI alone (condition F).

PEI + L-Dopa + HS;
Zo-1 - 6/8
Very high cell density and Zo-1 on almost the whole surface of the samples.
Presence of little defects.

The results presented here were obtained on the 20% w/w PEI membranes. The membranes prepared using four different PEI weight concentrations (10, 15, 20, 25 % w/w) had all good cell attachment. The membranes with 10 and 15% w/w PEI, however, were very thin and fragile, and difficult to handle. The membranes with a higher PEI content (25% w/w) had rather high auto-fluorescence, reducing the quality of the microscopy images. Besides, the application of HS coating on PEI was performed in one experiment. Since PEI is positively charged and HS is negatively charged, we hypothesized that obtaining a reproducible HS layer on a PEI membrane would be easier than on PES membrane. Our preliminary results indicate that the cell adhesion on PEI coated with HS is not better than on PEI alone. when the PEI membrane was hydrophilised with a L-Dopa coating and then coated with HS, we could obtain a good cell monolayer.

3.2.2. Cell attachment strength

Due to the low porosity of the PEI membranes, it was not possible to characterize them via transport experiments, neither for the evaluation of the coating quality nor for the cell function. There, we applied a new setup to measure the cell attachment strength. In theory, in case of the formation of a tight monolayer, the attachment strength of the cells to each other may be superior to the attachment to the surface [26]. Therefore, a difference in the measured attachment strength is expected, depending on the quality of the interactions between the cells and the substrate.

Table 4 shows representative photographs of mature ciPTEC, stained with calcein, grown on various membranes, after they were subjected to jetting. The PES membranes without coating were used as negative control, and the PES coated with L-Dopa/CIV as a positive control. The results of cell detachment experiments, namely the cleared radius for several membranes are presented, as well as the calculated attachment strength, T in $N \cdot m^{-2}$. It seems that the absolute values are not very different for the studied conditions. However, the cell detachment occurs with different mechanisms for the presented conditions. For the uncoated PES membrane and PES membrane coated with L-Dopa alone, we observed individual cell detachment: the cells are detached in a distance of a radius from the jetting impact. On the contrary, for the PES membranes coated with L-Dopa/CIV and for the PEI membranes, the cells detach in sheets. Several cells are detached under the jet and due to the high adhesion with the neighboring cells, a bigger area is cleared. As a result, the calculated attachment strength to the surface is apparently smaller. Therefore, these cases indicate high intercellular adhesion strength, superior to the forces of adhesion of each individual cell to the surface. These results indicate the achievement of a rather good cell monolayer for PEI membranes and for PES membranes coated with L-Dopa/CIV consistent to the findings of ZO-1 expression via immunochemistry (see Table 3). The detachment of cells from PEI coated with L-Dopa presented similar trend with the results obtained for PEI membrane alone, however the number of samples was not representative and the results are not presented here.

Table 4. Results of cell attachment strength. The cleared radius (CR) was measured for every jetting experiment (between 4 and 6 samples per case), as well as the resulting attachment strength, T . The median value is presented for both CR and T . Representative microscopy images of calcein-stained ciPTEC (green) grown on various flat membranes after jetting are presented.

PES	PES + LD	PES + LD + CIV	PEI
CR ~ 8 mm	CR ~ 5.5 mm	CR ~ 6.1 mm	CR ~ 5.5 mm
$T \sim 105 \text{ N}\cdot\text{m}^{-2}$	$T \sim 100 \text{ N}\cdot\text{m}^{-2}$	$T \sim 130 \text{ N}\cdot\text{m}^{-2}$	$T \sim 150 \text{ N}\cdot\text{m}^{-2}$

4. Discussion

In this study, we assessed the ability of two surfaces to support the formation of a ciPTEC monolayer, in the context of a BAK application. On the one hand, we coated PES membranes with HS, characterized the coating quality and compared it to the previously developed L-Dopa/CIV coating. We studied the monolayer formation via immunochemistry and assessed the transepithelial transport quality. We observed a positive effect of HS on the monolayer formation and cell function. On the other hand, we produced PEI membranes and observed a good monolayer formation, as well as a cell attachment strength similar to the original PES membranes coated with L-Dopa/CIV. Both strategies seem promising to achieve a ciPTEC monolayer.

4.1. HS coating

We characterized the HS coating prior to perform cell culturing experiments. The permeance slightly increased for both HS and L-Dopa/HS coated PES membranes indicating a hydrophilization of the membrane. This coating is different from the L-Dopa/CIV coating. Indeed, Schophuizen et al. [4] reported a strong decrease in the membrane permeance after the L-Dopa/CIV coating. Since the permeance of the PES membrane is preserved after the application of L-Dopa/HS, we were able to use these membranes for cell function experiments.

The immunochemistry results indicate that the L-Dopa/HS coating lead to the formation of a monolayer comparable to the one achieved on PES coated with L-Dopa/CIV. However, the inulin-FITC transport through membranes with cells is rather high in comparison with earlier studies with HFM [7, 8] (relative value of 75% versus 30 to 35 % previously [7, 8]). A similar trend was reported for OAT-expressing ciPTEC [27], with a relative inulin-FITC transport of 80% in flat configuration versus 10 to 20% on HFM [27]. The transport of inulin-FITC through the flat membranes (MWCO = 50 kDa, inulin flux $\sim 10 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$) is lower than through the HFM (MWCO > 160 kDa, inulin flux $\sim 1000 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$) due to the difference in membrane porosities. Namely, the concentration

gradient in the flat membranes is expected to be lower than in the HFM. In the case of the flat membrane with cells, due to the lower concentration gradient inside the flat membrane the driving force there is lower than in the HFM. Moreover, the inulin-FITC permeation experiments did happen under flow in HFM configuration, which accelerated its transport.

The creatinine transport confirms the trend observed during inulin-FITC permeation. The decrease of creatinine transport in the presence of cimetidine ($26 \pm 11 \%$, $P < 0.001$) for PES membranes coated with L-Dopa/HS is comparable to what was reported by Schophuizen *et al.* [4] $-18 \pm 0.2\%$ decrease, $P < 0.05$ – for PES membranes coated with L-Dopa/CIV. Therefore, the PES membranes coated with L-Dopa/HS seems to lead to a functional monolayer, as confirmed both by the inulin-FITC reduced transport and by the decrease of the creatinine transport in the presence of the inhibitor.

4.2. PEI membranes

The ciPTEC adhesion on PEI membranes was better in terms of cell density than on uncoated PES membranes, as indicated by immunochemistry. The reproducibility of cell monolayer formation on PEI and on PEI coated with L-Dopa was better than on HS-coated membranes. The cell attachment strength experiments indicated a sheet-detachment for monolayers grown both on PES coated with L-Dopa/CIV and on PEI. The cell – cell interaction seems to be good therefore upon application of the jet, the cell monolayer detaches as a whole sheet instead of individual cells. As a consequence, the cell attachment strength seems to be less due to the detachment of a bigger number of cells as a sheet. In the earlier study performed on HeLa cells grown on glass substrates [24], both individual and sheet-detachment phenomena were observed, depending on the cell density. The HeLa cell attachment strength to glass substrates was $T = 34 \pm 14 \text{ N}\cdot\text{m}^{-2}$. Our preliminary results performed on non-mature ciPTEC monolayer indicated an attachment strength of $T = 52 \pm 8 \text{ N}\cdot\text{m}^{-2}$, higher than for HeLa cells, and two to three times lower than for matured ciPTEC ($\sim 130 - 150 \text{ N}\cdot\text{m}^{-2}$). It is important to consider not only these numbers but also the cell detachment mechanism (individual or sheet). The cell-sheet

detachment phenomena however is only observed for matured ciPTEC and does indicate the formation of tight-junctions, confirming the immunostaining results.

5. Conclusion & outlook

In this work, the preliminary results of the formation of ciPTEC monolayer on PES coated with HS and PEI membranes are presented. Both the application of HS on PES and a PEI surface ameliorated ciPTEC adhesion and, when combined with the application of L-Dopa, led to the formation of a monolayer. The transepithelial transport tests indicated a barrier function of the monolayer as well as the function of the OCT2 on PES coated with L-Dopa/HS. It was not possible to perform transport experiments on PEI membranes due to their low porosity. The attachment strength experiments showed sheet-detachment phenomena on PES coated with L-Dopa/CIV and on PEI membranes suggesting that both could be good candidates for membranes for BAK applications. Future work, should include further studies of creating of a porous PEI membrane with tailored transport properties combination of PEI with HS coating, after optimization of both components.

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7. List of abbreviations

ASP ⁺ -	4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide
BAK -	bioartificial kidney device
BSA -	bovine serum albumin
CiPTEC -	conditionally immortalized human proximal tubule epithelial cells
CWF -	clean water flux
ECM -	extracellular matrix
GAG -	glycosaminoglycans
HBSS -	Hank's balanced salt solution
HFM -	hollow fiber membrane
HS -	Heparan sulfate
Inulin-FITC -	fluorescein isothiocyanate (FITC)-labelled-inulin
KHH -	Krebs-Henseleit buffer supplemented with HEPES (10 mM)
L-Dopa -	3,4-dihydroxy-L-phenylalanine
PEI -	polyetherimide
PES -	polyethersulfone
PG -	proteoglycans
SC -	sieving coefficient
PTEC -	proximal tubule epithelial cell
SEM -	scanning electron microscopy
TMP -	transmembrane pressure
ZO-1 -	zonula occludens-1

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9. Supplement

Figure S1. **Scheme of polyethyleimine:** (A) repeat unit, (B) linear polymer, (C) branched polymer.

Chapter 6

Polarized immune response in an upscaled bioartificial kidney device

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Abstract

The accumulation of protein-bound toxins in dialyzed patients is strongly associated with their high morbidity and mortality. The bioartificial kidney device (BAK), containing proximal tubule epithelial cells (PTEC) seeded on functionalized synthetic hollow fiber membranes (HFM), may be a powerful solution for the active removal of those metabolites. In our earlier studies, conditionally immortalized human PTEC (ciPTEC) seeded on polyethersulfone (PES) HFM have shown to express functional organic cationic transporter 2 (OCT2). Here, an upscaled three-dimensional BAK device capable of actively removing anionic uremic wastes is presented. We confirmed the quality of the ciPTEC monolayer by confocal microscopy and paracellular inulin-FITC leakage. The transport of indoxyl sulfate (IS), an anionic uremic toxin decreased by 40 to 50% in the presence of the inhibitor, proving the function of, among others, the organic anionic transporter 1 (OAT1). Furthermore, the immunosafety of our system was assessed by measuring the production of relevant cytokines upon lipopolysaccharide (LPS) stimulation. Upon LPS treatment, the concentration of pro-inflammatory cytokines was 10 fold higher in the extraluminal space, corresponding to the dialysate compartment, as compared to the intraluminal space. This favorable polarized secretion of immune response mediators together with the stability of ciPTEC monolayers in the upscaled device show that our approach could be very suitable for BAK engineering.

1. Introduction

Despite the ongoing progress in dialysis therapy, only small and middle-size xenobiotics can be eliminated. The removal of bigger size solutes and protein-bound toxins is limited [1, 2]. Recently, the accumulation of these protein-bound solutes has been strongly associated with the fatal outcome of the patients [3, 4]. Therefore, there is a strong need for novel strategies and concepts to remove these solutes [5]. The bioartificial kidney (BAK) may be a powerful solution for the removal of protein-bound toxins. The BAK aims at mimicking the functional kidney by making use of proximal tubule epithelial cells (PTEC), equipped with a broad range of transporters, which normally mediate the excretion of those solutes [6]. This device consists of “living membranes” comprising of tight monolayer of renal cells with preserved functional organic ion transporters, grown on an artificial porous hollow fiber membrane (HFM).

In recent years, several studies have presented BAK prototypes making use of human PTEC [7-14], showing a preserved phenotype and sometimes metabolic and/or endocrine functions *in vitro* or *in vivo*. However, mostly primary cell lines were used which are characterized by limited availability, donor to donor variation and the loss of phenotype or functionality upon culturing. The recently developed and well-characterized human conditionally immortalized PTEC (ciPTEC) line appears to be a suitable candidate for an efficient BAK system [15-21]. These cells are transduced with human telomerase (hTERT) that limits replicative senescence by telomere length maintenance. In addition, their proliferation is controlled by the temperature sensitive mutant of SV40 Large T antigen (SV40tsA58), allowing proliferation at 33 °C and differentiation in mature PTEC at 37 °C. Due to these modifications the cell line has high availability, limited senescence, and can be used up to a high passage number.

A recent study on small single HFM showed an active excretion of indoxyl sulfate (IS) and kynurenic acid (KA) by ciPTEC [18] through the concerted action of organic anion transporter-1 (OAT1), breast cancer resistance protein (BCRP) and multidrug resistance protein-4 (MRP4). This property of the ciPTEC

is of high importance for BAK application, considering that most of the protein-bound toxins are anionic molecules [22, 23]. In the present work, we used an upscaled “living membrane” to support the OAT1-expressing ciPTEC line. The transport properties and the quality and function of the grown ciPTEC monolayer were investigated, including the expression of zonula occludens-1 (ZO-1) protein and the diffusion of fluorescein isothiocyanate (FITC)-labelled-inulin (inulin-FITC). Furthermore, we studied the transport of an anionic uremic toxin, IS, mediated by the combined action of OAT1, BRCP and MRP4, in the absence or in the presence of the OAT1 inhibitor, probenecid.

A very important issue related to the clinical implementation of the BAK device is its safety. It is crucial to investigate whether the device with the human allogenic cells induces immune and inflammatory responses in the host. Besides, the high uremic toxins concentrations in kidney patients are often associated with inflammation, which may as well be detrimental for the BAK [24]. An assessment of the immunogenicity of the ciPTEC lines using flat membranes with a small surface area (1.12 cm²) showed that ciPTEC are lowly immunogenic *in vitro*, although able to secrete several pro-inflammatory cytokines that could potentially mediate a non-specific inflammatory response [25]. In this work, we also investigated the polarization of the production of pro-inflammatory and immune mediators by the ciPTEC on the developed upscaled system. We measured the release of the most relevant pro-inflammatory mediators – IL-6, IL-8 and TNF- α - and sHLA-class I without or with exposure to lipopolysaccharide (LPS) or interferon- γ (IFN- γ) in both – dialysate and blood – compartments of the system.

2. Materials and Methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) unless stated otherwise. MicroPES TF10 hollow fiber capillary

membranes (HFM) (wall thickness 100 μm , inner diameter 300 μm , max pore size 0.5 μm) were purchased from 3M - Membrana GmbH (Wuppertal, Germany).

2.2. Module preparation, HFM sterilization, coating and characterization

The modules were prepared following the protocol presented previously [20]. Briefly, MicroPES HFM were mounted into mini modules composed of Kartell PP T-shaped connectors (Fisher Scientific, Landsmeer, the Netherlands) and PTFE rigid semi-transparent tubing diameter 6-8 mm (VWR International B.V, Amsterdam, the Netherlands), see Figure 1. The use of PTFE tubing allowed the steam-sterilization of the modules at 121°C. A dual coating of L-Dopa and Collagen IV coating was applied to the fibers, at 37 °C on a shaking device, for a duration of 20h and 2h respectively [20]. The HFM transport properties (water permeability) was measured before and after sterilization and before and after coatings, in the absence of ciPTEC. In fact, we used an OSMO Inspector automated setup (Convergence B.V, Enschede, The Netherlands) to quantify the clean water (Merck Millipore, Billerica, MA) flux (CWF) through the HFM (J, in $\text{l}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$) as a function of the transmembrane pressure (TMP or ΔP , in bar). The membrane permeance (L, in $\text{l}\cdot\text{m}^{-2}\cdot\text{h}^{-1}\cdot\text{bar}^{-1}$) was calculated from the slope of this curve. Every pressure step was maintained for 30 min.

2.3. Cell culture and modules handling

The ciPTEC OAT-1 expressing urine-derived cell line [15, 21] was cultured at 33°C proliferating temperature and at 37°C maturation temperature in a ciPTEC complete medium. The latter was prepared as described previously [20] with the difference that CiPTEC were now always cultured in absence of antibiotics up to a maximum of 60 passages. The modules' handling and cell seeding was performed as reported previously [20].

Briefly, prior to cell seeding, modules were incubated for 1 h in ciPTEC complete medium. Proliferating 90% confluent ciPTEC were detached using Accutase

(StemPro® Accutase®, Life Technologies Europe BV, Bleiswijk, the Netherlands), centrifuged and suspended at a concentration of 2.0-2.5 million cells/ml in the ciPTEC complete medium. The modules' extraluminal space was completely filled with the cell suspension. To promote initial cell attachment, the modules were placed at 33°C, 5% CO₂ for 8 h, with a rotation of 90 degrees every 2 h. Afterwards, the modules were washed with the ciPTEC complete medium, provided with gas exchange filters and the cell proliferation was allowed for additional 64h. Finally, the temperature was changed to 37°C for 7 days to allow ciPTEC maturation. During the culture period, the ciPTEC were supplemented with fresh culture media every second day.

Figure 1. Upscaled "living membrane". (A) Picture of one module used for upscaled "living membrane" model. Three MicroPES hollow fiber membranes (HFM) within a housing composed of PTFE tubing, PP T-shaped connectors, silicone parts, PVDF Luer Lock fittings and caps. (B) SEM image of three MicroPES HFM. (C) Scheme of a transversal cut of one "living membrane". Not at proportional scale. Source: [20] with permission of Elsevier.

2.4. Immunocytochemistry

Matured ciPTEC were fixed using 2 w/v% paraformaldehyde in HBSS supplemented with 2 w/v% sucrose for 10 min and permeabilized in 0.3 v/v% triton X-100 in HBSS for 10 min. To prevent non-specific binding of antibodies, cells were exposed to block solution containing 2 w/v% bovine serum albumin, 2 v/v% fetal calf serum and 0.1 v/v% tween-20 in HBSS for 30 min. Cells were incubated with antibodies diluted in block solution against the tight junction protein zonula occludens 1 (ZO-1, 1:50 dilution, Invitrogen, Carlsbad, CA) for 90 min, followed by a simultaneous incubation with goat-anti-rabbit-Alexa 568 conjugate (1:200, Life Technologies Europe BV, Bleiswijk, The Netherlands) and Phalloidin-Atto 488 (1:500) for 30 min. Finally, DAPI nuclei staining (300 nM, Life Technologies Europe BV) was performed for 5 min. The modules were carefully cut open and the extracted fibers were mounted on microscopy slides using Dako fluorescent mounting media (Dako Netherlands B.V, Heverlee, Belgium). The slides were examined under the Nikon confocal A1/ super resolution N-STORM microscope (Nikon Instruments Europe B.V, Amsterdam, The Netherlands). Images were captured using the NIS-elements analysis software, version 4.40.000.

2.5. Transepithelial barrier function

Paracellular permeability in the mature living membranes was quantified following the previously described method [20]. After washing the modules with Krebs-Henseleit buffer supplemented with HEPES (10 mM; KHH buffer), inulin-FITC (0.1 mg/ml in KHH buffer) was perfused at 18 ml/h at 37°C for 15 min. The Inulin-FITC leakage was determined prior to perform functional IS transport. In some of the modules, the Inulin-FITC leakage was determined before and after the IS transport experiment and after the immune response experiment to assess the integrity of the monolayer after the functional tests.

2.6. Functional organic anion transport

Transepithelial transport of indoxyl sulfate (IS) through the HFM with matured ciPTEC was studied using a similar perfusion set-up as was used for the barrier function assay. First, fibers were pre-incubated in KHH buffer without or with probenecid (p) at concentrations 100 μ M (p100) or 500 μ M (p500) at 37°C for 15 min. Next, the fibers were perfused using 100 μ M IS in KHH buffer in the presence or absence of inhibitors for 10 min at a flow rate of 18 ml/h. Samples from both permeate and outlet were collected. Toxin concentrations were analyzed using a LC-MS/MS system (ThermoFisher Scientific, Breda, The Netherlands) following the method described by Mutsaers et al. [26].

2.7. Production of the pro-inflammatory cytokines and human leukocyte antigens

The apical and basolateral membranes of mature ciPTECs on upscaled HFM were exposed to the following treatments depending on the experimental set-up: interferon- γ (IFN- γ) 300 ng/ml, lipopolysaccharide (LPS) 10 μ g/ml. The production of IL-6, IL-8, TNF- α and soluble HLA-class I (sHLA-class I) molecules in various stimulatory conditions, was measured by means of enzyme-linked immuno-sorbent assays (ELISAs). Cell culture supernatants were collected from apical (external, volume ~ 3 ml) and internal (external, volume ~ 0.4 ml) compartments after exposure to various stimulatory conditions, centrifuged for 10 min at 240 x g, 4°C, and stored at -80°C. DuoSet® ELISA Development Systems kits (IL-6 #DY206, TNF- α #DY210, IL-8 #DY208; R&D systems, Abingdon, UK) and Human MHC class-I kit (Proteintech, Chicago, IL, USA) were used to quantify the cytokine and sHLA-class I levels respectively, in cell culture supernatants according to manufacturer's instructions. The optical density was determined immediately using the iMark Microplate Absorbance Reader (Bio-Rad, Japan) set to 450 nm. Each sample was measured in duplicates and quantification was done using Microplate Manager Software (version 6.0, Bio-Rad Laboratories, Hercules, CA, USA) capable of generating a four-parameter logistic (4-PL) curve-fit.

2.8. Data analysis

Every experiment was performed at least in duplicate. The number of samples (n) measured is indicated in each figure legend. The results are presented as mean $\pm$ standard deviation or standard error of the mean. Statistical analysis of the cell monolayer integrity and function was performed in the SPSS software (IBPM SPSS Statistics version 23.0) using a one-way analysis of variance (ANOVA) or Student's t-test, where appropriate. Statistical analysis of the production of pro-inflammatory cytokines was performed in the GraphPad Prism software (GraphPad software, version 5.03; La Jolla, CA, USA), using either unpaired two-tailed test (for differences between two compartments in the same module) or one way ANOVA followed by Tukey's multiple comparison test (for differences between treatments and different modules). A P-value of < 0.05 was considered significantly different.

3. Results

3.1. HFM characterization

The HFM were decontaminated with steam sterilization to prevent risks of infection and coated with L-Dopa and collagen IV to allow ciPTEC adhesion and function. Prior to performing cell culturing experiments the potential impact of sterilization and coating on the membrane transport properties were evaluated. Figure 2 shows the clean water flux (CWF) through the unsterilized or sterilized, and uncoated or coated HFM at different pressures. The steam sterilization did not affect the permeance of the uncoated membranes ($(19.2 \pm 0.9) \cdot 10^3 \text{ l} \cdot \text{m}^{-2} \cdot \text{h}^{-1} \cdot \text{bar}^{-1}$ unsterilized versus $(18.9 \pm 0.8) \cdot 10^3 \text{ l} \cdot \text{m}^{-2} \cdot \text{h}^{-1} \cdot \text{bar}^{-1}$ sterilized). The permeance was slightly decreased when the double coating was applied on the sterilized HFM ($(16.9 \pm 0.7) \cdot 10^3 \text{ l} \cdot \text{m}^{-2} \cdot \text{h}^{-1} \cdot \text{bar}^{-1}$) compared to the non-sterilized HFM ($(19.0 \pm 0.3) \cdot 10^3 \text{ l} \cdot \text{m}^{-1} \cdot \text{h}^{-1} \cdot \text{bar}^{-1}$), but remained high. This may be due to an increased wettability of the membranes after sterilization, which favors the coating deposition and results in a slight pore occlusion. The SEM analysis

shows no difference between the surfaces and cross-sections of the uncoated and coated HFM (supplemental figures S1 and S2). Overall, the results indicate that the high HFM permeance is preserved after sterilization and coating.

Figure 2. HFM permeability. Clean water fluxes (CWF) for unsterilized and sterilized HFM, with and without coating. The slope of the CWF as a function of the pressure gives the HFM permeance. Data are shown as mean $\pm$ standard deviation of three or four samples.

3.2. Cell monolayer integrity

Figure 3A and B show representative images of matured OAT-expressing ciPTEC cultured on MicroPES HFM. The applied L-Dopa (20 h) and collagen IV (2 h) coating supports the formation of a uniform ciPTEC monolayer. The abundant expression of the Zonula Occludens 1 (ZO-1) (Figure 3B) is demonstrative for the presence of tight junctions between the cells. In addition to a polarized epithelial barrier, the tight junction proteins contribute to fluid and ion homeostasis mediated by paracellular transport [27]. Besides, cytoskeleton (actin) staining (Figure 3A) shows that the cells have proper shape, structure and morphology, additionally confirming the epithelial character of the cell monolayer.

Figure 3. Monolayer quality of ciPTEC cultured on HFM. (A, B) Representative confocal microscopy images of ciPTEC cultured on HFM with in blue the DAPI staining of nuclei, in green the cytoskeleton and in red the immunostaining for ZO-1 (A, B). (C) Inulin-FITC paracellular leakage, ratio HFM with cells / HFM without cells. Data are presented as mean $\pm$ standard deviation of at least 4 samples. * $P < 0.001$ using a one-way ANOVA.

Inulin transport through the mature cell monolayer occurs in a non-active manner, via diffusion through the inter-cellular junctions or paracellularly. Therefore, the Inulin-FITC leakage test is representative of the monolayer tightness [17]. For a tight cell monolayer, Inulin-FITC leakage is expected to be a lot lower than through HFM without cells. Figure 3C presents the Inulin-FITC leakage through the tested HFM, for which the fibers without cells is set to 100%. The leakage of the HFM with a ciPTEC monolayer is as low as 31.8 ± 11.0 %. The data presented here arise from 4 experiments and 32 different modules.

3.3. Cell function – IS transport

We next assessed the activity of OAT1, BRCP and MRP by perfusing the ciPTEC monolayers grown on functionalized HFM modules with IS, an anionic uremic toxin, in the absence or presence of the OAT1 inhibitor, probenecid. Figure 4A shows the scheme of the experimental set-up and Figure 4B shows the IS transport results. The value of IS transport alone through the cell monolayer was set here at 100%. The IS transport is inhibited by ~40% by both concentrations of probenecid (IS transport becomes 52 ± 21 % and 66 ± 15 % of the original intensity, for p100 and p500 respectively), confirming the formation of a functional monolayer in the modules. Since the inhibition of IS transport was comparable for both concentrations of probenecid used, we have probably reached the saturation of the transporters. After the inhibition tests, we again measured the Inulin-FITC leakage through the HFM and found it very low 29.4 ± 20.1 % (see Figure 3C), suggesting cell monolayers were still intact.

Figure 4. Functional OAT1, BCRP and MRP4 transporters mediated IS transport. (A) Schematic presentation of the experimental set up of IS transepithelial transport in the absence (I) or presence (II) of probenecid. Adapted from [18]. (B) Quantification of IS transport (100 μ M) in the absence or presence of probenecid (concentration 100 μ M – p100 and 500 μ M – p500) in matured ciPTEC cultured on upscaled HFM. The perfusion lasted for 10 min. Data are normalized against IS transport in the absence of inhibitors and presented as mean $\pm$ standard deviation of at least 3 modules per case, from two independent experiments. * $P < 0.001$ using ANOVA.

3.4. Production of the pro-inflammatory cytokines

To assess whether the upscaled device mediates an inflammatory or immune response, we evaluated the release of IL-6, IL-8, TNF- α and sHLA-class I in both in the extraluminal (apical) and in the intraluminal (basolateral) compartments. Figure 5A shows a schematic representation of the experimental set-up in the case of exposure to LPS; an identical setup was used for the exposure to IFN- γ and the release of sHLA-class I. Figure 5B-E show the release of pro-inflammatory cytokines IL-6, IL-8 and TNF- α and the sHLA-class I.

Figure 5. Immune activation and production of pro-inflammatory cytokines by matured ciPTEC on upscaled BAK. (A) Schematic presentation of the experimental set up used for the study of the release of the pro-inflammatory cytokines, here exposure to LPS and measure of IL-6, IL-8 and TNF-α; an identical setup was used in case of IFN-γ treatment and sHLA-class I release. (B, C, D, E) Effect of 24 h basolateral or apical exposure to LPS (10 µg/ml) (B, C, D) or IFN-γ (300 ng/ml) (E) on production of pro-inflammatory cytokines (IL-6, IL-8 and TNF-α) and sHLA-class I in the apical

*and basolateral compartments. Data are presented as mean $\pm$ (standard error mean; SEM) of at least 3 modules per case, from two independent experiments. * $P < 0.05$ and *** $P < 0.001$ using either unpaired two-tailed test (for differences between two compartments in the same module) or one way ANOVA followed by Tukey's multiple comparison test (for differences between treatments and different modules).*

Prior to exposure to LPS, the concentrations of IL-6 and IL-8 were found to be 2 to 3 times higher in the extraluminal compartment ($(27 \pm 11) \cdot 10^3$ pg/ml and $(10 \pm 2) \cdot 10^3$ pg/ml respectively) than in the intraluminal compartment ($(9 \pm 10) \cdot 10^3$ pg/ml and $(4 \pm 1) \cdot 10^3$ pg/ml respectively). When the ciPTECs were directly exposed to LPS, the concentration of IL-6 and IL-8 increased in the extraluminal compartment (more than 10 times – $(496 \pm 654) \cdot 10^3$ pg/ml and $(103 \pm 16) \cdot 10^3$ pg/ml respectively). When LPS was administrated intraluminally, the extraluminal concentration of IL-6 and IL-8 increased slightly less (more than 5 times – $(100 \pm 37) \cdot 10^3$ pg/ml and $(67 \pm 8) \cdot 10^3$ pg/ml respectively), most likely due to the presence of the HFM. On the other hand, the intraluminal concentration of IL-6 remained stable after both the extraluminal and intraluminal exposures to LPS ($(19 \pm 9) \cdot 10^3$ pg/ml and $(16 \pm 6) \cdot 10^3$ pg/ml respectively). In similar conditions, the intraluminal concentration of IL-8 slightly increases: 2 times after extraluminal exposure and 3 times after intraluminal exposure ($(9 \pm 4) \cdot 10^3$ pg/ml and $(13 \pm 4) \cdot 10^3$ pg/ml respectively).

The concentrations of TNF- α did not vary in all of the tested configurations, and remained very low (100-300 pg/mL), in agreement with our previous findings [25, 28]. If we however estimate the absolute amount of TNF- α in both compartments (based on the volume of each compartment, figure S3), we observe that the amount of TNF- α is higher in the extraluminal than in the intraluminal compartment.

Similarly to TNF- α , the absolute levels of sHLA-class I were rather low (concentrations < 20 pg/ml) compared to normal serum levels [29, 30]. Hence, in agreement with IL-6 and IL-8, the concentrations of sHLA-class I were 2 to 3 times higher in the apical than in the basolateral compartment (15 ± 10 pg/ml and 5 ± 1 pg/ml respectively). The apical concentration of sHLA-class I did not

change after the apical and basolateral exposures to IFN- γ (20 ± 11 pg/ml and 19 ± 11 pg/ml respectively), although a light (2-fold) increase can be observed in the basolateral compartment after the basolateral exposure (9 ± 5 pg/ml).

Overall, for all of the tested configurations, the concentration of IL-6, IL-8 and sHLA-class I was higher in the extraluminal compartment, corresponding to dialysate, compared to the intraluminal one, which would correspond to patient's circulation. This difference is characteristic of a polarization of the ciPTEC monolayer.

The above conclusion holds also if one estimates the absolute amounts of pro-inflammatory cytokines and sHLA-class in both compartments taking into consideration the volumes for each compartment. The data with absolute amounts of produced pro-inflammatory cytokines are presented in the supplementary figure S3.

Finally in order to assess the integrity of the cell monolayer during these tests, additional Inulin-FITC leakage tests were performed on the HFM after exposure to stimulatory conditions (LPS and IFN-gamma), which was found to be really low, 27.4 ± 14.9 %, see Figure 3C, and similar to that before the stimulatory experiments.

4. Discussion

In this study, we developed upscaled (4 cm^2) 3D modules containing double coated MicroPES HFM supporting OAT1-expressing ciPTEC. The quality of the ciPTEC monolayers was assessed via immunochemistry and Inulin-FITC leakage. Afterwards, for the first time in an upscaled BAK prototype, we proved the function of the cell monolayer by studying the active transport of one of the most important anionic uremic toxins, IS. Moreover, we investigated in detail the polarization of the ciPTEC monolayer by comparing the basolateral and apical production of the pro-inflammatory cytokines IL-6 and IL-8 and sHLA-

class I to prove polarized secretion of these mediators of inflammatory and immune response.

The ciPTEC monolayer was characterized after 7 days of maturation at 37°C in the upscaled modules. The confocal microscopy analysis demonstrates regular nuclei, homogeneous cell structure and morphology, and abundant expression of the tight junction protein ZO-1. This underlines the epithelial character of the ciPTEC monolayer, which was confirmed further by limited inulin-FITC diffusion. When compared to our previous studies, the relative Inulin-FITC leakage is in a similar range. Indeed, the earlier reported values - $29.6 \pm 10.2\%$ for an upscaled system [20] and $31.1 \pm 8.5\%$ [19] for a small scale system – are identical to the Inulin-FITC leakage percentage - $31.8 \pm 11.0\%$ - reported here. In addition, the paracellular Inulin-FITC leakage remained low after exposure to IS, LPS or IFN- γ , confirming the preservation of intact ciPTEC monolayers after the exposure to toxins and to inflammatory stimuli.

Our upscaled system demonstrated the active transport of IS, an anionic uremic toxin from the family of the protein bound toxins [23], reported as strongly linked to the fatal outcome in kidney patients [4, 31]. This active transport indicates the presence of functional OAT1, BCRP and MRP4 transporters. OAT1 transporter, expressed along the basolateral membrane of PTEC, is responsible for the uptake of anionic uremic metabolites [18, 21] and is crucial for their renal elimination. BCRP and MRP4 transporters, located on the apical membrane of PTEC, are responsible for the toxin excretion to the urine in the matured ciPTEC monolayer [18]. When the modules were incubated with probenecid, an inhibitor of those transporters, the transport of IS was reduced by approximatively 40%. In comparison to the study performed on a short single HFM [18], we also found a similar inhibition of the transport of IS (100 μ M) by probenecid 100 μ M: $48 \pm 31\%$ versus $55 \pm 6\%$ respectively, $P < 0.001$.

Finally, we studied the production of IL-6, IL-8, TNF- α and sHLA-class I, in basic conditions and in response to LPS and IFN- γ , which we used to mimic extreme inflammatory conditions. In the context of a BAK, the production of pro-

inflammatory cytokines is important for the activation of immune cells and propagation of inflammatory response usually present in CKD patients, which could have undesirable effects. In most of the literature studies on BAK [7-9, 13], the concentrations of cytokines were measured only from the plasma (apical) compartment *in vitro* or the blood (basolateral) compartment *in vivo*. In our work, we studied the concentrations of pro-inflammatory cytokines in both apical and basolateral compartments. Both TNF- α and sHLA-class I were detected at very low concentrations in both compartments. When we corrected the concentrations for the volume of the compartments, a more obvious difference could be observed between extraluminal and basolateral compartment. For IL-6 and IL-8, the produced amounts were higher, especially after LPS stimulation. Both in the absence and in the presence of LPS or IFN- γ , the secretion of IL-6, IL-8 and of sHLA-class I was significantly higher from the apical side -corresponding to dialysate compartment - compared to the basolateral secretion - corresponding to blood side.

Overall, our results prove that in the 3D environment of a BAK device, the ciPTEC are fully polarized and have a privileged secretion of cytokines and sHLA-class-I molecules towards the dialysate compartment, which would greatly reduce risks associated with eventual pro-inflammatory and immunogenic effects of ciPTEC.

5. Conclusion & outlook

This work presents the successful upscaling of the OAT1-expressing ciPTEC line using functionalized MicroPES HFM. CiPTEC grow in a uniform and tight monolayer, as shown by immunochemistry and low inulin-FITC transport. More importantly, ciPTEC demonstrate active organic anionic transport, crucial for the removal of uremic anionic toxins, such as IS. In addition, the ciPTEC were fully polarized since the release of pro-inflammatory cytokines IL-6 and IL-8 and of sHLA-class I was mainly oriented towards the apical side, or dialysate compartment, which is not in direct contact with the patient body fluid. Overall,

for the first time in an upscaled 3D system, we presented active transport of anionic uremic toxin and polarized favorable immune response of a uniform PTEC monolayer, making this system a very suitable candidate for further BAK applications.

The next step towards a functional BAK device is to culture the cells while exposing them to a unidirectional flow with relative shear stress to mimic the natural kidney proximal tubule physiology. There is evidence that cell metabolism is stimulated when culturing cells under dynamic conditions [32-36], which suggests that better toxin removal could be achieved. Moreover, a ciPTEC-based BAK device should also ensure a sufficient toxin clearance for a prolonged session. Here, the upscaled “living membrane” has been tested for 10 min. Therefore, future work should evaluate longer clearance periods, using plasma or blood samples from CKD patients.

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7. List of abbreviations

BAK -	bioartificial kidney device
BCRP -	breast cancer resistance protein
ciPTEC -	conditionally immortalized human proximal tubule epithelial cells

CWF -	clean water flux
ECM -	extracellular matrix
HBSS -	Hank's balanced salt solution
HFM -	hollow fiber membrane
IL -	Interleukin
Inulin-FITC -	fluorescein isothiocyanate (FITC)-labelled-inulin
KHH -	Krebs-Henseleit buffer supplemented with HEPES (10 mM)
L-Dopa -	3,4-dihydroxy-L-phenylalanine
LPS -	lipopolysaccharide
MRP -	multidrug resistance-associated protein
OAT -	organic anionic transporter
OCT -	organic cationic transporter
PES -	polyethersulfone
PTEC -	proximal tubule epithelial cell
SEM -	scanning electron microscopy
SHLA -	soluble human leukocyte antigens
TNF- α -	tumor necrosis factor α
ZO-1 -	zonula occludens-1

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9. Supplement

9.1. SEM images of sterilized uncoated and coated HFM

Figure S1. SEM images of MicroPES HFM. Magnification x900 and x950. (A, B, C) uncoated and (D, E, F) with L-Dopa and collagen IV double coating. HFM (A, D) outside surface, (B-E) inside surface and (C, F) cross-section.

Figure S2. SEM images of MicroPES HFM. Magnification x9000. (A, B, C) uncoated and (D, E, F) with L-Dopa and collagen IV double coating. HFM (A, D) outside surface, (B-E) inside surface and (C, F) cross-section.

9.2. Absolute values of produced pro-inflammatory cytokines

Figure S3. Immune activation and production of pro-inflammatory cytokines by matured ciPTEC on upscaled BAK. Absolute values, corrected for the volume of the compartments. (A) Schematic presentation of the experimental set up used for the study of the release of the pro-inflammatory cytokines, here exposure to LPS and measure of IL-6, IL-8 and TNF-α; an identical setup was used in case of IFN-γ treatment and sHLA-class I release. (B, C, D, E) Effect of 24 h basolateral or apical

*exposure to LPS (10 µg/ml) (B, C, D) or IFN-γ (300 ng/ml) (E) on production of pro-inflammatory cytokines (IL-6, IL-8 and TNF-α) and sHLA-class I in the apical and basolateral compartments. Data are presented as mean ± (standard error mean; SEM) of at least 3 modules per case, from two independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 using either unpaired two-tailed test (for differences between two compartments in the same module) or one way ANOVA followed by Tukey's multiple comparison test (for differences between treatments and different modules*

Chapter 7

Conclusions and Outlook

1. General conclusions

The development of cell based bioartificial kidney device (BAK) could improve existing dialysis therapies for the removal of protein-bound toxins. A key requirement for the BAK is the formation of a “living membrane” consisting of a tight renal cells monolayer with preserved functional organic ion transporters, on an artificial porous membrane.

The main aim of this thesis was to develop an upscaled BAK prototype, making use of conditionally immortalized human proximal tubule epithelial cells (ciPTEC), seeded on functionalized hollow fiber membranes (HFM). The aim and general structure of this thesis was presented in **chapter 1**.

In **chapter 2**, the kidney physiology was briefly presented. Current renal replacement therapies (RRT) were reviewed and the needs for a BAK were formulated. After a short history review of the BAK, the requirements for this concept were presented. In fact, we highlighted that there is a strong need for a reliable and consistent cell line, for HFM with tailored transport and surface properties but also for device related logistics such as cost-effective manufacturing, storage, and distribution process.

Chapter 3 presented the successful upscaling of a “living membrane” comprising of functionalized MicroPES HFMs supporting ciPTEC. This living membrane, developed under static culturing conditions, exhibited a uniform, reproducible and tight ciPTEC monolayer, on the outside of the HFM, as shown by immunochemistry and reduced inulin-FITC transport. Our work showed that PTEC cultured in this upscaled system featured active organic cationic transporters (OCT), crucial for the removal of uremic cationic metabolites.

In **chapter 4**, the internal surface of commercially available MicroPES HFM was functionalized and seeded with ciPTEC. This configuration is preferred for the development of a clinically relevant BAK due to a better preservation of the cell monolayer against friction and to a greater similarity with the natural proximal tubule. After optimizing the coating on the inside of the HFM, the ciPTEC proliferation and monolayer formation was investigated for different seeding

conditions. The successful seeding parameters for the formation of the tight barrier were identified. In comparison to other studies, the procedure developed here presented two advantages: a single cell infusion and short proliferation time.

In **chapter 5**, we investigated the ability of alternative surfaces to support the formation of a living membrane. Preliminary results for PES membranes coated with HS, as well as PEI membranes are presented. Both surfaces improved the ciPTEC adhesion and, when combined with the application of L-Dopa, led to the formation of a monolayer. The transepithelial transport tests indicated a barrier function of the monolayer as well as the function of the OCT2 on PES coated with L-Dopa/HS. It was not possible to perform transport experiments on PEI membranes due to their low porosity. Cell attachment strength experiments indicated good cell-cell interaction for both PES membranes coated with L-Dopa/CIV and for PEI membranes. As a result, both studied surfaces – (i) PES coated with L-Dopa/HS and (ii) PEI membranes - could be good candidates for membranes for BAK applications.

Finally, **chapter 6** presented the successful upscaling of a “living membrane” of organic anionic transporter 1 (OAT1)-expressing ciPTEC on functionalized MicroPES HFM. The uniform and tight ciPTEC monolayer demonstrated active OAT, crucial for the removal of uremic anionic toxins, such as indoxyl sulfate (IS). In addition, the ciPTEC were fully polarized since the release of pro-inflammatory cytokines (IL-6 and IL-8) and of sHLA-class I was mainly oriented towards the apical side (dialysate compartment), which would not be in direct contact with the patient body fluid.

2. Outlook

2.1. Direct next steps *in vitro*

This thesis has presented an upscaled system for a BAK application. However there are still several steps to take to achieve a clinically relevant BAK device. First of all, if one would like to pursue in the directions indicated in chapters 3, 4 and 6, the direct next step towards a functional BAK device is to finalize the investigation of the presented prototypes *in vitro*.

- (i) We have cultured the cells statically, and it could be advantageous to culture the cells while exposing them to a unidirectional flow with relative shear stress to mimic the natural kidney proximal tubule physiology. There is evidence [1] the ciPTECs brush-border barrier height and therefore surface available for uremic metabolites uptake increases significantly when culturing cells under shaking conditions in a 2D environment. This result, supported by similar evidence for other cell lines [2-5], suggests better toxin removal under dynamic conditions.
- (ii) Moreover, a ciPTEC-based BAK device should also ensure a sufficient toxin clearance for a prolonged session. In chapters 3 and 6, the upscaled “living membrane” has been tested for 10 to 30 min using isolated toxins. Therefore, future work should evaluate longer clearance periods, using plasma or blood samples from CKD patients.
- (iii) Finally, the used ciPTEC present functional OCT or OAT, therefore a combination of both cell lines in 2 systems used in series should be evaluated, unless a cell line expressing all of the transporters simultaneously is being developed.

2.2. Estimated clinical size of a BAK

Prior to performing an *in vivo* study, a BAK with a clinically relevant size should be constructed. In order to have an estimate of the needed size BAK, one can consider:

- the absolute values of cleared IS (concentration 100 μM), reported by Jansen et. al [6] for OAT-expressing ciPTEC grown on single short HFM: 44 to 73 $\mu\text{l}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$, corresponding to a clearance of 0.26 to 0.44 $\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$;
- uremic plasma concentrations of IS reported in ESRD patients – 129 $\text{pmol}\cdot\mu\text{l}^{-1}$ [7], corresponding to an amount of 450 μmol IS per patient;
- the time of a dialysis session – 4 h.

Based on the information presented above and if we assume that the *in vitro* data obtained here can extrapolated to *in vivo*, the needed BAK surface for one patient would be in the range of 260 – 450 cm^2 . As already mentioned, this estimated value is extrapolated from the clearance of a single toxin, IS, and needs to be calculated for a complex uremic mixture. Indeed, the IS clearance is reduced in the presence of competitors.

The BAK surface of 260 – 450 cm^2 is approximatively one hundred times bigger than the prototype presented in chapters 3, 4 and 6. In the chapters 3 and 6, 100 cm^2 of 90% confluent non mature ciPTEC are necessary in order to achieve a cell monolayer on the external surface of the BAK. It corresponds to 1 m^2 of 90% confluent non mature ciPTEC for a final size device, if we preserve the same module packing density. In the chapter 4, 35 cm^2 of 90% confluent non mature ciPTEC are necessary in order to achieve a cell monolayer on the internal surface of the BAK, which is three time less cells. Therefore, the BAK with a cell monolayer on the inside of the HFM is directly more advantageous in terms of costs.

2.3. A possible way to achieve a BAK of clinical size

We have presented successful upscaling of BAK with ciPTEC seeded on the external surface of the HFM, in **chapters 3 and 6**, respectively expressing OCT and OAT. As explained in **chapter 4**, a cell monolayer achieved on the internal side of the HFM would be highly beneficial for a BAK, both for protection of the cell monolayer and increased similarity with the configuration of the natural nephron. Moreover, combined with the estimation in the previous paragraph, the cell monolayer achieved on the internal surface is more economical. Consequently, the next steps should focus on this configuration. A combination of parameters to achieve a cell monolayer on the inside surface of HFMs was presented showing that we need to perform a cell seeding in 1 time with a cell concentration of $1\text{--}2.5 \cdot 10^6 \text{ cells} \cdot \text{ml}^{-1}$, 4 h of attachment, 4 days of proliferation and 7 days of maturation. A longer initial attachment time of 6 or 8 h, instead of 4 h, could possibly lead to shorter proliferation time and is therefore recommended for further work.

In order to have a system easier to handle and to add an additional mechanical protection to the cells, the use of a multibore HFM could be advantageous. The use of multibore HFM has already been reported for bioartificial organs, for (i) Liver, hepatocytes, [8] and for pancreas [9].

In our laboratory, the multibore fibers have been earlier prepared for the encapsulation of pancreatic cells [9]. Briefly, the multibore hollow fibers were fabricated by dry-wet spinning via immersion precipitation using a specially designed spinneret. The polymer dope solution was a blend of 15wt% polyethersulfone (PES, Ultrason E6020) and 10wt% polyvinylpyrrolidone (PVP K90, Sigma Aldrich) dissolved in N-methylpyrrolidone (NMP) (Acros organic). The polymer dope and bore solution (water) were pumped through the spinneret and after a 6-cm air gap, the nascent multibore hollow fiber was immersed into the water coagulation bath, where phase separation occurred and the fiber was formed. The collected hollow fiber membranes were washed with demineralized water in order to remove remaining solvent traces and stored in demineralized water until further use. To increase the membrane

porosity, the PVP of some of the developed fibers was washed with 4000 ppm sodium hypochlorite aqueous solution (NaClO, Fluka) for 24 hours. Subsequently, the membranes were washed and stored in demineralized water. Prior to drying, the membranes were immersed in a 25 vol.% glycerol solution for 24 h to protect the hollow fiber structure and morphology during air drying.

Our preliminary results using PES multibore coated on the inside with L-Dopa and collagen IV showed good formation of tight junctions, see Figure 1. Besides, taking into account the preliminary results of chapter 5, and the rising costs of the nature-derived products used for the membranes functionalization, a multibore PEI HFM (or a PES HFM with a PEI coating) with tailored transport properties [10-13] could be a good option for a BAK. Finally, performing modelling studies of a BAK could assist in designing a BAK for optimal PTEC function and longevity. In fact, modelling could aid optimizing the dimensions of the hollow fiber, the packing of the HFM, the flows of dialysate and body fluid, the control of nutrients and oxygen distribution. In our laboratory in collaboration with the EU Marie Curie ITN-TheLink (grant agreement 642890), the first steps have been undertaken to validate a BAK model.

Figure 1. (A) Scanning electron microscopy images of cross-section of PES multibore hollow fiber membrane. (B and C) confocal images of longitudinal section of multibore HFM functionalized with L-Dopa and collagen IV supporting matured ciPTEC. In blue the cell nuclei, in green the expression of the tight-junction protein ZO-1.

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Summary

Summary

The development of cell based bioartificial kidney device (BAK) could improve existing dialysis therapies for the removal of protein-bound toxins. A key requirement for the BAK is the formation of a “living membrane” consisting of a tight renal cells monolayer with preserved functional organic ion transporters, on an artificial porous membrane.

The main aim of this thesis is to develop an upscaled BAK prototype, making use of conditionally immortalized human proximal tubule epithelial cells (ciPTEC), seeded on functionalized hollow fiber membranes (HFM). The aim and general structure of this thesis are presented in **chapter 1**.

Chapter 2 introduces the background of the kidney anatomy and function. It gives an overview of the current renal replacement therapies (RRT), their advantages and limitations. After a short history review of the BAK, the requirements for this concept are presented. In fact, we highlight that there is a strong need (i) for a reliable and consistent cell line, (ii) for HFM with tailored transport and surface properties, (iii) but also for device related logistics such as cost-effective manufacturing, storage, and distribution process.

Chapter 3 presents the upscaling of a “living membrane” for a BAK device. First, the development of a double L-Dopa and collagen IV coating on the external surface of a commercially available hollow fiber membrane (HFM) module is reported and the HFM transport properties are studied. Secondly, Organic Cationic Transporter (OCT)-expressing ciPTEC are seeded on the functionalized HFM and the quality of the obtained monolayer is studied. Immunochemistry results and reduced inulin-FITC transport prove that this living membrane, developed under static culturing conditions, exhibits a uniform, reproducible and tight ciPTEC monolayer, on the outside surface of the HFM. Finally, our work shows that ciPTEC cultured in this upscaled system feature active OCT, crucial for the removal of uremic cationic metabolites.

Chapter 4 investigates strategies for achieving a good quality OCT2-expressing ciPTEC monolayer on the inside surface of the polymeric HFM. This

configuration could be preferred for the development of a clinically relevant BAK due to a better preservation of the cell monolayer against friction and to a greater similarity with the natural proximal tubule. We first optimize the functionalization of the internal surface of the polymeric fiber to achieve high transport of metabolites. Secondly, we investigate several cell seeding parameters in order to achieve a tight ciPTEC monolayer on the inside surface of the HFM. The successful seeding parameters for the formation of the tight barrier are identified. In comparison to other studies, the procedure developed here presents two advantages: a single cell infusion and a short proliferation time.

Chapter 5 investigates the ability of alternative flat surfaces to support the formation of a “living membrane”. Following our hypothesis, not only collagen IV but also other elements of the natural kidney epithelial extra cellular matrix can be used to coat membranes to support ciPTEC. Moreover, we investigate the ability of a positively charged polymer membrane to support the adhesion of the negatively charged cells. Preliminary results for PES membranes coated with HS, as well as for PEI membranes are presented. Both surfaces improve the ciPTEC adhesion and, when combined with the application of L-Dopa, lead to the formation of a monolayer. The transepithelial transport tests indicate a barrier function of the monolayer as well as the function of the OCT2 on PES coated with L-Dopa/HS. Cell attachment strength experiments indicate good cell-cell interaction for both PES membranes coated with L-Dopa/CIV and for PEI membranes. As a result, both studied surfaces – (i) PES coated with L-Dopa/HS and (ii) PEI membranes - could be good candidates for membranes for BAK applications.

Chapter 6 presents the successful upscaling of a “living membrane” of organic anionic transporter 1 (OAT1)-expressing ciPTEC on functionalized MicroPES HFM. The uniform and tight ciPTEC monolayer, achieved on the external side of the HFM, demonstrates active OAT, crucial for the removal of uremic anionic toxins, such as indoxyl sulfate (IS). Furthermore, we study the immune response of the ciPTEC. The ciPTEC are fully polarized: the pro-inflammatory cytokines are mainly released towards the apical - dialysate - compartment,

which would not be in direct contact with the patient body fluid. This would greatly reduce risks associated with eventual pro-inflammatory and immunogenic effects of the ciPTEC.

Finally **chapter 7** presents the general conclusions and reflections on the future directions in the development of a clinically relevant BAK.

Samenvatting

Samenvatting

De ontwikkeling van een biokunstmatige nier (Bioartificial kidney: BAK) is één van de mogelijke verbeteringen van de huidige bestaande dialyse behandelmethoden voor het verwijderen van proteïne-gebonden gifstoffen. Een essentiële eis voor de goede werking van een biokunstmatige nier is de vorming van een “levend membraan” op een poreus support (een membraan). De levende laag bestaat uit een monolaag van functionele niercellen die belangrijke eigenschappen behouden zoals functioneel transport van organische ionen.

Het doel van dit onderzoek is het opschalen van een BAK prototype membraan naar module grootte van meerdere vierkante centimeters. Hierbij wordt in dit onderzoek gebruik gemaakt van gemodificeerde niercellen: onsterfelijke proximaal tubulair epitheliaal cellen (ciPTEC) die gekweekt worden op holle vezel membranen die specifiek deze cellen hechten. Het support-membraan is hiervoor gefunctionaliseerd. **Hoofdstuk 1** formuleert het doel van het onderzoek en zet de algemene aanpak van dit onderzoek uiteen.

Hoofdstuk 2 geeft een korte samenvatting van de functie van de nieren in het lichaam. Daarnaast worden de huidige bestaande behandelmethodes bij nierfalen besproken met hun voor- en nadelen. De eisen voor een succesvolle biokunstmatige nier worden geformuleerd en deze zijn: (i) een betrouwbare cellen soort met de juiste eigenschappen moet beschikbaar zijn; (ii) een gefunctionaliseerd membraan is benodigd die selectief deze niercellen kan hechten en goede vloeistof-transport eigenschappen behoudt; (iii) het levend membraan moet kosten-effectief en betrouwbaar geproduceerd, getransporteerd en bewaard kunnen worden.

Hoofdstuk 3 onderzoekt het opschalingsproces van het levende membraan voor een bio kunstmatige nier. Ten eerste wordt een commercieel verkrijgbaar holle vezel dialyse membraan (HFM) module gefunctionaliseerd met een dubbele L-dopa en collageen IV coating. De transport eigenschappen voor vloeistoffen worden voor en na deze behandeling vergeleken. Ten tweede wordt een ciPTEC cellenlaag op het membraan gekweekt onder

statische omstandigheden die selectief organische cationen transporteert. De kwaliteit en uniformiteit van de aangebrachte laag wordt hierna bestudeerd. Immunochemie experimenten en verminderd insuline-transport experimenten tonen aan dat het levende membraan een uniforme en dichte ciPTEC cellenlaag heeft die reproduceerbaar aan te brengen is. Ten slotte wordt aangetoond dat het opgeschaalde system actief selectief organisch cationisch transport heeft, wat cruciaal is voor het verwijderen van uremische kationische metabolieten vanuit het lichaam.

Hoofdstuk 4 onderzoekt de strategieën voor het aanbrengen van eenzelfde selectieve ciPTEC monolaag aan de binnenzijde van een hollevezel membraan. Deze configuratie kan in de praktijk de voorkeur hebben vanwege een betere bescherming tegen frictie en een grotere gelijkenis met de natuurlijke 'proximale buis'. Ten eerste wordt het functionaliserings proces geoptimaliseerd voor het maximaliseren van de transport eigenschappen (van metabolieten) door het membraan, met behoud van reproduceerbaarheid en uniformiteit. Ten tweede worden verschillende parameters in het cel kweek proces van de ciPTEC laag gevarieerd om een dichte monolaag te bereiken die reproduceerbaar te maken is. De best gevonden parameters voor het vormen van een dichte barrière zijn bepaald. In vergelijking met andere vergelijkbare studies is de heeft de ontwikkelde procedure twee voordelen: enkele cell infusie en een korte proliferatie tijd.

Hoofdstuk 5 onderzoekt de mogelijkheid van andere (vlakke) support materialen voor de vorming van het levende membraan. De hypothese stelt dat naar collageen IV ook andere elementen uit de natuurlijke nier epitheliale extra cellulaire matrix, zoals heparaan sulfaat (HS) kunnen worden gebruikt om membranen te functionaliseren om ciPTEC cellen te hechten. Verder wordt de mogelijkheid onderzocht om een positief geladen oppervlak polymeer membraan te gebruiken voor de adhesie van negatief geladen cellen. De eerste resultaten van polyethesulfon (PES) en Polyether imide (PEI) membranen die gecoat zijn met HS worden gepresenteerd. Beide gefunctionaliseerde oppervlaktes verbeteren de hechting van ciPTEC, en wanneer gecombineerd met L-Dopa, leidt dit tot de vorming van een monolaag. De transepitheliale

transport testen indiceren dat deze laag ook een barrière functie heeft. Cell-hechtings sterkte experimenten resulteren in een goede cel-cel interactie voor zowel PES gecoated met L-DOPA/CIV en voor PEI membranes. Het resultaat is dat beide geteste oppervlakten i) PES coated met L-DOPA/HS en ii) PEI membranes goede kandidaten zijn voor BAK applicaties.

Hoofdstuk 6 presenteert een alternatieve opschalingsroute van een levend membraan met een anionic transporter-expressing (OAT) ciPTEC op een gefunctionaliseerde microPES holle vezel membraan. De uniforme en dichte ciPTEC monolaag die aan de buitenzijde van de hollevezel membraan is aangebracht bewijst dat OAT actief is, wat cruciaal is voor de verwijdering van uremische anionische toxinen (zoals indoxyl sulfaat) uit het lichaam. Verder zijn de ciPTEC volledig gepolariseerd. De pro-inflammatoire cytokines worden voornamelijk aan de dialysaat zijde van het membraan vrijgelaten. Deze zijde is niet in contact met het bloed van de patient, en vermindert het risico op eventuele pro-inflammatoire en immunogenische effecten van de ciPTEC.

In **hoofdstuk 7** worden de algemene conclusies besproken en de mogelijke vervolgroutes voor verdere ontwikkeling van een biokunstmatige nier genoemd.

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Natalia Vladimirovna Chevtchik, July 2017

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About the author

Natalia Vladimirovna Chevtchik was born in Minsk, USSR (currently Belarus) on December 16th 1987. She moved to Lyon, France, in 1994 with her parents. She studied in Lyon and obtained her Scientific Baccalaureate with distinction in 2005. She obtained her Master of Engineering in 2010 from

I.N.S.A. Lyon, with specialization in Materials Science and Polymers. She obtained the same year a double Master from the University of Lyon on Innovative Polymers. Interested by the medical applications of polymers, she joined Sofradim Medtronic (formerly Covidien) in Trévoux, France. After completing her master thesis, she became in September 2010 Engineering Project Leader at Sofradim. She developed manufacturing process for implantable products for muscular hernia repair. Driven by the will to work on highly innovative products, she moved to the R&D department and then decided to go back to academia and start a PhD program.

She left Sofradim and joined in August 2013 the BST group at the University of Twente, Enschede, the Netherlands for a Marie Curie ITN Fellowship. She conducted her research on the development of an upscaled bioartificial kidney under the supervision of Prof. Dr. Stamatialis. At the end of her PhD contract, she joined Bond3d high Performance Technology where she is Polymer Expert since February 2017.

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