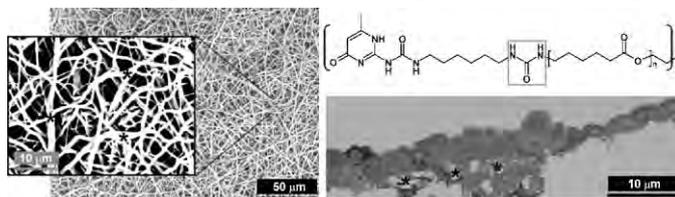


The Use of Fibrous, Supramolecular Membranes and Human Tubular Cells for Renal Epithelial Tissue Engineering: Towards a Suitable Membrane for a Bioartificial Kidney^a

Patricia Y. W. Dankers,^{*b} Jasper M. Boomker,^b Ali Huizinga-van der Vlag, Frank M. M. Smedts, Martin C. Harmsen, Marja J. A. van Luyn

A bioartificial kidney, which is composed of a membrane cartridge with renal epithelial cells, can substitute important kidney functions in patients with renal failure. A particular challenge is the maintenance of monolayer integrity and specialized renal epithelial cell functions *ex vivo*. We hypothesized that this can be improved by electro-spun, supramolecular polymer membranes which show clear benefits in ease of processability. We found that after 7 d, in comparison to conventional microporous membranes, renal tubular cells cultured on top of our fibrous supramolecular membranes formed polarized monolayers, which is prerequisite for a well-functioning bioartificial kidney. In future, these supramolecular membranes allow for incorporation of peptides that may increase cell function even further.



P. Y. W. Dankers, J. M. Boomker, A. Huizinga-van der Vlag, M. C. Harmsen, M. J. A. van Luyn
Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, Hanzeplein 1, NL-9713 GZ Groningen, The Netherlands
Fax: +31 40 247 8367; E-mail: p.y.w.dankers@tue.nl
P. Y. W. Dankers
Current address: Institute for Complex Molecular Systems, and Laboratory of Chemical Biology, Eindhoven University of Technology, P.O. Box 513, NL-5600 MB Eindhoven, The Netherlands
F. M. M. Smedts
Laboratory of Pathology, Foundation of Collaborating Hospitals of Eastern Groningen, Grintweg 71, NL-9675 HJ Winschoten, The Netherlands

Introduction

End-stage renal disease is a life-threatening condition that affects many patients worldwide. Whilst a kidney transplantation is the best solution on the long term, the majority of patients depends on hemodialysis therapy; a method which removes waste products and excess water by dialysis of the blood against a defined salt solution over a semi-permeable membrane. Although this technique can sufficiently remove small molecules and waste products, it is inadequate for the removal of large and protein-bound waste products, which subsequently accumulate in the body and cause uremic toxicity. It has been shown that the selectivity of solute transport over the dialysis membrane can be enhanced by the use of renal epithelial cells that are cultured on top of these conventional dialysis membranes.^[1] This concept of cell-aided solute transport has been developed into a bioartificial kidney.^[1] Placed in series with a conventional hemofiltration module, such

^a Supporting information for this article is available at the bottom of the article's abstract page, which can be accessed from the journal's homepage at <http://www.mbs-journal.de>, or from the author.

^b P. Y. W. Dankers and J. M. Boomker contributed equally to this work.

bioartificial kidney was shown to replace critical endocrine and metabolic renal functions.^[2]

Initially, to develop a bioartificial kidney renal epithelial cell lines were used from porcine or canine origin, i.e. respectively Lewis lung cancer-porcine kidney 1 (LLC-PK1) cells and Madin-Darby canine kidney (MDCK) cells. Although, confluent layers of MDCK cells on polycarbonate (PC) membranes initially displayed active Na^+ transport, functional properties could not be maintained after 2 weeks.^[3] Moreover, the loss of function was associated with the aberrant distribution of Na^+/K^+ ATPase, multilayer growth, and necrosis.^[3] Reabsorption of water, glucose and sodium could be maintained up to 10 d when LLC-PK1 cells were used.^[4] Importantly, the type of membrane material and the extracellular matrix (ECM) protein coating on the membrane used, appeared to be critical for the adhesion and functional differentiation of LLC-PK1 cells.^[5]

Humes et al. applied human renal epithelial cells in a conventional hollow fiber cartridge to replace endocrine and metabolic renal functions in uremic dogs.^[6] During 24 h the epithelial cells in the cartridge displayed ammonia excretion, and 1,25-dihydroxyvitamin D3 conversion and to a limited extent electrolyte transport. Although the aforementioned studies clearly outline the feasibility of the application of cells for selective solute transport, their performance critically depends on complete cell coverage of the cartridge membranes, the subsequent maintenance of an epithelial cell monolayer as well as the expression of relevant solute transporter proteins.

Exposure of renal epithelial cells to an atypical physico-chemical environment, e.g. fluctuations of glucose levels, lactate accumulation, hypoxia or hyperoxia, an aberrant ECM, and lack of heterotypic cell interactions, contribute to monolayer disruption and subsequent dedifferentiation events. Culturing of renal epithelial cells under organotypic perfusion culture conditions might help to maintain differentiated renal epithelial cell characteristics.^[7]

It is a great challenge to improve the bioartificial kidney in such a way that within the device epithelial cells can easily adhere to the membrane, form monolayers, and encounter a regenerative, organotypic renal niche. In addition, the production requirements for such a device impose the use of easily processable polymeric materials. These conditions might be accomplished by using supramolecular, ureido-pyrimidinone (UPy)^[8] modified polymers electro-spun into fibrous membranes (Figure 1). The monomers or short oligomers in supramolecular polymers are held together via strong, directed, non-covalent interactions.^[9] They behave as macromolecules at ambient temperatures, but display low molecular weight properties at elevated temperatures. Therefore, benefits are shown in their ease of processability and in their tunable mechanical properties.^[10] In addition, these supramolecular polymers allow for a modular approach to bioactive membranes by simple incorporation of bioactive modalities functionalized with the same supramolecular unit.^[10,11] Here, we apply UPy-moieties connected to LMW polycaprolactone, i.e. oligocaprolactone,

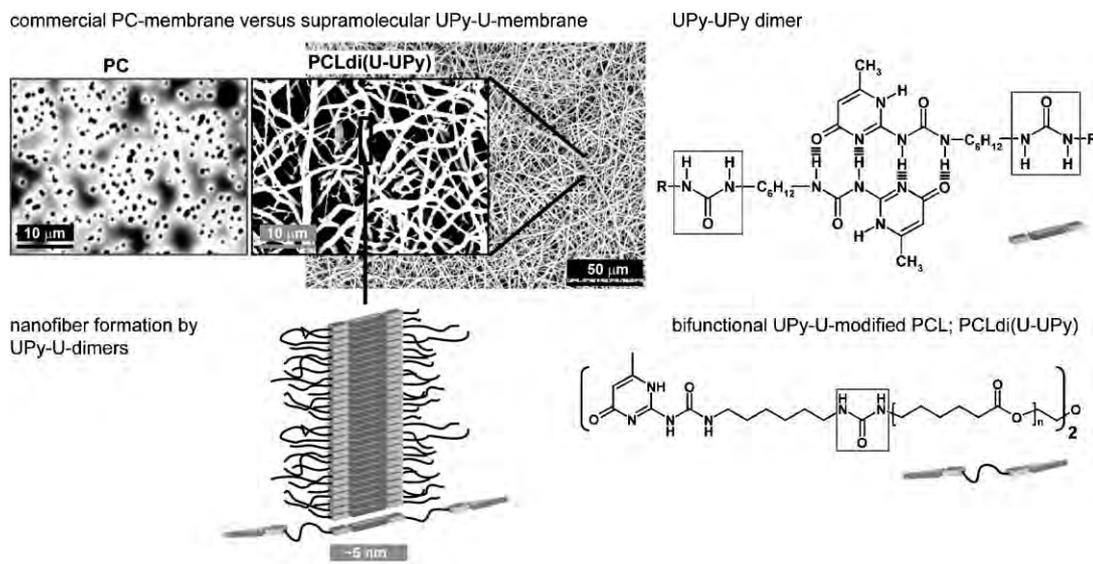


Figure 1. Membrane design: fibrous, supramolecular membranes and commercial microporous membranes. Four-fold hydrogen bonding ureido-pyrimidinone (UPy) moieties form dimers, and stack in lateral direction via additional hydrogen bonding between urea (U) functionalities forming nanofiber structures. These UPy-U-groups were used to end-functionalize short polycaprolactones ($\overline{M}_n = 2000 \text{ g} \cdot \text{mol}^{-1}$) in order to obtain supramolecular UPy-U-polymers (PCLdi(U-UPy)). These supramolecular polymers were electro-spun into fibrous, supramolecular UPy-U-membranes composed of microfibrils. Commercial flat microporous PC membranes were used as reference membranes.

via an urea (U) functionality as supramolecular units. It has been shown that different polymers modified with a UPy-moiety via a urea form nano-fibers in the lateral direction via additional hydrogen bonding between the urea groups and π - π interactions between the UPy-U-dimers, and therefore behave as thermoplastic elastomers (Figure 1).^[12]

Our study set-up implies the functional read-out of human primary tubular epithelial cells (PTEC) cultured on two-dimensional (2D) fibrous, supramolecular PCLdi(U-UPy) membranes in comparison to PTEC cultured on 2D commercially available microporous PC membranes. As first proof of principle the PTEC were cultured for a short period of time, i.e. 1 week, both under static and perfusion conditions. Their capability to form monolayers, biochemical performance and ability to maintain epithelial cell specific gene expression were investigated.

Experimental Part

Preparation and Characterization of UPy-U-Membranes

The bifunctional UPy-U-modified LMW polycaprolactone base polymer, PCLdi(U-UPy), $\overline{M}_n = 2.7 \text{ kg} \cdot \text{mol}^{-1}$, was synthesized as described before.^[13] A home-built electro-spinning set-up equipped with a KD Scientific syringe pump and a high voltage source was used. The membranes were collected on a glass plate covering the ground plate. PCLdi(U-UPy) meshes were prepared from solutions varying between 25 and 30 wt.-% PCLdi(U-UPy) in 5 wt.-% water in tetrahydrofuran (THF) by slow addition of the polymer to the solvent mixture. The resulting solution was spun at a feed rate of 20–30 $\mu\text{L} \cdot \text{min}^{-1}$. The tip-to-target distance was 12 cm and the applied voltage was 15–17.5 kV. Circular membranes with a diameter of 12 mm were punched from the PCLdi(U-UPy) meshes. These circular membranes were clamped in membrane holders (Minucells & Minutissue). As reference membranes, circular commercial PC membranes (Millipore) were used. The electro-spun, fibrous PCLdi(U-UPy) membranes and the commercial microporous PC membranes were studied with scanning electron microscopy (SEM) on a Philips XL30 FEG E-SEM under high vacuum.

Isolation and Culture of PTEC

Human PTEC were isolated from renal cortical tissue obtained from patients that underwent unilateral nephrectomy after diagnosis of a urethral or renal tumor. These patients were informed as to the nature of the study and signed a consent form according to Dutch legal requirements. Isolation, culture and characterization were performed as described in the Supporting Information. The

cells used for seeding on the membranes have been frozen, and have been cultured as from isolation for in total 7–8 d in the case of passage 1, or for in total 11–14 d in the case of passage 2.

Cell Seeding on Membranes

The electro-spun PCLdi(U-UPy) and reference PC membranes were used. First, the membranes were wetted in complete medium and transferred to 24-well plates containing 200 μL complete medium per well. Then, the PTEC were counted after suspension in complete medium, and $1.5 \cdot 10^5$ cells were seeded per membrane in 100 μL complete medium. Cells were left to adhere on the membranes for 1 h at 37 °C and 5% CO₂ in a humidified atmosphere. Subsequently, 700 μL complete medium was added per well, and the cells were precultured for 4 d at 37 °C and 5% CO₂ in a humidified atmosphere under static culture conditions, after which confluency was reached. Medium was changed every 2–3 d when cultured under static culture conditions.

Perfusion Set-up

The single chamber perfusion system (Minucells & Minutissue) used, has been developed and described by Minuth et al.^{[7b], [14]} When cells were cultured in the perfusion systems 1% bufferall (Sigma-Aldrich) was added to the complete medium to maintain a pH 7.4 at ambient CO₂ levels. In addition, also 0.5 $\mu\text{g} \cdot \text{ml}^{-1}$ fungizone (Bristol Meyers Squibb) was added. The medium was pumped through the containers by a peristaltic pump (Ismatec) at a rate of 1 $\text{mL} \cdot \text{h}^{-1}$. Medium was pumped from 4 °C cooled sterile storage glass bottles equipped with a filter-vented screw cap, and medium was collected at room temperature in similar glass bottles. Since the system was used in a flow cabinet outside an incubator, the temperature was maintained at 37 °C using a heating plate (Medax). Glucose and lactate levels in the culture medium were monitored for 72 h, showing that under static culture conditions the glucose level was decreased and the lactate level was increased in time, while both levels stayed constant in time in the perfusion system.

Study Set-up

After preculturing of the PTEC on the membranes for 4 d, 24 precultured membranes were put in the single chamber perfusion system. The side of the membrane without the cells, i.e. the basal side, was put at the inlet side. The perfusion container was put on the heating plate in the horizontal position. The experimental set-up in detail is shown in the Supporting Information in Table S1. The whole procedure was carried out in duplicate for the

PCLdi(U-UPy) membranes, and in duplicate for the PC membranes. For each experiment, PTEC from 3 different donors were seeded on in total 72 membranes. The PTEC were precultured on these 72 membranes for 4 d, after which 24 membranes were pretreated for the appropriate analyses (control), 24 membranes were left under static conditions for 7 additional days (static culture), and 24 membranes were put in the perfusion system for 7 d (perfusion). PTEC were cultured for in total 11 d under static culture conditions (static culture: 11 d). The PTEC in the perfusion system were first cultured for 4 d under static conditions and then 7 d in perfusion (perfusion: 4 + 7 d). The PTEC cultured on the membranes were characterized with: toluidin blue staining on 3 μm longitudinal sections, transmission electron microscopy (TEM) on longitudinal ultrathin slices, fluorescent immunostainings [anti-epithelial cell adhesion molecule (anti-EpCAM), and anti-aquaporin 1 (anti-AQP1)], resazurin cell viability assay, brush border enzyme activity assays, and reverse transcriptase polymerase chain reaction (RT-PCR) on several transcripts.

Toluidin Blue Staining: Monolayer Formation

Monolayer formation of the cells on membranes was evaluated. After culturing, the cells were washed twice with phosphate-buffered saline (PBS), fixed in 2% glutaraldehyde (Merck) at 4 $^{\circ}\text{C}$, again washed twice with PBS, and dehydrated through a graded series of ethanol. The samples were embedded in Technovit 7100 (Heraeus Kulzer). Semithin 3 μm longitudinal sections were stained with 1% toluidin blue (Fluka Chemical) in 1% sodium tetraborate (Merck).

TEM: Cell-Cell Contacts and Brush Borders

After culturing, the cells were washed twice with PBS, fixed in 2% glutaraldehyde at 4 $^{\circ}\text{C}$. Then, the cells were again washed twice with PBS, washed with 6.8% sucrose in PBS, and post-fixed in 1% osmium tetroxide (Merck) with 1.5% tetrapotassium hexacyanoferrate (Merck) in PBS at 4 $^{\circ}\text{C}$. The cells were washed with distilled water, dehydrated through a graded series of ethanol, and embedded in EPON 812 (Serva Feinbiochemica). Ultrathin sections were cut on a Sorvall microtome (Sorvall) and contrasted with uranyl acetate (Merck) and lead citrate (Merck), and evaluated with a Philips 201 TEM (Philips).

Fluorescent Immunostaining

For fluorescent immunostaining, PTEC (cultured on collagen I coated cover slips, or on the membranes) were washed twice with PBS, and subsequently fixed in 2% paraformaldehyde (Merck) for 10 min at 21 $^{\circ}\text{C}$. The fixed

cells were stored in PBS at 4 $^{\circ}\text{C}$ until further use. Non-specific binding sites were blocked with 5% animal serum and 2% bovine serum albumin in PBS for 30 min. Endogenous biotin was blocked with a biotin blocking kit (Dako). Then, the PTEC were incubated with the following primary antibodies for 1 h, and the appropriate secondary antibodies for 30 min: mouse anti-EpCAM (MOC31; undiluted hybridoma supernatant; Serotec) with secondary antibody rabbit anti-mouse-fluorescein isothiocyanate (FITC) (1:100; Dako), and rabbit anti-AQP1 (1:100; Chemicon International) with secondary antibody goat anti-rabbit-biotin

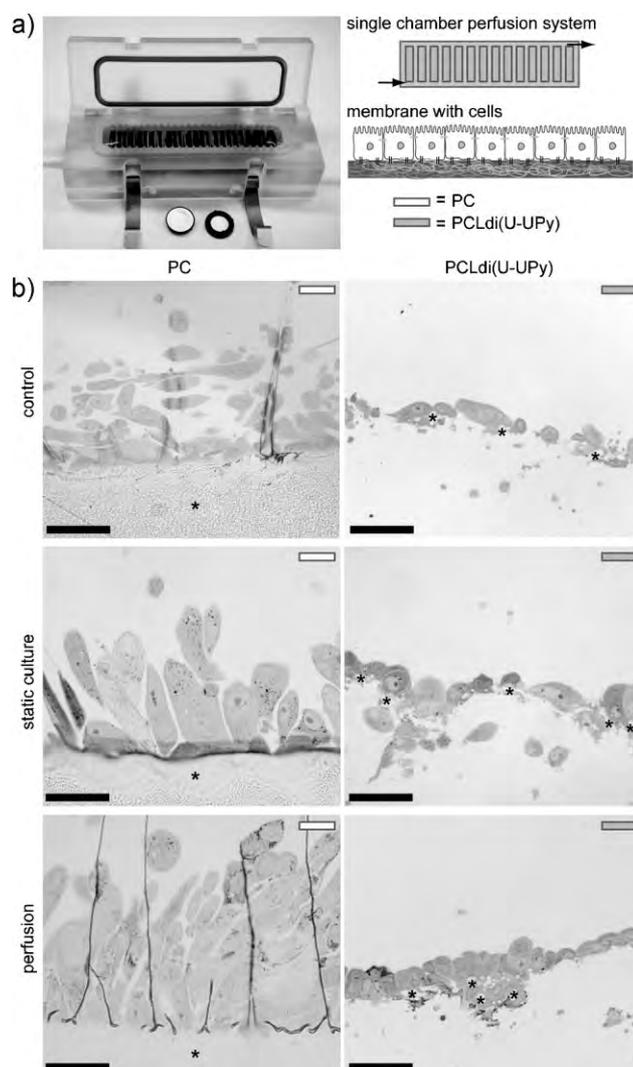


Figure 2. Single chamber perfusion. (a) The single chamber perfusion system with membrane holders is shown. In this system, PTEC were cultured on membranes of PCLdi(U-UPy), and as reference on microporous membranes of PC. (b) Toluidin blue staining of sections of PTEC cultured on the membranes. PTEC were precultured on the membranes until confluency was reached (control). Then they were cultured under static culture conditions or in the single chamber perfusion system for 7 d. The PC membrane and PCLdi(U-UPy) fibers are indicated with *. Scale bars represent 10 μm .

(1:400; Dako) and additional streptavidin-FITC (1:400; Dako) for 30 min. Staining with 4,6-diamidino-2-phenylindole (DAPI; 1:5000; Sigma Aldrich) was used to visualize cell nuclei. The samples were subsequently embedded in CitiFluor (Agar Scientific). Fluorescence microscopy was performed using a Leica DMLB microscope, Leica DC300F camera and Leica Qwin 2.8 software (Leica Microsystems).

Resazurin Assay: Cell Viability

For the assessment of cell viability a resazurin assay was used. The cells were incubated for 2 h at 37 °C in 800 μ L culture medium per well containing $4.4 \cdot 10^{-5}$ M resazurin (Sigma Aldrich). Viable cells convert non-fluorescent resazurin into fluorescent resorufin ($\lambda_{\text{ex}} = 540$ nm, $\lambda_{\text{em}} = 590$ nm) which was measured with a Varioskan plate reader (Thermo Fisher Scientific) in duplicate in 200 μ L. The assays were carried out under static culture conditions.

Brush Border Enzyme Activity

Brush border enzyme activities were measured in cell extracts that were prepared by freeze-thawing at -20 °C in 400 μ L $5 \cdot 10^{-3}$ M Tris-HCl/well, 0.9% NaCl, pH 7.4. γ -Glutamyl transferase activity was determined by kinetic colorimetric measurement of the transfer of γ -glutamyl residue from L-glutamic acid 5-(3-carboxy-4-nitroanilide) to glycyl-glycine yielding γ -glutamyl glycyl-glycine (Sigma Aldrich) and 3-carboxy-4-nitroaniline. Alkaline phosphatase activity was determined by kinetic colorimetric measurement of the conversion of 4-nitrophenyl phosphate (Sigma Aldrich) into 4-nitrophenol. Alanine aminopeptidase activity was determined by kinetic colorimetric measurement of the conversion of L-alanine-4-nitroanilide (Sigma Aldrich) into L-alanine and 4-nitroaniline. The brush border enzyme activities were standardized with respect to the amount of protein in the samples which was assessed with the Bradford assay using bovine serum albumin as reference.^[15] A Varioskan plate reader was used (Thermo Fisher Scientific).

RT-PCR Analysis

RNA was extracted from the PTEC (cultured on collagen I coated culture dishes, or on the membranes) with the use of the RNeasy Micro Kit (Invitrogen). For the

reverse transcriptase reaction, 0.5 U RNase inhibitor (Fermentas), 10^{-3} M dNTPs (Fermentas), 500 ng T18 primer, and 10 U Moloney Murine Leukemia Virus Reverse Transcriptase (Fermentas) in reverse transcriptase buffer was added to 200 ng of DNase-treated RNA. For the amplification reaction, an amount of cDNA equivalent to 10 ng RNA was added to a reaction mixture containing 2.5 U Taq DNA polymerase (Fermentas), $2 \cdot 10^{-4}$ M dNTPs, $1.5 \cdot 10^{-3}$ M MgCl_2 and 10^{-6} M gene-specific primers. The cDNA was amplified using 35 cycles. In Table S2, Supplementary Information, a list of primer sequences that were used in this study can be found.

Statistical Analysis

Differences in data were evaluated with the Kruskal-Wallis test followed by Dunns post-hoc multiple comparison analysis (for each membrane between the control, static and perfusion culture group). Differences between two groups were analyzed with a one-tailed Mann-Whitney test for the control, static and perfusion culture group between the membranes PC and PCLdi(U-UPy). In all cases a 95% confidence interval was used. All data is expressed as mean \pm standard error of mean. Probabilities of $P < 0.05$ were

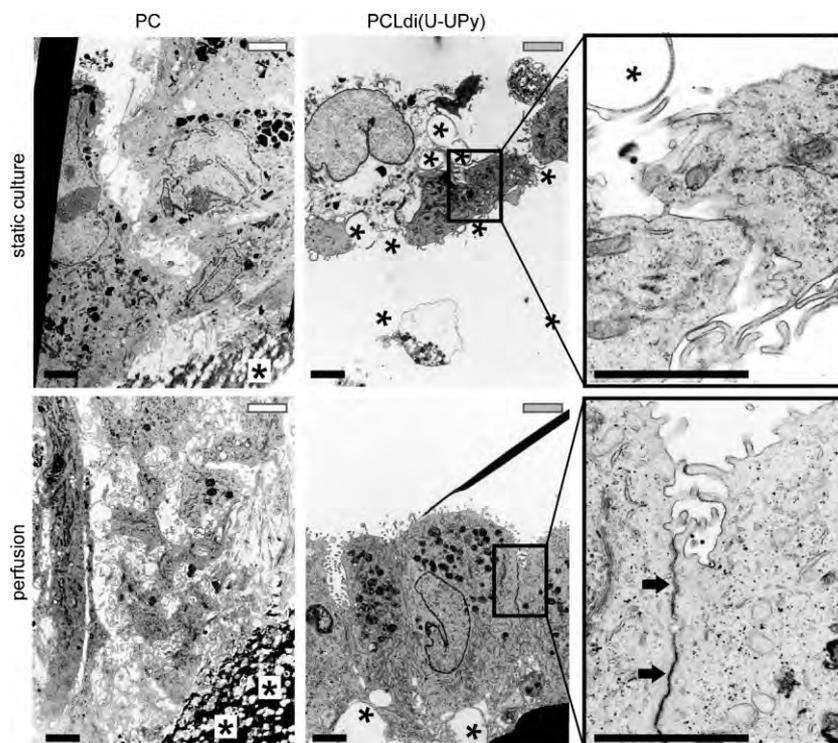


Figure 3. Visualization of cell-cell junctions of PTEC cultured on PC and PCLdi(U-UPy) membranes by TEM. PTEC were precultured on these materials until confluency was reached. Confluent cultures were continued in static conditions with regular medium replacements (static) or in the single chamber perfusion system (perfusion) for another 7 d. The PC membrane and PCLdi(U-UPy) fibers are indicated with *. Scale bars represent 1 μ m.

considered to be statistically significant; $P < 0.05$ is depicted as *, $P < 0.01$ is shown as **, $P < 0.001$ is indicated as ***.

Results and Discussion

Fibrous, Supramolecular Membranes

Many techniques have been applied for manufacturing membranes to be used for tissue engineering purposes.^[16] Examples of these techniques are: foaming, polymer casting, salt leaching, freeze-drying, sintering and electro-spinning. In living organisms, tissue organisation and proper function, requires cells to be adhered to a micro-fibrous ECM. These ECM microfibrils comprise of nanofiber assemblies. Both nanofiber and microfiber structures can be made using electro-spinning techniques.^[17] Therefore, electro-spinning is very suitable to construct scaffolds that resemble the natural ECM. We have chosen to use electro-spinning^[17] to produce a fibrous membrane, and to investigate its usability for the culture of human tubular epithelial cells in order to ultimately arrive at a membrane which can be applied in a bioartificial kidney device.

Fibrous, supramolecular meshes were made by electro-spinning of PCLdi(U-UPy), which is composed of oligocaprolactone functionalized with two urea-UPy (U-UPy) units at both ends of the 2 kDa oligomer (Figure 1). The electro-spun micro-fibers had typical fiber diameters of 0.1–1 μm , and the thickness of the membranes was 10–30 μm . As reference membranes, commercial PC membranes were used with pore sizes of 0.4 μm . The thickness of the membranes was 7–22 μm .

The benefits of the supramolecular membranes lie in several unique properties of the supramolecular polymers used. First, we propose that the electro-spun microfibrils are formed in a hierarchical fashion, and therefore resemble the hierarchical structure of the ECM. They are proposed to be composed of many nanofibers (of approximately 5 nm in size) that are formed by stacking of the UPy-UPy dimers in the lateral direction assisted by the additional urea functionalities (Figure 1).^[12] Additionally, these supramolecular interactions allow for a modular approach to easy introduction of bioactivity into these supramolecular, fibrous membranes. This can be conveniently performed by simple mixing of UPy-modified bioactive compounds with the UPy-functionalized oligocaprolactones. Ultimately the 2D supramolecular membranes have to be translated into a

3D configuration in order to be applied in a bioartificial kidney device. This might not encounter too many problems because these supramolecular polymers are easy to process due to the dynamic nature of the four-fold hydrogen bonding UPy-moiety.^[10,18]

Human PTECs

Human renal PTECs were used to engineer kidney epithelial tissue on the supramolecular, fibrous membranes. The PTECs were successfully isolated from human kidneys derived from different donors. These early passage PTECs could be cultured on collagen I in wells plates in serum free medium in the presence of epidermal growth factor. After isolation and culturing of the PTECs in wells plates, they were characterized. They were stained for tubular-segment specific lectins,^[19] i.e. *Lotus tetragonolobus* (LTA) for proximal tubuli, *Peanut agglutinin* (PNA) for distal tubuli, and *Dolichos biflorus* (DBA) for collecting duct and cells of the loop of Henle. It was shown that the culture was heterogeneous and consisted of cells derived from all tubular segments (Supporting Information; Figure S1). The cells formed continuous monolayers with tight junctions between the cells. These tight junctions were visualized by staining for EpCAM and zona occludens 1 (ZO1). Furthermore, a subset of cells expressed specialized transporter

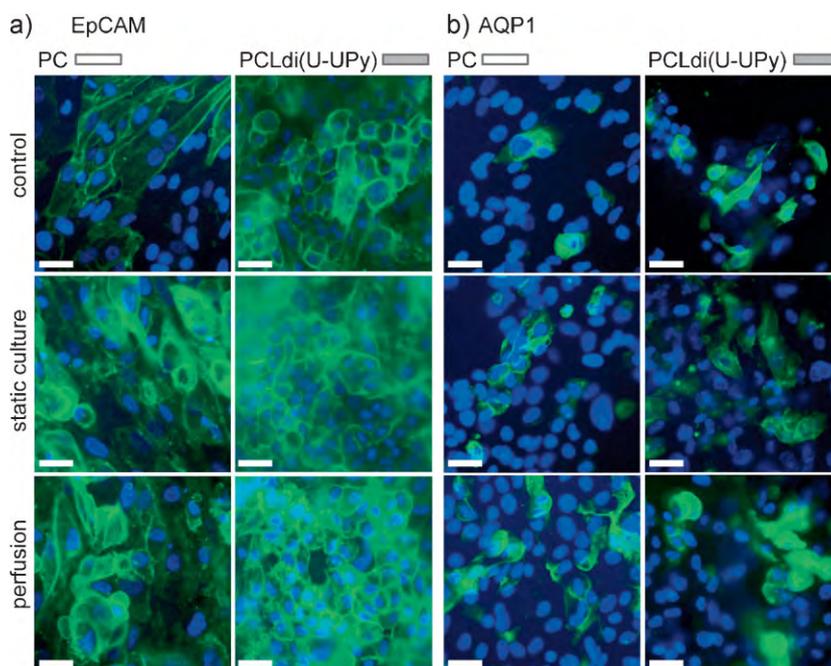


Figure 4. Characterization of renal tubular epithelial cell phenotype of PTEC on PC and PCLdi(U-UPy) meshes using fluorescence microscopy. PTEC were precultured on the membranes until confluency was reached (control). Then they were cultured under static culture conditions or in the single chamber perfusion system for 7 d. Antigens EpCAM and AQP1 were visualized using FITC-conjugates (green). Nuclei were stained with DAPI (blue). Scale bars represent 10 μm .

proteins, such as glucose transporter protein 1 (GLUT1), Na⁺/K⁺ ATPase and AQP1. Nevertheless, cytoplasmic staining for E-cadherin, the presence of the mesenchymal marker vimentin, and the near absence of the dedifferentiation marker α -smooth muscle actin (α SMA) suggest that the majority of cells had lost at least part of their epithelial phenotype. This was also reflected in the loss of the expression of several tubular epithelial genes over a 14 d culture period (Supporting Information; Table S1). Hence, we conclude that our PTEC cultured in wells plates contain cells from different nephron segments, and that they lose several specialized tubular epithelial genes in time. Therefore, it is necessary that these PTEC are cultured on membranes that resemble the ECM.

Renal Epithelial Tissue Engineering

Evaluation of PTEC cultured on the electro-spun, supramolecular membranes of PCLdi(U-UPy) was performed to investigate whether these meshes are suitable as epithelial cell carriers, and ultimately can be used in a bioartificial kidney set-up. As reference membranes, commercial PC membranes were used. Their microporous structure resembles that of conventional dialysis membranes which have similar micropores, but are generally made of polysulfone and have in the past been used in a bioartificial kidney.^[2] Furthermore, possible PTEC maturation was studied under continuous medium perfusion in a single chamber perfusion set-up for 7 d (Figure 2(a)). The perfusion conditions were applied to study the cells on the membranes in a more physiological relevant environment.

After 7 days of static culture, monolayers of PTEC were found on the electro-spun PCLdi(U-UPy) membranes; however a few cells migrated into the membranes (Figure 2(b)). We propose to overcome this cell migration via fine-tuning of the electro-spinning parameters. In contrast, PTEC did not form monolayers on PC membranes but grew in densely packed multilayers. The integrity of the epithelial monolayer on the electro-spun meshes was improved by perfusion, indicating the importance of flow and medium refreshment. This could be confirmed by the presence of adherent junctions between the epithelial cells,

which was clearly visible with TEM (Figure 3). Unexpectedly, these clear morphological differences were not mirrored by the presence of differentiation markers, such as the cell adhesion molecule EpCAM and the specialized renal proximal tubular cell marker AQP1; PTEC on both membranes behaved similar with this respect (Figure 4).

Besides that, cell viability and activity was studied by the mitochondrial conversion of resazurin into fluorescent resorufin (Figure 5(a)). During the 7 d culture period no large differences in mitochondrial activity between the cell cultures on the two membranes were found, indicating that the cells remained viable and active throughout the experiment. However, after perfusion a slight increase in metabolic activity of the cells cultured on PC membranes was detected, which might be caused by the presence of a higher amount of cells in the densely packed multilayers (Figure 5(a)). In addition, the functional activity of three

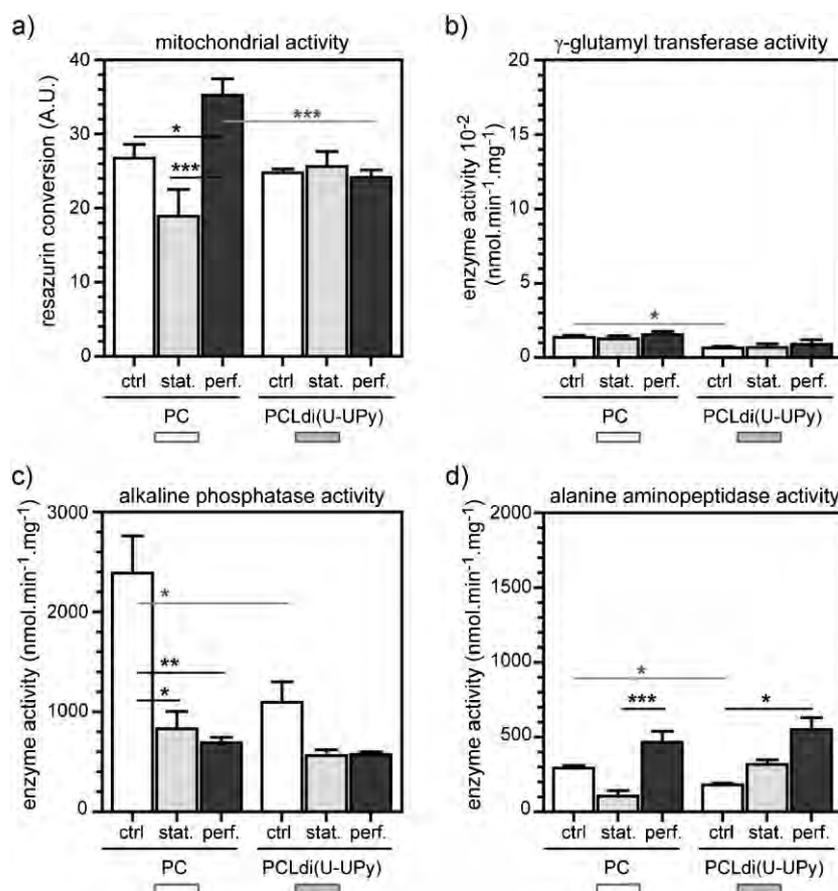


Figure 5. Biochemical characterization of PTEC cultured on PC and PCLdi(U-UPy) membranes. Activity measurements of the brush border enzymes γ -glutamyl transferase, alkaline phosphatase and alanine aminopeptidase were performed on PTEC cultured on the membranes. PTEC were precultured on the membranes until confluency was reached (ctrl). Then they were cultured under static culture conditions (stat.) or in the single chamber perfusion system (perf.) for additional 7 d. Statistical differences were determined by two methods: i. for each membrane between the control, static and perfusion culture group (depicted with a black line), and ii. for each group between the membranes PC and PCLdi(U-UPy) (indicated with a grey line).

Table 1. Epithelial-specific gene expression profiles of PTEC cultured on PC and PCLdi(U-UPy) membranes. (a) The presence of each gene transcript is expressed as the amount of present gene transcript per total amount of different donors used, i.e. 3. Time points at which bands were detected in all samples, part of the samples, or none of the samples are indicated in white, grey or black, respectively. (b) A summary is shown of the total amount of specific genes that were always positive, variable, and always negative.

(a)

| Family | Gene | Protein | PC | | | PCLdi(U-UPy) | | |
|--|---------|--|---------|--------|-----------|--------------|--------|-----------|
| | | | control | static | perfusion | control | static | perfusion |
| Glucose transporters | SLC5A1 | SGLT1 | 2/3 | 1/3 | 0/3 | 2/3 | 2/3 | 2/3 |
| | SLC5A2 | SGLT2 | 3/3 | 2/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| | SLC2A1 | GLUT1 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| Peptide transporters | SLC15A1 | PEPT1 | 3/3 | 1/3 | 2/3 | 2/3 | 3/3 | 3/3 |
| | SLC15A2 | PEPT2 | 3/3 | 3/3 | 3/3 | 0/3 | 3/3 | 3/3 |
| Organic anion/cation transporters | SLC22A1 | OCT1 | 3/3 | 3/3 | 2/3 | 3/3 | 3/3 | 3/3 |
| | SLC22A6 | OAT1 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| | SLC22A8 | OAT3 | 1/3 | 1/3 | 1/3 | 1/3 | 2/3 | 0/3 |
| HCO ₃ ⁻ transporter | SLC4A4 | NBC1 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 1/3 |
| ATP-driven transporters | ATP1B1 | Na ⁺ /K ⁺ ATPase | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| | ATP6 | H ⁺ ATPase | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| | SLC9A3 | Na ⁺ /H ⁺ ATPase | 0/3 | 2/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| HPO ₄ ²⁻ transporter | SLC34A1 | NaPi IIa | 0/3 | 0/3 | 0/3 | 3/3 | 1/3 | 1/3 |
| Water channel | AQP1 | Aquaporin 1 | 0/3 | 2/3 | 1/3 | 3/3 | 3/3 | 3/3 |
| ECM proteins | COL1A1 | Collagen I | 3/3 | 2/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| | COL3A1 | Collagen III | 2/3 | 2/3 | 3/3 | 0/3 | 1/3 | 1/3 |
| | COL4A1 | Collagen IV | 1/3 | 0/3 | 1/3 | 3/3 | 3/3 | 3/3 |
| | LAMA1 | Laminin | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| Other markers | Gp330 | Megalyn | 1/3 | 1/3 | 1/3 | 1/3 | 1/3 | 1/3 |
| | CYP27B1 | DH-vitD3 hydroxylase | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| | TJP1 | Zona occludens 1 | 3/3 | 2/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| | TGFB1 | TGFβ | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| | HNF1A | HNF1 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |

(b)

| Genes | PC | | | PCLdi(U-UPy) | | |
|-----------------|---------|--------|-----------|--------------|--------|-----------|
| | control | static | perfusion | control | static | perfusion |
| Always positive | 15 | 11 | 14 | 16 | 17 | 16 |
| Variable | 5 | 10 | 6 | 4 | 5 | 5 |
| Always negative | 3 | 2 | 3 | 3 | 1 | 2 |

membrane-bound brush border enzymes, i.e. γ -glutamyl transferase, alkaline phosphatase, and alanine aminopeptidase, appeared to be variable (Figure 5(b), (c), (d)). Whereas on both membranes the activity of γ -glutamyl transferase remained consistently low, the activity of alkaline phosphatase slightly dropped, and the activity of alanine aminopeptidase slightly increased. However, no significant

differences in brush border enzyme activity were observed between PC and PCLdi(U-UPy) membranes at the end of the culture period.

Also the expression of 23 relevant tubular epithelial genes was monitored (Table 1). As it appeared, the expression of several epithelial transporter genes was lost and could not be totally regained by perfusion culture. Only

significant differences between the two membranes with respect to the expression of these genes were seen under static culture conditions. Interestingly, some genes that were not or hardly expressed in PTEC cultured on the PC membranes, appeared to be regulated in cells cultured on electro-spun PCLdi(U-UPy), e.g. peptide transporter PEPT1, water channel AQP-1, and basement membrane protein collagen IV. However, also the reverse situation was found; gene expression of organic anion transporter OAT3 and interstitial ECM protein collagen III was more abundant in PTEC cultured on PC membranes. This indicates that the type of membrane clearly affects the gene expression profile of PTEC. The differential regulation of collagen IV and III on the fibrous PCLdi(U-UPy) membranes suggests that PTEC on these novel membranes are more prone to depositing a basement membrane instead of interstitial ECM, than cells cultured on microporous PC membranes. So, expression of these epithelial specific genes in PTEC cultured on the supramolecular, fibrous membranes indicates that these membranes are able to partly maintain epithelial specific marker expression.

We demonstrated that fibrous supramolecular membranes have superior properties over conventional microporous membranes with respect to ability of PTEC to form polarized monolayers on these membranes. This finding implies that application of these supramolecular membranes in a bioartificial kidney set-up might ameliorate its function, while multilayers were formed in previous studies.^[3] Introduction of ECM derived bioactive signals into these supramolecular membranes may prevent the loss of differentiated features and may even help to restore the expression of the differentiated properties.^[20] The importance of bioactive signals has been addressed in previous studies in which the influence of ECM derived peptides on the behaviour of proximal tubular epithelial cells seeded on ECM coated polycarbonate membranes was studied.^[21] It has been shown that a synthetic peptide containing the cell adhesion sequence RGD (Arg-Gly-Asp) induces higher cell densities, monolayer formation, and higher digoxin transport.^[21]

Conclusion

We conclude that fibrous, supramolecular PCLdi(U-UPy) membranes, in contrast to microporous commercial PC membranes, induce the formation of PTEC monolayers after a one week culture period. Furthermore, gene expression analysis suggested that PTEC on these fibrous membranes better preserved their renal epithelial phenotype. These results show that fibrous, supramolecular membranes can be applied in 2D for renal epithelial tissue engineering. Since for a well-functioning bioartificial kidney a renal epithelial cell monolayer is critical, we propose to use the

fibrous PCLdi(U-UPy) membranes as base materials in a bioartificial kidney set-up. Additionally, it is recommended to include bioactive modularities into a novel membrane design for a bioartificial kidney, in order to improve the differentiation of renal epithelial cells even further.

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