Biomaterials 32 (2011) 723–733

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Bioengineering of living renal membranes consisting of hierarchical, bioactive supramolecular meshes and human tubular cells

Patricia Y.W. Dankers [a,b,*,1], Jasper M. Boomker [a,1], Ali Huizinga-van der Vlag [a], Eva Wisse [b], Wilco P.J. Appel [b], Frank M.M. Smedts [c], Martin C. Harmsen [a], Anton W. Bosman [d], W. Meijer [b], Marja J.A. van Luyn [a]

[a] Department of Pathology and Medical Biology, Stem Cells and Tissue Engineering Group, University of Groningen, University Medical Center Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands
[b] Institute for Complex Molecular Systems, and Laboratory of Chemical Biology, Eindhoven University of Technology, P.O. Box 513, 5600 MB Eindhoven, The Netherlands
[c] Laboratory of Pathology, Foundation of Collaborating Hospitals of Eastern Groningen, Grintweg 71, 9675 HJ Winschoten, The Netherlands
[d] SupraPolix Research Center, Horsten 1, NL-5612 AX Eindhoven, The Netherlands

ARTICLE INFO

Article history:
Received 7 July 2010
Accepted 3 September 2010
Available online 12 October 2010

Keywords:
Bioactivity
ECM (extracellular matrix)
Epithelial cell
Membrane
Self-assembly
Kidney

ABSTRACT

Maintenance of polarisation of epithelial cells and preservation of their specialized phenotype are great challenges for bioengineering of epithelial tissues. Mimicking the basement membrane and underlying extracellular matrix (ECM) with respect to its hierarchical fiber-like morphology and display of bioactive signals is prerequisite for optimal epithelial cell function *in vitro*. We report here on a bottom-up approach based on hydrogen-bonded supramolecular polymers and ECM-peptides to make an electro-spun, bioactive supramolecular mesh which can be applied as synthetic basement membrane. The supramolecular polymers used, self-assembled into nano-meter scale fibers, while at micro-meter scale fibers were formed by electro-spinning. We introduced bioactivity into these nano-fibers by intercalation of different ECM-peptides designed for stable binding. Living kidney membranes were shown to be bioengineered through culture of primary human renal tubular epithelial cells on these bioactive meshes. Even after a long-term culturing period of 19 days, we found that the cells on bioactive membranes formed tight monolayers, while cells on non-active membranes lost their monolayer integrity. Furthermore, the bioactive membranes helped to support and maintain renal epithelial phenotype and function. Thus, incorporation of ECM-peptides into electro-spun meshes via a hierarchical, supramolecular method is a promising approach to engineer bioactive synthetic membranes with an unprecedented structure. This approach may in future be applied to produce living bioactive membranes for a bio-artificial kidney.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

In epithelial organs such as the pancreas, lungs, salivary glands, and kidneys the appropriate signals for differentiation, maintenance of epithelial cell polarity, and epithelial function are substantially provided by the basement membrane and the underlying matrix [1]. The major protein components of the basement membrane are collagen type IV, and laminin [1,2]. Both molecules self-assemble into large supramolecular structures forming a hierarchically organized network of nano-fibers anchored together by nidogen/entactin and perlecan [1,3,4]. Minor components of the basement membrane include fibronectin, osteonectin, fibulin, and agrin. The underlying extracellular matrix (ECM) consists mainly of fibrous proteins such as different collagen types, and proteoglycans [4]. Besides providing physical support, the constituents of the basement membrane interact with cell-surface receptors such as integrins, syndecans and glycosaminoglycan receptors, and invoke intracellular signalling [5].

Bioengineering of epithelial tissues through reconstruction of the ECM's hierarchical fiber-like structure from nano-meter to micro-meter scale [2,4], as well as the incorporation of complex bioactive cues of the natural basement membrane, is a major challenge. The heterogeneity of basement membranes proves that the composition of the ECM is critical for epithelial function. Hence, different mixtures of several natural ECM proteins might fulfil the need of regulating cell specific responses. Nevertheless, their use is limited

* Corresponding author. Institute for Complex Molecular Systems, and Laboratory of Chemical Biology, Eindhoven University of Technology, P.O. Box 513, 5600 MB Eindhoven, The Netherlands. Tel.: +31 40 247 5620; fax: +31 40 247 8367.
E-mail address: p.y.w.dankers@tue.nl (P.Y.W. Dankers).
[1] P.Y.W.D. and J.M.B. contributed equally to this work.

0142-9612/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2010.09.020

due to poor availability and processability, and there is little control of their composition and stability. Thus, for epithelial tissue engineering one would prefer to use materials that show no batch-to-batch differences, are conveniently processable, and defined in composition [6]. Additionally, they have to be free-standing and stable *ex vivo* in the presence of cells and media. Therefore synthetic materials might be the solution. It has been shown that nano-fibrillar scaffolds produced by electro-spinning are beneficial in maintaining and regulating specific cells *in vitro*. For example, self-renewal of embryonic stem cells was promoted by synthetic meshes [7], and also *in-vivo*-like organizations were found when different kinds of cells were cultured on these synthetic meshes [8]. However, the cells did not receive specific bioactive signals, but were only influenced by the topology provided by the fiber-like structure. In addition, combinations of natural polymers and synthetic bioactive peptides as membrane materials have been used to promote cell growth and differentiation [9]. These synthetic peptides regulated specific cell responses; nevertheless the membranes were made by freeze-drying and therefore lack the fiber-like structure. Besides these covalent polymeric structures, also supramolecular nano-fibers formed by peptide amphiphiles might be applied as basement membrane and ECM mimics [10]. These nano-fibers display high amounts of bioactive signals which render them applicable to instruct cells. Another advantage is their dynamic nature at a molecular level, which is common in a natural micro-environment [11,12]. However, these nano-fibers formed hydrogels with low mechanical strength, which makes them not directly suitable for application as free-standing, stable membranes.

Therefore, we propose here a synthetic, self-assembly approach to stable hierarchical, fibrous bioactive membranes by means of directed supramolecular interactions between LMW hydrogen-bonded polymers and bioactive peptides (Fig. 1A, B). It has been shown in a modular approach that mixing of supramolecular polymers [13,14] and peptides [15] with the dimerizing four-fold hydrogen bonding ureido-pyrimidinone (UPy) unit [16] lead to bioactive supramolecular biomaterials [17]. Furthermore, different polymers modified with a UPy-moiety via a urea (U) functionality have been shown to form nano-fibers in lateral direction via additional hydrogen bonding between the urea groups and $\pi-\pi$ interactions between the UPy-U-dimers [18–20]. Using electro-spinning as a convenient processing technique, we show here a bottom-up approach in which we combine bioactive biomaterials construction in a modular fashion with the introduction of different ECM-derived bioactive peptides in the UPy-U-nano-fibers in a stable manner (Fig. 1).

In order to show the bioactivity of our supramolecular membranes, we aimed at bioengineering of a living kidney membrane based on our bioactive mesh and human renal primary tubular epithelial cells (PTEC) (Fig. 1C). Therefore, the ECM-derived bioactive peptides used in this study, were selected for their presence in kidney basement membranes and in the underlying ECM. Coatings of laminin and collagen IV have been shown to be beneficial for the maintenance of differentiated monolayers of human proximal tubular epithelial cells [21]. Accordingly, we selected laminin, collagens I and IV, and fibronectin as main cell-binding ECM-components for instructing renal epithelial cells. To show the proof-of-principle we used a mixture of the cell-binding motifs present in these ECM-components i.e. the ECM-derived peptides [22]: GRGDS (Gly-Arg-Gly-Asp-Ser) [22,23] present in laminin, collagen I and IV, and fibronectin; PHSRN (Pro-His-Ser-Arg-Asn) [24,25] derived from fibronectin; YIGSR (Tyr-Ile-Gly-Ser-Arg) [26,27] from laminin, and DGEA (Asp-Gly-Glu-Ala) [28] present in collagen I and IV were employed in our investigations. These sequences are able to bind and activate several integrins [29,30] expressed on renal tubular epithelial cells [31,32], except for YIGSR which is a ligand for 67LR (67 kDa laminin) receptor [33].

Our bioactive, supramolecular polymers were assembled in a hierarchical fashion and processed into fibrous, bioactive membranes, and as proof-of-principle their long-term biological performance was read-out by studying the behaviour of human primary tubular epithelial cells with respect to their capacity to form monolayers, their brush border enzyme activity and gene expression profile. The living renal membranes were assessed at organotypical culture conditions in a double chamber perfusion bioreactor.

2. Materials and methods

2.1. Synthesis of UPy-U-prepolymer and UPy-U-peptides

These compounds were synthesized in a similar manner as reported in the supplementary information and in the references [15,34].

2.2. Preparation of electro-spun membranes

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 w/w% PCLdi(U-UPy) in 5 w/w% water in tetrahydrofuran (THF) by slow addition of the prepolymer to the solvent mixture. The resulting solution was spun at a feed rate of 20–30 μL/min. The tip-to-target distance was 12 cm and the applied voltage was 15–17.5 kV. Bioactive PCLdi(U-UPy) meshes were prepared from a 5 w/w% water in THF solution containing 34 w/w% PCLdi(U-UPy) and 0.875 mol% of each UPy-U-peptide **1**-**4**, i.e. in total 3.5 mol% UPy-U-peptide. This solution was made by dissolution of the UPy-U-peptides in water and dissolution of the prepolymer in THF in a similar manner as described above, after which the two solutions were mixed. Overnight all solvent was evaporated and the residue was redissolved in 5 w/w% water in THF. The resulting solution was spun at a feed rate of 30 μL/min. The tip-to-target distance was 12 cm and the applied voltage was 15 kV. Circular membranes with a diameter of 12 mm were punched from the PCLdi(U-UPy) and bioactive PCLdi(U-UPy) meshes. These circular membranes were clamped in membrane holders (Minucells & Minutissue).

2.3. Characterization of UPy-U-materials

Bioactive PCLdi(U-UPy) films were analysed with Atomic Force Microscopy (AFM). A solution containing PCLdi(U-UPy) and 2 mol% UPy-peptide **1** in 5 w/w% water in THF was drop cast on mica plates and dried at 40 °C *in vacuo* for 16 h. AFM images were recorded at ambient conditions using a Digital Instrument Multimode Nanoscope IV operating in the tapping regime mode using silicon cantilever tips (PPP-NCH-50, 204–497 kHz, 10–130 N/m). A scanner 5962EV was used with scan rates between 0.5 and 1 Hz. All images were subjected to a first-order plane-fitting procedure to compensate for sample tilt. (Bioactive) PCLdi(U-UPy) meshes were analysed with Scanning Electron Microscopy (SEM) on a Philips XL30 FEG E-SEM under high vacuum.

Thermal properties of the non-active and bioactive PCLdi(U-UPy) meshes were measured with differential scanning calorimetry on a Perkin Elmer Differential Scanning Calorimeter Pyris 1 with Pyris 1 DSC Autosampler and Perkin Elmer CCA7 cooling element under a nitrogen atmosphere with heating and cooling rates of 10 °C/min.

2.4. Extraction experiment

The extraction of UPy-U-peptides **1**-**4** out of the bioactive PCLdi(U-UPy) membranes was investigated with reversed phase liquid chromatography–mass spectroscopy (RPLC-MS) measurements, and was performed in duplicate. The membranes were incubated in 1 mL water/membrane at 37 °C for 4 h. Then the water was removed, and again 1 mL water/membrane was added. The membranes were incubated at 37 °C for additional 20 h, after which the water was removed. The UPy-U-peptide concentrations were measured with RPLC-MS after 4 h and after 24 h. RPLC-MS was performed on a system consisting of the following components: Shimadzu SCL-10A VP system controller with Shimadzu LC-10AD VP liquid chromatography pumps (with an Alltima C18 3u (50 mm × 2.1 mm) reversed phase column and gradients of water-acetonitrile supplemented with 0.1% formic acid), a Shimadzu DGU-14A degasser, a Thermo Finnigan surveyor autosampler, a Thermo Finnigan surveyor PDA detector and a Finnigan LCQ Deca XP Max. Calibration was performed by quantification of the parent ions of the UPy-U-peptides using different concentrations of UPy-U-peptide (0.2×10^{-6} M, 0.5×10^{-6} M, 1×10^{-6} M, 2×10^{-6} M and 5×10^{-6} M). The surface area of the corresponding peak in the total ion count was calculated with the ICIS algorithm. UPy-U-peptide **2** was not extracted into the water.

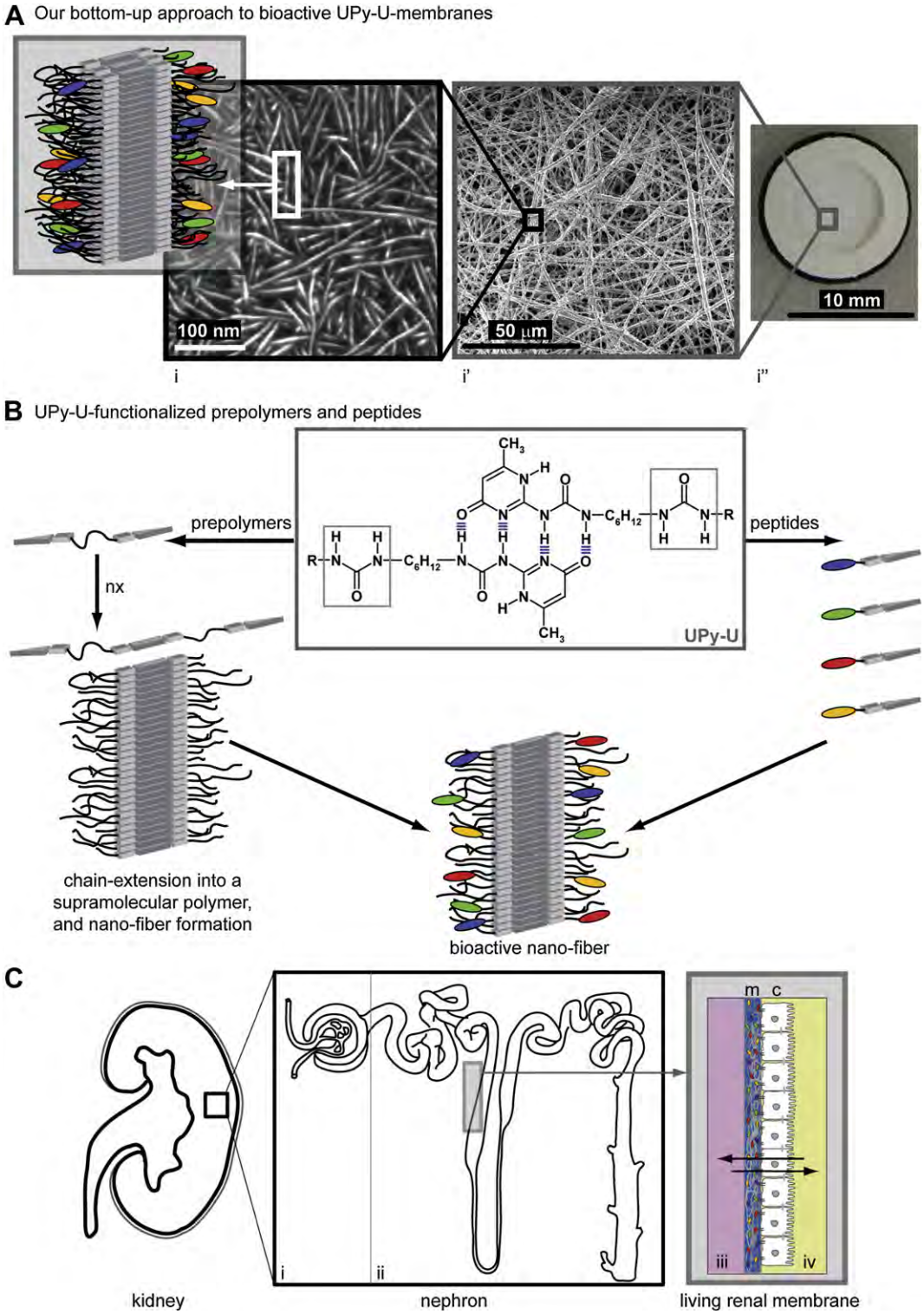

Fig. 1. Our bottom-up approach to hierarchical, bioactive supramolecular membranes. (A, B) **i.** Self-assembled bioactive nano-fibers of UPy-U-stacked dimers are formed by UPy-U-oligomers and UPy-U-peptides (AFM phase image: PCLdi(U-UPy) with 2 mol% **1**, scan size = 400 nm, $\Delta\varphi = 8^{\circ}$). **i'.** Electro-spun micro-fibers of bioactive PCLdi(U-UPy) (SEM image). Bioactive nano-fibers in these electro-spun micro-fibers are proposed to be formed. **i''.** These micro-fibers are spun into meshes that are punched into circular membranes (optical image), on which primary tubular epithelial cells are cultured. (C) Schematic representation of a kidney, one of its million nephrons, and a living membrane which can be considered to mimic a part of the renal tubular system. **i.** = glomerulus, **ii** = tubular system, **iii** = blood side, **iv** = pre-urine side, **c** = tubular epithelial cells, **m** = synthetic supramolecular bioactive mesh/membrane.

2.5. Isolation and culture of primary tubular epithelial cells

Human primary tubular epithelial cells (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 supplementary information and in the reference [35]. A heterogeneous population of human primary tubular epithelial cells was obtained as described [35], and used in this study.

2.6. Cell seeding on membranes

Non-active PCLdi(U-UPy) and bioactive PCLdi(U-UPy) meshes were used. First, the membranes were wetted in complete medium and transferred to 24-well plates containing 200 μL complete medium per well. Then, 1.5×10^5 PTEC 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% CO_2 in a humidified atmosphere. Subsequently, 700 μL complete medium was added per well, and the cells were precultured for 4 days at 37 °C and 5% CO_2 in a humidified atmosphere at static culture conditions, after which confluency was reached. Medium was changed every 2–3 days when cultured at static culture conditions.

2.7. Double chamber perfusion set-up

The double chamber perfusion system (Minucells & Minutissue) which was used in this study has been developed and described by Minuth et al [36–38]. When cells were cultured in the perfusion system 1% bufferall (Sigma–Aldrich) was added to the complete medium to maintain a pH 7.4 at ambient CO_2 levels. In addition, also 0.5 μg/mL fungizone (Bristol Meyers Squibb) was added. The medium was pumped through the containers by a peristaltic pump (Ismatec) at a rate of 1 mL/h. 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). Both heating plate and perfusion container were put in a vertical position, and medium was pumped into the container at the bottom, to allow possibly formed air bubbles to escape. Glucose and lactate levels in the culture medium were monitored for 72 h, showing that at 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.

2.8. Study set-up

After preculturing of the PTEC on the membranes for 4 days, static culture conditions were continued for 3 non-active and 3 bioactive PCLdi(U-UPy) membranes for 15 days. Additionally, 3 non-active and 3 bioactive PCLdi(U-UPy) membranes were put in the double chamber perfusion system and also cultured for additional 15 days. So, PTEC were cultured for in total 19 days at static culture conditions (static culture: 19 days). The PTEC in the perfusion system were first cultured for 4 days at static conditions and then 15 days in perfusion (perfusion: 4 + 15 days). Three independent experiments were performed in triplicate. The confluency after 4 days of preculturing on both the non-active and bioactive PCLdi(U-UPy) membranes (control) was tested using carboxy fluorescein diacetate succinimidyl ester (CFSE; 1:1000; Molecular Probes) staining and a resazurin cell viability assay. The PTEC cultured on the membranes (static culture and perfusion) were characterized with: toluidin blue staining on 3 μm thick longitudinal sections, fluorescent immunostaining (anti-ZO1; 1:200; BD Biosciences), resazurin cell viability assay, brush border enzyme activity assays, and RT-PCR on several transcripts (Suppl. Info; Table S1). Fluorescence microscopy was performed using a Leica DMLB microscope, Leica DC300F camera and Leica Qwin 2.8 software (Leica Microsystems).

2.9. Toluidin blue staining: monolayer formation

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

2.10. 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 μL culture medium per well containing 44 μM resazurin (Sigma Aldrich). Viable cells convert non-fluorescent resazurin into fluorescent resorufin ($\lambda_{ex} = 540$ nm, $\lambda_{em} = 590$ nm) which was measured with a Varioskan plate reader (Thermo Fisher Scientific) in duplicate in 200 μL. The assays were carried out at static culture conditions.

2.11. Brush border enzyme activity

Brush border enzyme activities were measured in cell extracts that were prepared by freeze-thawing at −20 °C in 400 μL/well 5 mM Tris–HCl, 0.9% NaCl, pH 7.4. γ-glutamyl transferase activity was determined by kinetic colorimetric measurement of the transfer of γ-glutamyl 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 [39]. A Varioskan plate reader was used (Thermo Fisher Scientific).

2.12. Gene expression analyses

RNA was extracted with the use of the RNeasy Micro Kit (Invitrogen) from the PTEC cultured on the membranes. For the reverse transcriptase reaction, 0.5 U RNase inhibitor (Fermentas), 1 mM dNTPs (Fermentas), 500 ng T18 primer, and 10 U Moloney Murine Leukemia Virus Reverse Transcriptase (Fermentas) in reverse transcriptase buffer were 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), 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 1 μM gene-specific primers. The cDNA was amplified for 35 cycles. In Suppl. Info; Table S1, a list of primer sequences that were used in this study are shown.

2.13. Statistical analysis

Differences between two groups were analysed with a two-tailed Mann–Whitney test with a 95% confidence interval. Data is expressed as mean ± standard error of mean. Probabilities of $P < 0.05$ were considered to be statistically significant; $P < 0.05$ is depicted as *, $P < 0.01$ is shown as **, $P < 0.001$ is indicated as ***.

3. Results

3.1. Hierarchical, bioactive supramolecular membranes

The electro-spinning technique has been studied and used extensively for processing of many natural and synthetic polymers [40,41]. However, electro-spinning of low molecular weight (LMW) compounds has not been explored; difficulties arise in the production of the fibers and/or the fibers formed are not stable. Here, we propose that using a mixture of molecules that form supramolecular polymers by self-assembly induce fiber formation upon arrival at the collecting plate. Supramolecular meshes were made by electro-spinning using PCLdi(U-UPy) as base prepolymer. This supramolecular prepolymer is composed of oligocaprolactone functionalized with two urea-UPy (U-UPy) units at both ends of the 2 kDa oligomer (Fig. 2A). To make bioactive meshes, we synthesized four different ECM-derived peptides all with a molecular weight of approximately 1 kDa, and modified them with UPy-U-moieties [15], i.e. UPy-U-GGG-GRGDS **1**, UPy-U-GGG-PHSRN **2**, UPy-U-GGG-YIGSR **3**, and UPy-U-GGG-DGEA **4** (Fig. 2B). Bioactive UPy-U-materials were obtained by mixing these peptides in a 1:1:1:1 ratio with the PCLdi(U-UPy) prepolymer in solution, ending up with in total 3.5 mol% of UPy-U-peptide. Hierarchical, (bioactive) supramolecular membranes could be made by electro-spinning from solution containing PCLdi(U-UPy), or both PCLdi(U-UPy) and the UPy-U-peptides (Fig. 2C). The electro-spun micro-fibers had typical fiber diameters of 0.1–1 μm, while the thickness of the membranes was 10–50 μm.

As stated, the quadruple hydrogen bonding moieties form dimers, and take care of chain-extension of the polymer chains resulting in virtual high molecular weight supramolecular polymers (Fig. 1B). Evenly important is the finding that these dimers stack in the lateral direction into nano-fibers by π–π interactions between the UPy-dimers and additional hydrogen bonds between the urea (U) functionalities (Fig. 1A and B). This has been shown for

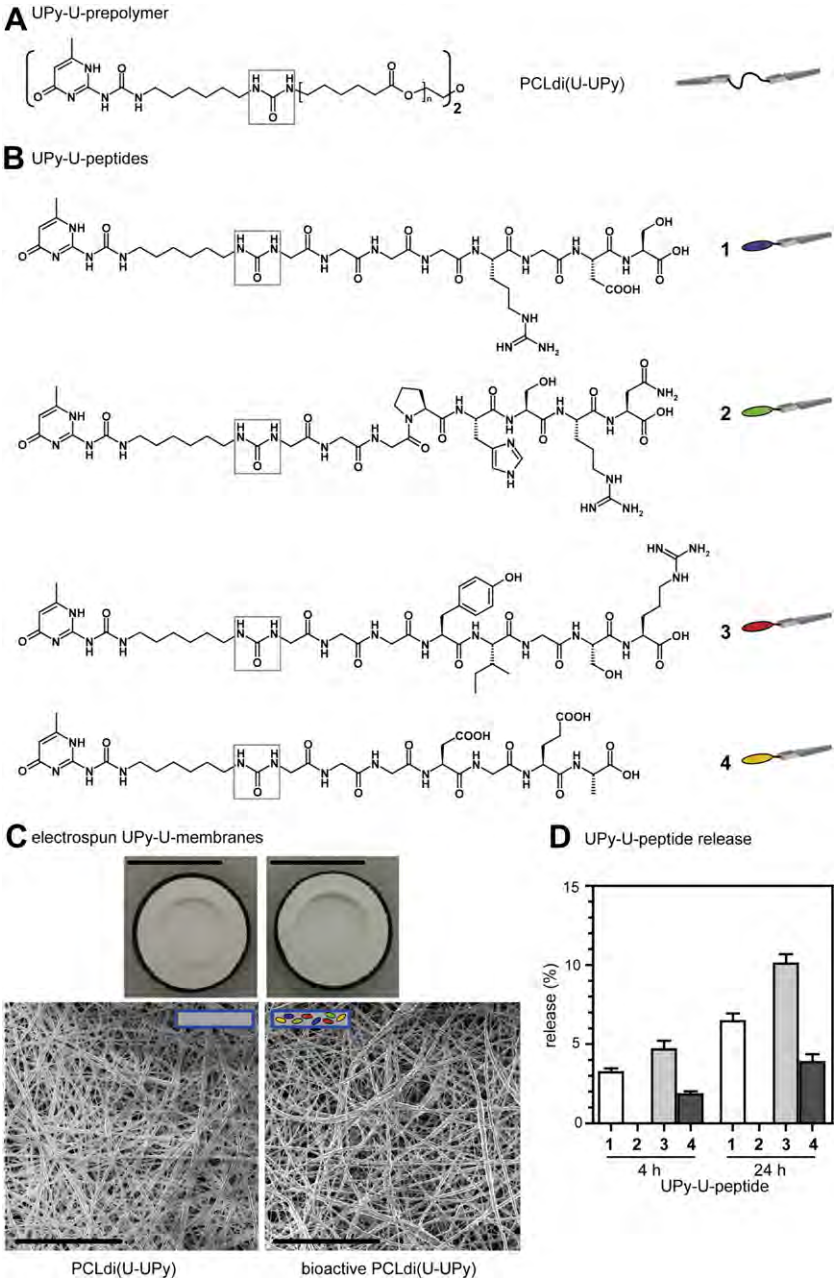

Fig. 2. The chemistry towards a synthetic bioactive supramolecular membrane. (A) Oligocaprolactone ($M_n = 2000$ g/mol) bifunctionalized with UPy-U-groups was used as base prepolymer: PCLdi(U-UPy). (B) Bioactive peptides were also functionalized with UPy-U-units resulting in UPy-U-GGG-GRGDS **1**, UPy-U-GGG-PHSRN **2**, UPy-U-GGG-YIGSR **3**, and UPy-U-GGG-DGEA **4**. (C) Supramolecular membranes were made by electro-spinning from solution containing PCLdi(U-UPy), or both PCLdi(U-UPy) and UPy-U-peptides **1**-**4**, i.e. bioactive PCLdi(U-UPy). Optical images of the circular membranes with scale bars representing 10 mm, and SEM images with scale bars representing 50 μm are shown. (D) Extraction behaviour of UPy-U-peptides **1**-**4** from the bioactive membranes after 4 and 24 h incubation in water. UPy-U-peptide **2** was not extracted into the water.

polymer films [18–20], but is also proposed to occur in the electro-spun micro-fibers. With differential scanning calorimetry the thermal properties of the meshes can be determined, which has been investigated for PCLdi(U-UPy) films before [20]. Here, we show that similar behaviour is found for both the non-active and bioactive PCLdi(U-UPy) membranes (Suppl. Info; Fig. S1). The PCL part melts between 59 and 62 °C in both non-active and bioactive meshes. More important an additional melting transition is detected around 128–129 °C, which is attributed to the melting of the UPy-U-nano-fibers, which proves the presence of the nano-fiber structures in the electro-spun micro-fibers (Fig. 1A).

Incorporation of the UPy-U-peptides into the fibers was investigated with extraction experiments in water. Between 90 and 100% of each UPy-U-peptide was still present in the membrane after 24 h of extraction, indicating stable incorporation of the UPy-U-peptides within the nano-fibers of the supramolecular polymer (Fig. 2D). Easy processing by electro-spinning of LMW supramolecular prepolymers and bioactive peptides led to the formation of bioactive, supramolecular membranes with a hierarchical fiber structure (Fig. 1A).

3.2. Living renal membranes: formation of monolayers and tight junctions

Supramolecular electro-spun membranes of PCLdi(U-UPy) with our bioactive peptides have been shown to be suitable epithelial cell carriers, during a 7-day culture period [35]. However, these

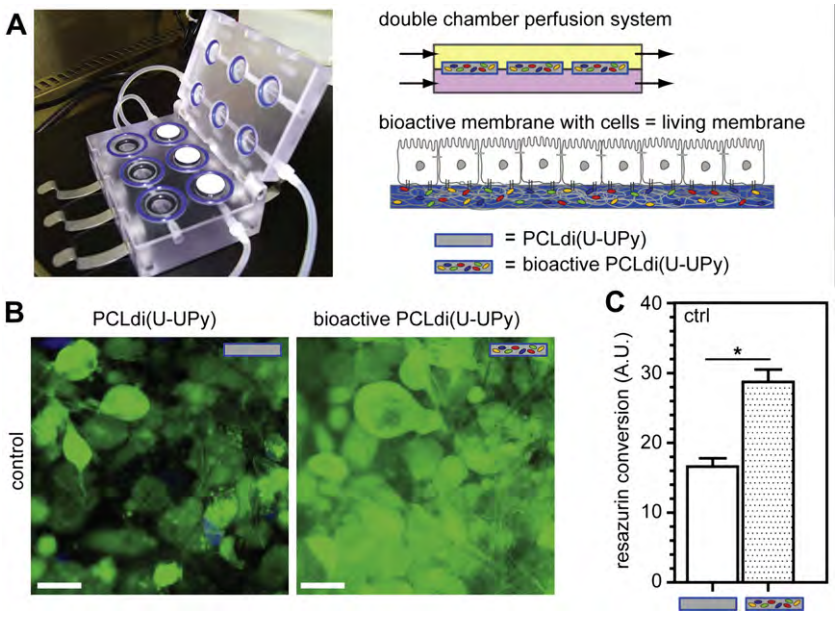

Fig. 3. (A) The double chamber perfusion system used. Both membrane types, the bioactive PCLdi(U-UPy) and the non-active PCLdi(U-UPy) membranes, were investigated in parallel using both sides of the culture system. (B) PTEC were precultured on the membranes until confluency was reached (control). CFSE staining was performed to visualize the cells, and (C) mitochondrial activity was measured to determine the viability of the cells in the control situation.

fiber structures did not support complete PTEC differentiation and function. We hypothesized that in order to obtain a living renal membrane consisting of PTEC and a synthetic mesh we need both a hierarchical fiber-like structure and bioactivity. Epithelial cells need bioactive signals from the basement membrane and ECM they encounter. Therefore, we introduced such bioactive cues by means of a mixture of several UPy-U-modified ECM-derived peptides. PTEC were cultured on bioactive electro-spun PCLdi(U-UPy) meshes containing these bioactive ECM-derived UPy-U-peptide sequences. In order to prove the necessity and activity of the peptides, a long-term culture experiment of 19 days was performed. The PTEC were cultured at static conditions, and at organotypical conditions using basolateral and apical flows in a double chamber perfusion system (Fig. 3A). In this system the flow rate was set at 1 mL/h which was sufficient to keep glucose levels constant and avoid accumulation of lactate.

After 4 days of PTEC preculturing at static conditions, their viability was evaluated by CFSE staining and a mitochondrial activity assay in which the conversion of resazurin to resorufin is measured (Fig. 3B and C). Large differences were found between the non-active and bioactive electro-spun meshes, PCLdi(U-UPy) and bioactive PCLdi(U-UPy), respectively. CFSE staining revealed that more viable cells adhered to bioactive than to non-active PCLdi (U-UPy) membranes, which was confirmed by the mitochondrial activity assay showing higher activity on the bioactive membranes. Although after 7 days of culturing at static conditions monolayer formation on non-active membranes has been shown [35], monolayer integrity on these non-active membranes was lost after prolonged culturing for 19 days (Fig. 4A). Cellular debris and possible cell in-growth into the non-active membranes were clearly visible. Nevertheless, on the bioactive membranes the monolayer was intact at the end of the culture period after 19 days. The toluidin blue sections show that perfusion culture did not seem to have large influence on the monolayer formation and presence of cellular debris when compared to static conditions (Fig. 4A). However, clear differences between perfusion and static conditions, and between the non-active and bioactive membranes, were seen by fluorescent immunostaining for the zona occludens 1 (ZO1) protein as marker for tight junctions (Fig. 4B). ZO1 was only visible between a few PTEC on the non-active membranes when using perfusion conditions, whereas PTEC cultured on the bioactive membranes showed high intercellular ZO1 expression both at static and perfusion conditions. Besides that, the cells cultured at static conditions appear to be smaller than those cultured in the perfusion system; which is clearly visible on the bioactive membranes. Both the toluidin blue sections and the ZO1 stainings show that our bioactive supramolecular membranes induce renal epithelial cells to form a tight monolayer which is prerequisite for a well-functioning renal epithelium.

3.3. *Living renal membranes: brush border enzyme activity and epithelial specific gene profiles*

Renal epithelial cells should not only adhere, survive and form monolayers, but they should also express specialized transporter proteins such as aquaporins, glucose transporters, peptide transporters, various organic cation and anion transporters, and bulk transporter proteins such as megalin. Phenotyping of the primary tubular epithelial cells cultured on our supramolecular membranes was done by gene expression profiling and by measuring the activity of brush border enzymes such as γ-glutamyl transferase, alkaline phosphatase, and alanine aminopeptidase that are expressed on well-differentiated renal tubule epithelial cells.

First, the mitochondrial activity of the PTEC cultured on the membranes was studied after 19 days. The mitochondrial activity on bioactive PCLdi(U-UPy) was higher than on non-active PCLdi(U-UPy) membranes; both at static and perfusion conditions (Fig. 5A). Remarkably, at static culture conditions the mitochondrial activity on the bioactive membranes was higher than at perfusion conditions. This might be linked to the assumed higher number of cells on the bioactive membranes when cultured at static conditions, which was observed in the ZO1 staining (Fig. 4B). In addition, the activity of three membrane-bound brush border enzymes was studied. The activity of γ-glutamyl transferase and alanine aminopeptidase was detected to be higher in cells cultured on bioactive membranes than on non-active membranes (Fig. 5B and D).

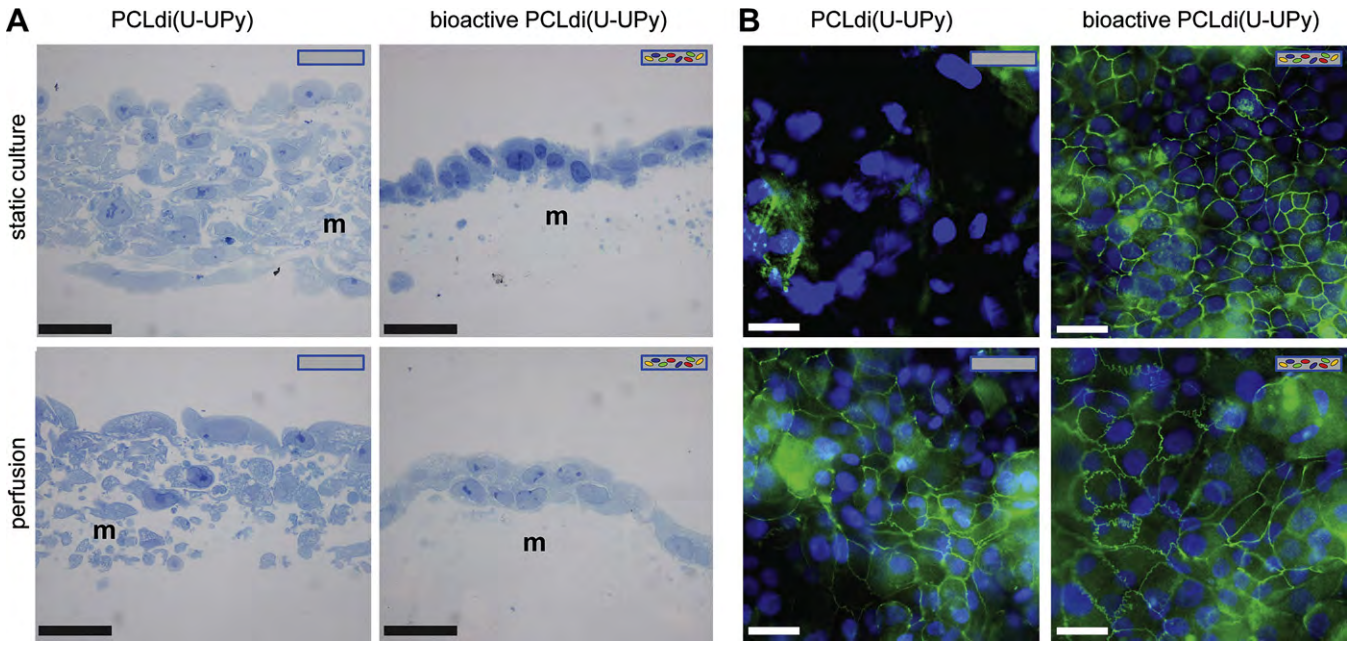

Fig. 4. Formation of monolayers and tight junctions: bioactive PCLdi(U-UPy) versus PCLdi(U-UPy) membranes. PTEC were precultured on the membranes until confluency was reached after 4 days. Then, the cells were cultured at static culture conditions or in the double chamber perfusion system for 15 days. (A) Toluidin blue staining of sections of PTEC cultured on the membranes **m**. (B) Fluorescence micrographs of ZO1 tight junctions (FITC-conjugate, green) and nuclei (DAPI, blue). All scale bars represent 10 μm.

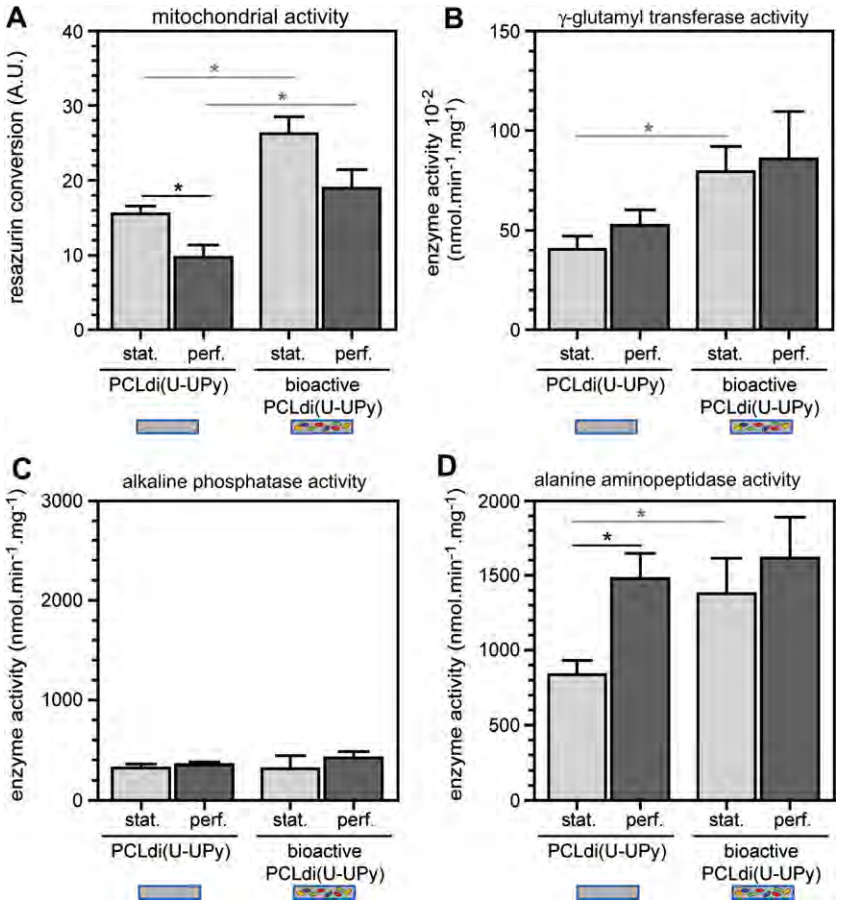

Fig. 5. Biochemical characterization of the cells cultured on non-active and bioactive PCLdi(U-UPy) membranes using activity measurements of brush border enzymes. PTEC were precultured on the membranes until confluency was reached. Subsequently, they were transferred to the double chamber perfusion system (perf.) or continued in static culture (stat.) for additional 15 days. Statistical differences were determined by two methods: i. for each membrane between the static and perfusion culture group (depicted with a black line), and ii. for each group between the PCLdi(U-UPy) and bioactive PCLdi(U-UPy) membranes (indicated with a grey line).

However, this difference was only seen in static cultures since in perfusion culture the activity levels of these enzymes were probably already increased by perfusion.

The expression of 23 genes was monitored representing the tubular epithelial function and differentiation status of the cells (Table 1). PTEC cultured on non-active PCLdi(U-UPy) membranes at static conditions did not express several of the genes representing tubular epithelial function, including important peptide transporter proteins, organic anion/cation transporters, and ATP-driven transporters. Additionally, most of these genes could not be induced by perfusion culture. The gene expression profile of PTEC grown on the bioactive PCLdi(U-UPy) membranes at static conditions was comparable to that of PTEC cultured on the non-active membranes; with the exception of the gene expression of the organic anion/cation transporter OCT1, and the megalin receptor, that actually were expressed. Nevertheless, on the bioactive membranes, perfusion culture could induce almost this complete set of genes. Only megalin and the glucose transport protein SGLT1 were variable expressed. Besides that, ECM gene expression was investigated by monitoring collagen type I, type III, type IV and laminin. The observation that the genes encoding laminin and collagen IV were expressed in PTEC cultured on the bioactive membranes after perfusion suggests that the PTEC are capable of depositing basement membrane specific proteins on these membranes. The constitutive expression of TGFβ and the gain of collagen III expression indicate that at perfusion culture conditions PTEC on bioactive membranes have engaged in an active remodelling process resembling wound healing.

Epithelial-to-mesenchymal transition (EMT) might occur when renal epithelial cells are isolated from kidneys, and are subsequently not cultured at organotypical conditions [42–44]. We showed that the PTEC cultured on bioactive membranes at perfusion conditions expressed important epithelial specific transporter genes. Nevertheless, investigation of the expression of

Table 1
Epithelial specific gene expression profiles of PTEC cultured on bioactive PCLdi(U-UPy) versus PCLdi(U-UPy) membranes.

A

Family	Gene	Protein	PCLdi(U-UPy)		bioactive PCLdi(U-UPy)	
			static	perfusion	static	perfusion
Glucose transporters	SLC5A1	SGLT1	2/3	0/3	3/4	1/3
	SLC5A2	SGLT2	3/3	3/3	4/4	3/3
	SLC2A1	GLUT1	3/3	3/3	4/4	3/3
Peptide transporters	SLC15A1	PEPT1	2/3	3/3	3/4	3/3
	SLC15A2	PEPT2	1/3	2/3	3/4	3/3
Organic anion/cation transporters	SLC22A1	OCT1	2/3	3/3	4/4	3/3
	SLC22A6	OAT1	0/3	0/3	0/4	3/3
	SLC22A8	OAT3	0/3	0/3	0/4	3/3
HCO_3- transporter	SLC4A4	NBC1	3/3	3/3	4/4	3/3
ATP-driven transporters	ATP1B1	Na^+/K^+ATPase	3/3	3/3	4/4	3/3
	ATP6	H^+ATPase	3/3	3/3	4/4	3/3
	SLC9A3	Na^+/H^+ ATPase	0/3	0/3	1/4	3/3
HPO_4^{2-} transporter	SLC34A1	NaPi IIa	0/3	1/3	0/4	3/3
Water channel	AQP1	Aquaporin 1	3/3	3/3	4/4	3/3
ECM proteins	COL1A1	Collagen I	3/3	3/3	4/4	3/3
	COL3A1	Collagen III	0/3	0/3	0/4	2/3
	COL4A1	Collagen IV	3/3	1/3	2/4	3/3
	LAMA1	Laminin	3/3	2/3	4/4	3/3
Other markers	Gp330	Megalin	0/3	0/3	4/4	1/3
	CYP27B1	DH-vitD3 hydroxylase	3/3	3/3	4/4	3/3
	TJP1	Zona occludens 1	3/3	0/3	4/4	3/3
	TGFB1	TFGβ	3/3	3/3	4/4	3/3
	HNF1A	HNF1	3/3	3/3	4/4	3/3

B

Genes	PCLdi(U-UPy)		bioactive PCLdi(U-UPy)	
	static	perfusion	static	perfusion
always posilive	13	12	14	20
variable	4	4	5	3
Always negative	6	7	4	0

Qualitative PCR analyses of PTEC cultured on the membranes are shown. (A) PTEC were precultured on the membranes until confluency was reached. Then they were cultured at static culture conditions or in the double chamber perfusion system for 15 days. The presence of each gene transcript is expressed as the amount of present gene transcripts per total amount of samples used. Time points at which bands were detected in all samples, part of the samples, or none of the samples are indicated in green, orange or red, respectively. (B) A summary is shown of the total amount of specific genes that are always positive, variable, and always negative.

mesenchymal marker genes, SM22α, αSMA, and vimentin, showed that PTEC at all conditions in our cultures did express these genes (Suppl. Info; Fig. S2). However, at all conditions studied, the PTEC expressed the epithelial marker E-cadherin, and a few to all of the transporter proteins discussed, indicating that they display an intermediate phenotype (Table 1 & Suppl. Info; Fig. S2). Furthermore, semi-quantitative analysis of these EMT markers and the epithelial marker E-cadherin using densitometry on the gel bands showed that SM22α is down-regulated and E-cadherin is up-regulated at perfusion conditions. This indicates that EMT can be controlled in our cultures.

4. Discussion

The creation of a bio-artificial matrix that resembles the natural basement membrane and ECM as the foundation for engineered tissues has been the subject of extensive research in regenerative medicine and tissue engineering sciences [45,46]. In this study, we provide the proof-of-principle that such a bio-artificial matrix can be created from ECM peptide-bearing supramolecular polymers electro-spun into membranes. The great benefits of using supramolecular polymers particularly lie in the fact that these polymers are held together by directed, non-covalent interactions, and in that way resemble the natural ECM more closely. Furthermore, this supramolecular approach allows the meshes to be constructed in a modular, hierarchical fashion, and totally defined in composition [11,12]. Using electro-spinning we were able to obtain stable, free-standing synthetic membranes composed of micro-fibers which consist of nano-fiber structures in which the bioactive ECM-derived peptides could be intercalated.

In order to investigate the biofunctionality of our synthetic bioactive supramolecular membranes we aimed at bioengineering living renal membranes consisting of the bioactive membranes and human renal primary tubular epithelial cells. Primary renal cells exhibit recondite functions, including the transport of many electrolytes and organic compounds, and dihydroxy-vitamin D3 activation, which are usually progressively lost during culture. Consequently, our challenges were to regain and maintain the differentiated and functional epithelial phenotype of PTEC after primary isolation and culture. Moreover, the functional heterogeneity of renal tubular epithelial cells in the nephron reflects the diversity of the basement membrane composition, which shows that their phenotype is dictated by the underlying basement membrane [47]. Therefore, we propose that these renal tubular epithelial cells rapidly dedifferentiate *in vitro* because they do not receive appropriate signals from the ECM. Several studies have shown that ECM-components can differentially regulate the behaviour of renal epithelial cells on artificial scaffolds [21,48,49]. Accordingly, we have chosen to apply ECM-peptides derived from different ECM-proteins, i.e. collagen I, IV, fibronectin, and laminin, which are known to bind to integrins and the laminin receptor 67LR [33], respectively. As proof-of-principle, we used a mixture of these ECM-derived peptides. To study the behaviour of the PTECs on these bioactive membranes in more detail and to elucidate cell signalling mechanisms, all peptides have to be studied separately and/or in different mixtures and ratios, which will be subject to further research.

In addition to bioactive stimulation of the cells, also physicochemical properties of the environment are of great importance. Fluctuations in glucose levels and electrolyte content, as well as the accumulation of waste products such as lactate, which frequently occur in static culture set-ups, can trigger dedifferentiation of renal epithelial cells [50,51]. Perfusion culture avoids these fluctuations and can be a critical factor for epithelial differentiation and function [52]. Accordingly, in our experimental set-up we also applied perfusion conditions. Additionally, the influence of pre-urine and blood plasma on the cells and membranes is very important, and will be investigated in future.

Recently, we have shown that our non-active PCLdi(U-UPy) supramolecular membranes were suitable as PTEC carrier for at least 7 days [35]. Here, we aimed at long-term, i.e. 19 days, preservation of epithelial phenotype through the use of bioactive PCLdi (U-UPy) membranes. We showed that the PTEC lost many of their features on the non-active membranes, indicating that solely a fibrous structure is not sufficient in a long-term culture set-up. Excitingly, after a 19 days culture period, PTEC cultured on the bioactive membranes showed enhanced differentiation, which could even be further improved using perfusion conditions. However, mesenchymal markers were also expressed, indicating that we have to adjust and fine tune our design.

Currently, we are performing the fine tuning of our supramolecular membrane design in several ways. We are investigating whether we can make bilayers of densely packed fibers, and open, coarse structures to mimic the basement membrane and underlying ECM even better. Additionally, applying renal epithelial cells and endothelial cells in one construct might be an interesting mimic of the renal tubular-blood vessel transition. Our supramolecular approach easily enables us to address different biofunctionalities at both sides of the membrane. Another level of complexity can be introduced by coupling polysaccharides to the supramolecular membranes. Polysaccharides play an important role in the basement membrane and ECM, since they can bind growth factors and facilitate the activation of growth factor receptors. This supramolecular system allows for an easy change in composition of the membranes by modifying the nature of the bioactives, which in future might lead to even superior membranes which can exert complex functions in a spatio-temporal way. Therefore, these synthetic basement membrane mimics are suggested to be highly relevant for those applications requiring long-term viability and functionality of renal epithelial cells *in vitro*, such as nephrotoxicity testing [53] and bio-artificial kidneys [54–59].

5. Conclusions

We have successfully developed living renal membranes composed of bioactive, free-standing supramolecular membranes and human primary tubular epithelial cells. Low molecular weight supramolecular prepolymers could be conveniently processed into micro-fiber structures which are proposed to be composed of supramolecular nano-fibers. Bioactivity was introduced by supramolecular intercalation of UPy-modified ECM-derived peptides, designed for stable binding, into these nano-fibers. This resulted in hierarchical, bioactive membranes of micro-fibers composed of bioactive nano-fiber structures. We showed that these bioactive membranes induced human primary tubular epithelial cells to form tight monolayers, even after a long-term culturing period of 19 days. In contrast, this was not seen on the non-active membranes, on which the cells lost their monolayer integrity after 19 days. Furthermore, the bioactive membranes helped to support and maintain renal epithelial phenotype and function. In addition, epithelial differentiation could even be further enhanced using perfusion conditions. Our approach mimicked both the biological and the physicochemical properties of the organotypical renal epithelial cell environment in one culture set-up.

Acknowledgements

We thank Jolanda Spiering for synthesizing the UPy-modified oligocaprolactone, Jan Kolijn for electro-spinning the membranes, Jan van der Wijk for supplying the tissues, Jasper Koerts, Marja

Brinker, Jelleke Dokter, Björne Mollet, and Saskia de Rond for technical assistance, and Guido Krenning, Diana Ploeger, Ruud Bank and Eliane Popa for useful discussions. This work is supported by SupraPolix, the Council for Chemical Sciences of the Netherlands Organization for Scientific Research (CW-NWO), and the Dutch Kidney Foundation.

Appendix

Figures with essential color discrimination. Figs. 1–5 in this article are difficult to interpret in black and white. The full color images can be found in the on-line version at doi:10.1016/j.biomaterials.2010.09.020

Appendix. Supplementary data

The supplementary data associated with this article can be found in the on-line version at doi:10.1016/j.biomaterials.2010.09.020

References

[1] LeBleu VS, MacDonald B, Kalluri R. Structure and function of basement membranes. Exp Biol Med 2007;232(9):1121–7.
[2] Yurchenco PD, Schittny JC. Molecular architecture of basement-membranes. Faseb J 1990;4(6):1577–90.
[3] Kalluri R. Basement membranes: structure, assembly and role in tumour angiogenesis. Nat Rev Cancer 2003;3(6):422–33.
[4] Alberts B, Johnson A, Lewis L, Raff M, Roberts K, Walter P. Molecular biology of the cell. Garland Sci; 2007. 5th ed.
[5] Rosso F, Giordano A, Barbarisi M, Barbarisi A. From cell-ECM interactions to tissue engineering. J Cell Physiol 2004;199(2):174–80.
[6] Place ES, Evans ND, Stevens MM. Complexity in biomaterials for tissue engineering. Nat Mat 2009;8(6):457–70.
[7] Nur-E-Kamal A, Ahmed I, Kamal J, Schindler M, Meiners S. Three-dimensional nanofibrillar surfaces promote self-renewal in mouse embryonic stem cells. Stem Cells 2006;24(2):426–33.
[8] Schindler M, Ahmed I, Kamal J, Nur-E-Kamal A, Grafe TH, Chung HY, et al. A synthetic nanofibrillar matrix promotes in vivo-like organization and morphogenesis for cells in culture. Biomaterials 2005;26(28):5624–31.
[9] Mochizuki M, Yamagata N, Philp D, Hozumi K, Watanabe T, Kikkawa Y, et al. Integrin-dependent cell behavior on ECM peptide-conjugated chitosan membranes. Pept Sci 2007;88(2):122–30.
[10] Silva GA, Czeisler C, Niece KL, Beniash E, Harrington DA, Kessler JA, et al. Selective differentiation of neural progenitor cells by high-epitope density nanofibers. Science 2004;303(5662):1352–5.
[11] Collier JH. Modular self-assembling biomaterials for directing cellular responses. Soft Mat 2008;4(12):2310–5.
[12] Dankers PYW, Meijer EW. Supramolecular biomaterials: a modular approach towards tissue engineering. Bull Chem Soc Jpn 2007;80(11):2047–73.
[13] Dankers PYW, Zhang Z, Wisse E, Grijpma DW, Sijbesma RP, Feijen J, et al. Oligo (trimethylene carbonate)-based supramolecular biomaterials. Macromolecules 2006;39(25):8763–71.
[14] Dankers PYW, van Leeuwen ENM, van Gemert GML, Spiering AJH, Harmsen MC, Brouwer LA, et al. Chemical and biological properties of supramolecular polymer systems based on oligocaprolactones. Biomaterials 2006;27(32):5490–501.
[15] Dankers PYW, Adams P, Löwik D, van Hest JCM, Meijer EW. Convenient solid-phase synthesis of ureido-pyrimidinone modified peptides. Eur J Org Chem 2007;22:3622–32.
[16] Sijbesma RP, Beijer FH, Brunsveld L, Folmer BJB, Hirschberg JHKK, Lange RFM, et al. Reversible polymers formed from self-complementary monomers using quadruple hydrogen bonding. Science 1997;278(5343):1601–4.
[17] Dankers PYW, Harmsen MC, Brouwer LA, van Luyn MJA, Meijer EW. A modular and supramolecular approach to bioactive scaffolds for tissue engineering. Nat Mat 2005;4(7):568–74.
[18] Botterhuis NE, van Beek DJM, van Gemert GML, Bosman AW, Sijbesma RP. Self-assembly and morphology of polydimethylsiloxane supramolecular thermoplastic elastomers. J Polym Sci Polym Chem 2008;46(12):3877–85.
[19] Kautz H, van Beek DJM, Sijbesma RP, Meijer EW. Cooperative end-to-end and lateral hydrogen-bonding motifs in supramolecular thermoplastic elastomers. Macromolecules 2006;39(13):4265–7.
[20] Wisse E, Spiering AJH, Dankers PYW, Mezari B, Magusin PCMM, Meijer EW. Peptide-modified nanofibers in a multi-component supramolecular thermoplastic elastomer, Biomater, submitted for publication.
[21] Zhang H, Tasmin F, Ying JY, Zink D. The impact of extracellular matrix coatings on the performance of human renal cells applied in bioartificial kidneys. Biomaterials 2009;30(15):2899–911.
[22] Hersel U, Dahmen C, Kessler H. RGD modified polymers: biomaterials for stimulated cell adhesion and beyond. Biomaterials 2003;24(24):4385–415.
[23] Pierschbacher MD, Ruoslahti E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature 1984;309 (5963):30–3.
[24] Aota S, Nomizu M, Yamada KM. The short amino acid sequence Pro-His-Ser-Arg-Asn in human fibronectin enhances cell-adhesive function. J Biol Chem 1994;269(40):24756–61.
[25] Leahy DJ, Aukhil I, Erickson HP. 2.0 ANG crystal structure of a four-domain segment of human fibronectin encompassing the RGD loop and synergy region. Cell 1996;84(1):155–64.
[26] Graf J, Ogle RC, Robey FA, Sasaki M, Martin GR, Yamada Y, et al. A pentapeptide from the laminin-B1 chain mediates cell-adhesion and binds the 67000-laminin receptor. Biochemistry 1987;26(22):6896–900.
[27] Massia SP, Rao SS, Hubbell JA. Covalently immobilized laminin peptide Tyr-Ile-Gly-Ser-Arg (YIGSR) supports cell spreading and colocalization of the 67-kilodalton laminin receptor with alpha-actinin and vinculin. J Biol Chem 1993;268(11):8053–9.
[28] Staatz WD, Fok KF, Zutter MM, Adams SP, Rodriguez BA, Santoro SA. Identification of a tetrapeptide recognition sequence for the alpha-2-beta-1-integrin in collagen. J Biol Chem 1991;266(12):7363–7.
[29] Clark EA, Brugge JS. Integrins and signal transduction pathways: the road taken. Science 1995;268(5208):233–9.
[30] Stupack DG, Cheresh DA. Get a ligand, get a life: integrins, signaling and cell survival. J Cell Sci 2002;115(19):3729–38.
[31] Simon EE, McDonald JA. Extracellular-matrix receptors in the kidney cortex. Am J Physiol 1990;259(5):F783–92.
[32] Simon EE, Liu CH, Das M, Nigam S, Broekelmann TJ, McDonald JA. Characterization of integrins in cultured human renal cortical tubule epithelial-cells. Am J Physiol - Ren Physiol 1994;267(4):F612–23.
[33] Nelson J, McFerran NV, Pivato G, Chambers E, Doherty C, Steele D, et al. The 67 kDa laminin receptor: structure, function and role in disease. Biosci Rep 2008;28(1):33–48.
[34] Dankers PYW. Supramolecular biomaterials - introducing a modular approach. thesis; [Dissertation].
[35] Dankers PYW, Boomker JM, Huizinga-van der Vlag A, Smedts FMM, Harmsen MC, van Luyn MJA. The use of fibrous, supramolecular membranes and human tubular cells for renal epithelial tissue engineering: towards a suitable membrane for a bioartificial kidney. Macromolec Biosc; 2010;. doi:10.1002/mabi.201000146.
[36] Minuth WW, Sittinger M, Kloth S. Tissue engineering: generation of differentiated artificial tissues for biomedical applications. Cell Tissue Res 1998;91(1):1–11.
[37] Schumacher K, Strehl R, de Vries U, Minuth WW. Advanced technique for long term culture of epithelia in a continuous luminal-basal medium gradient. Biomaterials 2002;23(3):805–15.
[38] Minuth WW, Denk L, Glashauser A. A modular culture system for the generation of multiple specialized tissues. Biomaterials 2010;31 (11):2945–54.
[39] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[40] Cui WG, Zhou Y, Chang J. Electrospun nanofibrous materials for tissue engineering and drug delivery. Sci Technol Adv Mater 2010;11(1):1–11.
[41] Yang JY, Zhan SH, Wang N, Wang XM, Li Y, Ma WY, et al. A mini review: electrospun hierarchical nanofibers. J Disp Sci Technol 2010;31(6):760–79.
[42] Liu Y. Epithelial to mesenchymal transition in renal fibrogenesis: pathologic significance, molecular mechanism, and therapeutic intervention. J Am Soc Nephrol 2004;15:1–12.
[43] Neilson EG. Setting a trap for tissue fibrosis. Nat Med 2005;11(4):373–4.
[44] Rastaldi MP. Epithelial-mesenchymal transition and its implications for the development of renal tubulointerstitial fibrosis. J Nephrol 2006;19(4):407–12.
[45] Griffith LG, Naughton G. Bodybuilding: the bionic human: tissue engineering - current challenges and expanding opportunities. Science 2002;295 (5557):1009–14.
[46] Hench LL, Polak JM. Bodybuilding: the bionic human: third-generation biomedical materials. Science 2002;295(5557):1014–7.
[47] Miner JH. Renal basement membrane components. Kidney Int 1999;56 (6):2016–24.
[48] Ip TK, Galletti PM, Aebischer P. Effects of attachment substrates on the growth and differentiation of Llc-Pk1 cells. Vitro Cell Develop Biol 1990;26(2):162–8.
[49] Kanai N, Fujita Y, Kakuta T, Saito A. The effects of various extracellular matrices on renal cell attachment to polymer surfaces during the development of bioartificial renal tubules. Artif Organs 1999;23(1):114–8.
[50] Gstraunthaler G, Seppi T, Pfaller W. Impact of culture conditions, culture media volumes, and glucose content on metabolic properties of renal epithelial cell cultures - are renal cells in tissue culture hypoxic? Cell Physiol Biochem 1999;9(3):150–72.
[51] Minuth WW, Steiner P, Strehl R, Kloth S, Tauc M. Electrolyte environment modulates differentiation in embryonic renal collecting duct epithelia. Exp Nephrol 1997;5(5):414–22.
[52] Minuth WW, Schumacher K, Strehl R. Renal epithelia in long term gradient culture for biomaterial testing and tissue engineering. Bio-Med Mater Eng 2005;15(1–2):51–63.
[53] Pfaller W, Gstraunthaler G. Nephrotoxicity testing in vitro - what we know and what we need to know. Environ Health Persp 1998;106:559–69.

[54] Ip TK, Aebischer P. Renal epithelial-cell-controlled solute transport across permeable membranes as the foundation for a bioartificial kidney. Artif Organs 1989;13(1):58–65.
[55] Humes HD, MacKay SM, Funke AJ, Buffington DA. Tissue engineering of a bioartificial renal tubule assist device: in vitro transport and metabolic characteristics. Kidney Int 1999;55(6):2502–14.
[56] Humes HD, Buffington DA, MacKay SM, Funke AJ, Weitzel WF. Replacement of renal function in uremic animals with a tissue-engineered kidney. Nat Biotechn 1999;17(5):451–5.
[57] Tumlin J, Wali R, Williams W, Murray P, Tolwani AJ, Vinnikova AK, et al. Efficacy and safety of renal tubule cell therapy for acute renal failure. J Am Soc Nephrol 2008;19(5):1034–40.
[58] Tiranathanagul K, Brodie J, Humes HD. Bioartificial kidney in the treatment of acute renal failure associated with sepsis. Nephrology 2006;11 (4):285–91.
[59] Fissell WH, Manley S, Westover A, Humes HD, Fleischman AJ, Roy S. Differentiated growth of human renal tubule cells on thin-film and nanostructured materials. Asaio J 2006;52(3):221–7.