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


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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-spin and 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.

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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 micrometer 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 fulfill the need of regulating cell specific responses. Nevertheless, their use is limited...
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 π–π 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, collagen 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-Glu-Asp-Ser) [23,24] 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-spin 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 mg/mL of each Upy-U-peptide 1-4, i.e. in total 3.5 mg/mL 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-) and bioactive PCLdi(U-)peptide 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 mg/mL Upy-U-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 5962EZ 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 CAC 7 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 spectrometry (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: Shimagdu SCL-10A VP system controller with Shimadzu LC-10AD VP liquid chromatography pumps (with an Alltima C18 3μ (50 mm 2.1 mm) reversed phase column (PPP-NCH-50, 204 μM, 0.5 μM, 1 × 10−5 M, 2 × 10−5 M and 5 × 10−5 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%, scan size = 400 nm, Δθ = 8°). ii. 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. iii. 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. After preculturing of the PTEC on the membranes for 4 days, 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) was added to the complete medium to maintain a pH 7.4 at ambient CO₂ levels. In addition, also 0.5 µg/mL fungizone (Bristol Meyers Squibb) was added. The medium was pumped through the bioreactor 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). PTEC were cultured in the perfusion system for 4 days at static conditions and then 15 days in perfusion (perfusion: 4 + 15 days). Three independent experiments were performed in triplicate. The confluence 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 and a resazurin assay. 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 CO₂ levels. In addition, also 0.5 µg/mL fungizone (Bristol Meyers Squibb) was added. The medium was pumped through the bioreactor 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.

2.9. 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 (λex = 540 nm, λem = 590 nm) which was measured with a Vario-scan 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% Tris–HCl, 0.5% 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. Alkaline 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 µM 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 µM dNTPs, 1.5 µM MgCl₂, 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 depicted as **, and P < 0.001 is depicted 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 oligocaprolicactone functionalized with two urea-UPy (U-UPy) units as base prepolymer. As stated, the quadruple hydrogen bonding moieties 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 oligocaprolicactone 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-spin 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 (Fig. 1B). Upon arrival at the collecting plate. Supramolecular meshes 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-spin micro-fibers had typical fiber diameters of 0.1–1 µm, while the thickness of the membranes was 10–50 µm.

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polymer films [18–20], but is also proposed to occur in the electro-spin 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) meshes (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-spin 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
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 perfusion 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 an 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 occulden 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 transporters, 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>
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<th>Family</th>
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Table 1
Epithelial specific gene expression profiles of PTEC cultured on bioactive PCLdi(U-UPy) versus PCLdi(U-UPy) membranes.

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.
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 SM22a 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 cultivating 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

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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


