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Renal Epithelial Monolayer Formation on Monomeric and Polymeric Catechol Functionalized Supramolecular Biomaterials

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Induction of a functional, tight monolayer of renal epithelial cells on a synthetic membrane to be applied in a bioartificial kidney device requires for bio-activation of the membrane. The current golden standard in bio-activation is the combination of a random polymeric catechol (L-DOPA) coating and collagen type IV (Col IV). Here the possibility of replacing this with defined monomeric catechol functionalization on a biomaterial surface using supramolecular ureido-pyrimidinone (UPy)-moieties is investigated. Monomeric catechols modified with a UPy-unit are successfully incorporated and presented in supramolecular UPy-polymer films and membranes. Unfortunately, these UPy-catechols are unable to improve epithelial cell monolayer formation over time, solely or in combination with Col IV. L-DOPA combined with Col IV is able to induce a tight monolayer capable of transport on electrospun supramolecular UPy-membranes. This study shows that a random polymeric catechol coating cannot be simply mimicked by defined monomeric catechols as supramolecular additives. There is still a long way to go in order to synthetically mimic simple natural structures.

The bio-artificial kidney combines hemodialysis membranes with a functional tight monolayer of renal proximal tubule epithelial cells (PTECs) which are capable of filtering uremic toxins out of the blood into the pre-urine, while retaining essential molecules and salts.[1,2] Recent strives have been made in uniting anti-fouling hemodialysis filtration hollow fiber membrane with cells by combining two types of coating. First the membrane is coated with polymeric L-3,4-dihydroxyphenylalanine (L-DOPA) which is physically absorbed to the surface.[3,4] Catechols in the resulting layer are able to become reactive o-quinones which are able to react to nucleophiles such as lysines or cysteines, found in proteins, though e.g. Michael-type addition or via Schiff basereactions.[5] In addition, it is proposed that changes in hydrophobicity also contribute to favorable protein adherence.[3,6] The primary coating is followed by a collagen type IV (Col IV) coating, the resulting synergistic combination allows for the formation of a tight monolayer of renal PTECs which are capable of uremic toxin transport over the membrane.[7–12] In spite of the success of this coating strategy, still several laborious coating steps are required, which are based on random uncontrolled material deposition. A defined total synthetic off-the-shelf hollow fiber membrane is preferred.

Modular supramolecular chemistry allows for the circumvention of laborious biomaterial post-processing to introduce complex bio-activity.[13,14] Carefully designed supramolecular moieties are able to form self-complementary non-covalent interactions, which allow for a modular approach in biomaterial design.[14–16] Ureido-pyrimidinone (UPy)-moieties have been extensively used as a basis for supramolecular biomaterials.[13,17] UPy-moieties are able to dimerize through hydrogen bonding and assemble through π–π stacking to form modular nanometer sized fibers.[18,19] Modification of polymers and additives with UPy-moieties allows for the integration of both as one biomaterial. Previously, we have shown that incorporation of UPy-functionalized cell adhesive peptides in UPy-modified polycaprolactone (PCLdiUPy) based electrospun membranes resulted in tight monolayer formation of primary human PTEC.[20] However, replacement of peptides with smaller reactive moieties like catechols to induce cell attachment would be synthetically more accessible and easier to scale up.

Researchers are moving away from random polymeric L-DOPA coatings toward more defined systems with monomeric catechols at the biomaterial surface.[21–23] Work by Choi et al. demonstrated that monomeric catechols conjugated at the surface of PCL-poly(ethylene glycol) (PEG) electrospun fibers increased cell attachment and migration compared to

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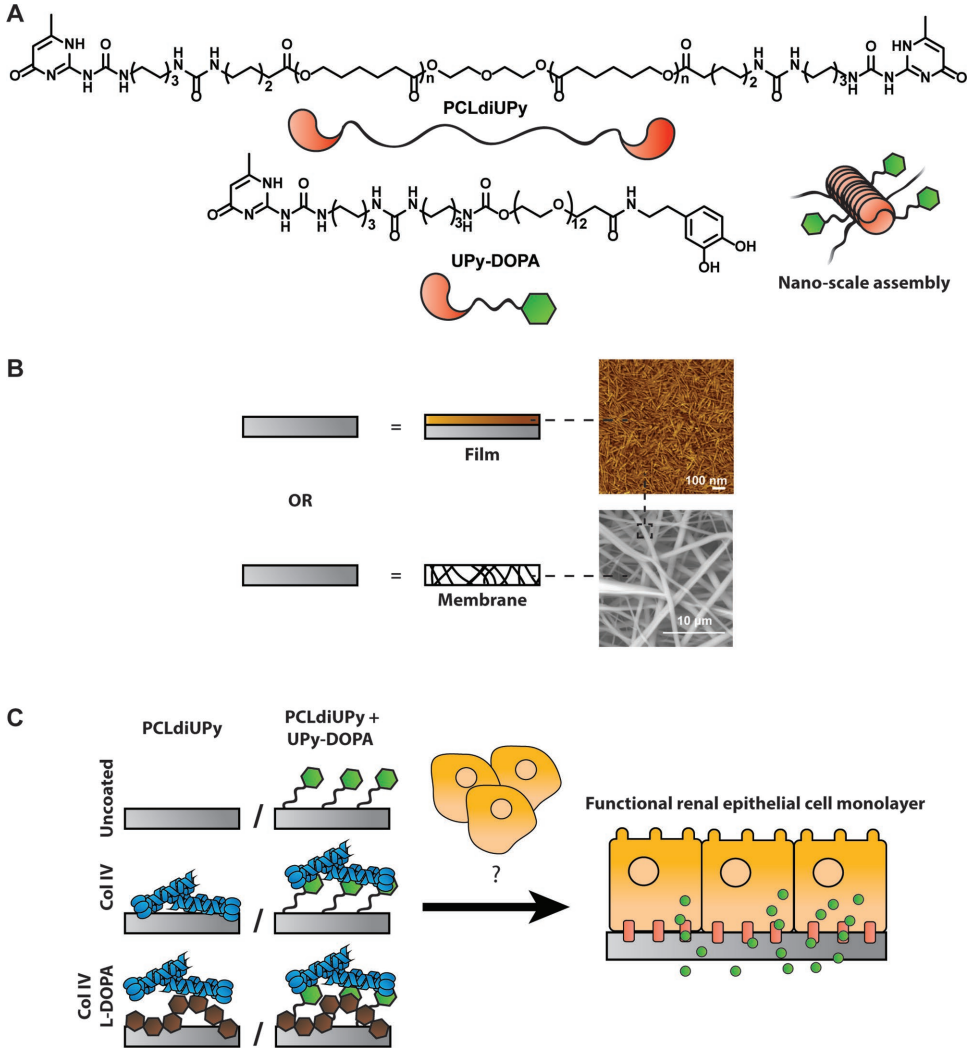

Scheme 1. Chemical structures and schematic representation of the experimental conditions. A) Chemical structures of UPy-polycaprolactone (PCLdiUPy) and UPy-modified catechol (UPy-DOPA) with a schematic representation of UPy-assembly. B) Employed substrates, films, and membranes with characteristic AFM and SEM images showing the material surface. C) Experimental conditions which were investigated for their potential to induce a functional renal epithelial cell monolayer.

physically absorbed polymeric dopamine coating.[22] Our group recently functionalized a hydrophobic non-cell adhesive UPy-priplast polymer film with a monomeric UPy-catechol additive via supramolecular incorporation. Robust incorporation via the UPy-catechol moiety into the UPy-priplast film was proven and induced cardiomyocyte attachment and differentiation.[23]

In this study it was investigated if the effect of monomeric catechol functionalization of PCLdiUPy by a dopamine was conjugated to a UPy-guest molecule (UPy-DOPA) could be translated from a polymer film model system, with nanofibrous assemblies at the surface formed by UPy–UPy stacking in the lateral direction, to electrospun membranes with micrometer-scale fibers (**Scheme 1**A,B). Moreover it was investigated whether monomeric catechol functionalization can aid the formation of a functional tight monolayer of PTECs and therefore replacing current physically absorbed polymeric dopamine coating (Scheme 1C).

In order to mimic the polydopamine coating traditionally used, we here designed and synthesized a synthetic analogue based on a UPy-group, C_6-urea-C_6 and OEG spacer and a reactive dopamine. For this end, an UPy-C_6-urea-C_6-OEG_{12}-COOH was synthesized as previously described by de Feijter et al. (Scheme S1, Supporting Information).[24] Conjugation of the dopamine to the UPy-synthon followed similar reaction as reported before.[23] Successful synthesis of the UPy-C6-Urea-C6-OEG12-DOPA was confirmed with NMR and LC-MS (Figure S2, Supporting Information). The final product was achieved with a yield of 71% (237 mg) and appeared as a fluffy white solid. Formulation of the compounds into films and membranes was performed by dissolving first PCLdiUPy in an organic solvent and subsequently UPy-DOPA was dissolved in the PCLdiUPy containing solution (chemical structures in Scheme 1A). Polymer films were cast and membranes were electrospun from these solutions.

Catechol incorporation and accessibility was assessed through an Arnow's staining, which turns orange in the presence of accessible catechols able to oxidize.[25] Both pristine PCLdiUPy films and membranes remained clear or white,

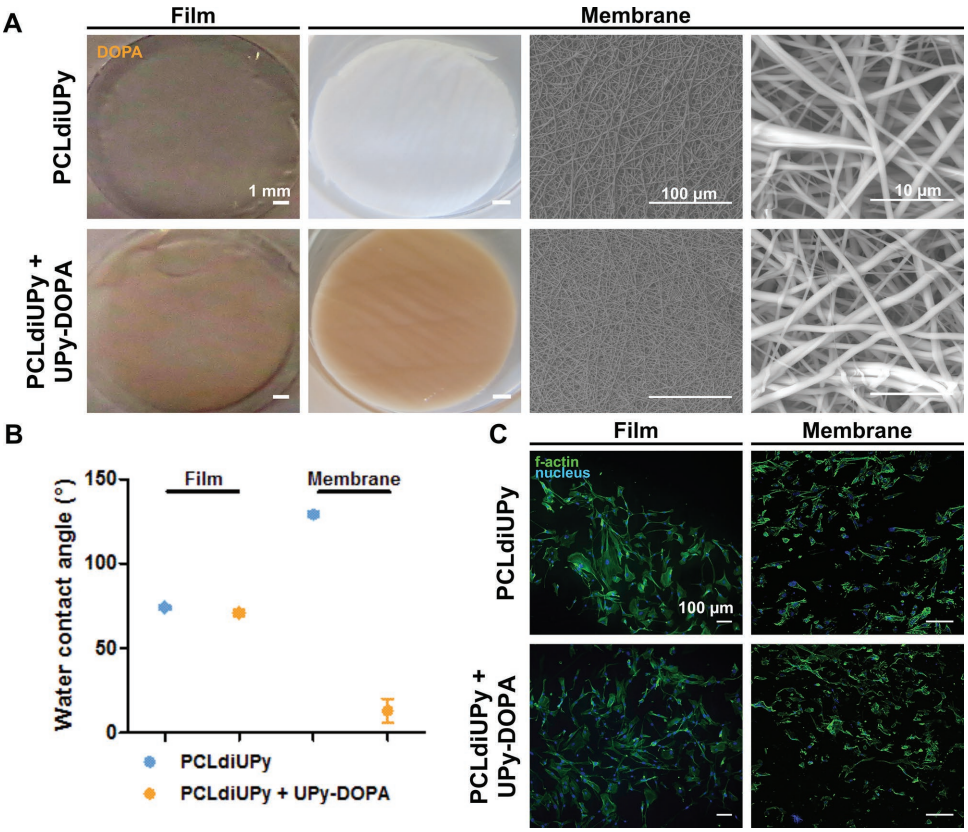

Figure 1. Incorporation of UPy-DOPA in PCLdiUPy and cell response to it. A) left panels and middle left panels show macroscopic images of polymer films and membrane, either pristine or with UPy-DOPA incorporation subjected to an Arnow's staining for catechols (orange). The middle right and right panels show scanning electron micrographs of the membranes. B) Water contact angle measurements of both pristine PCLdiUPy and UPy-DOPA incorporated films and membranes, $n = 3$, mean ± SEM. C) Representative microscopic images of ciPTEC adhesion on the films and membranes cultured for 8d, f-actin (green), and nucleus (blue).

respectively, but in both cases the addition of UPy-DOPA during material fabrication allowed for an orange color to appear (**Figure 1**A). To prevent cell infiltration into the electrospun membrane a design fiber diameter around 1 µm was selected. The addition of UPy-DOPA resulted in slight decrease in the fiber diameter compared to pristine PCLdiUPy of the electrospun membrane with the same spinning parameters, respectively 0.79 ± 0.32 µm versus 0.98 ± 0.32 µm (Figure 1A, **Table 1**). The fiber diameter correlated with the mean pore size found, 0.74 ± 0.01 µm versus 1.12 ± 0.02 µm, respectively (Table 1). The correlation between fiber diameter and mean pore size of electrospun membranes is dependent on the polymer employed and the fiber size, for electrospun PCL membranes a similar correlation has been reported as here.[26–28] Incorporation of UPy-DOPA resulted in an insignificant decrease in hydrophobicity from 74 ± 1° to 71 ± 2° in films (Figure 1B) in line with previous findings.[23] Interestingly however, a large decrease in hydrophobicity was observed when water was added on the membranes functionalized with UPy-DOPA, showing a decrease in water contact angle, from 129 ± 2° to 13 ± 7° ($p = 0.0041$) (Figure 1B). The water droplet was absorbed in UPy-DOPA functionalized membranes, as the water contact angle (WCA) measurement progressed. We hypothesize that this phenomenon is the result of an accumulation effect of the slight decrease caused by UPy-DOPA which is amplified due to the increase surface area in a fibrous membrane allowing absorption of the water into the membrane.

Conditionally immortalized RPTEC (ciPTECs) have been developed for applications such as the bio-artificial kidney with relevant transporter expression for the clearance of uremic toxins.[11,29–31] It is unknown if this cell line can function on supramolecular materials. ciPTECs showed adherence and spreading on both polymer films as membranes, it appeared that the cells have adopted a smaller size on the membranes than on the films (Figure 1C). It is hypothesized that the fibrous topology poses obstacles for the cells to evenly spread, while cells are left

Table 1. Properties of PCLdiUPy and PCLdiUPy + UPy-DOPA electrospun membranes. $n = 3$, mean ± SEM.

	Fiber diameter [µm]	Max pore size [µm]	Mean pore size [µm]	Smallest pore size [µm]
PCLdiUPy	0.98 ± 0.32	2.24 ± 0.08	1.12 ± 0.02	0.52 ± 0.02
PCLdiUPy + UPy-DOPA	0.79 ± 0.32	1.38 ± 0.06	0.74 ± 0.01	0.35 ± 0.01

unhindered on flat surfaces.[32,33] No clear beneficial effect of UPy-DOPA on cell adhesion or monolayer formation was observed in either film as electrospun membrane (Figure 1C). This is in line with previous research where cardiomyocyte progenitor cells showed only a slight increased cell attachment on PCLdiUPy functionalized with UPy-DOPA.[23] Moreover, only polymeric L-DOPA functionalization of polyethersulfone membranes did not favor tight monolayer formation of ciPTECs over 7 days of culture.[7] Previous studies did report dramatic increase in cell adhesion on previously poor cell adhesive materials surface functionalized with monomeric catechols.[22,23] PCLdiUPy has innate cell adhesive properties, that might overshadow the effect of monomeric catechols. Taken together, these results showed that UPy-DOPA is incorporated into the films and membranes, capable of oxidative reaction, but is unable to solely improve cell adhesion.

Monolayer formation did not improve after Col IV coating on PCLdiUPy films and membranes (**Figure 2**A,B), which is in line with the observation that Col IV alone is insufficient for monolayer formation and maintenance.[7,8] Unfortunately, the combination of UPy-DOPA and Col IV had no effect on monolayer formation for both films as membranes compared to only Col IV coating. The lack of effect could be due to the inability of the UPy-DOPA to react to the Col IV under coating conditions. Induced oxidation of the catechol with sodium periodate ($NaIO_4$) allows for the formation of reactive o-quinones at the surface which are able to react with nucleophiles, present in proteins.[5,34] An oxidative environment during Col IV coating allows, in theory, for the conjugation of the Col IV to the UPy-DOPA. However, no beneficial effect from an oxidative environment was observed when compared to sole Col IV coating (Figure 2A,B). The deposition of a polymeric L-DOPA coating followed by Col IV did allow for a monolayer to remain over a longer period of time (Figure 2A,B). The monolayer showed to be slightly tighter on a 2D surface than the rougher fibrous surface of the membrane, corresponding to the difference in cell spreading found before (Figure 1C).Overall, these results suggested that a random polymerized L-DOPA coating is superior over the monomeric UPy-DOPA in monolayer formation and maintenance.

The ineffectiveness of UPy-DOPA could be due to low level surface presentation, although the presence of accessible catechols was shown by an Arnow's staining. Additionally, the employed concentration of 10 mol% of UPy-DOPA is twice as high as was previously shown to be effective in improving cell adhesion of different cells.[23] Moreover, the chemistry of the base polymer, in this case PCLdiUPy, might also have an effect on the DOPA activity. Importantly, modularity of the UPy-system allows for redesign of the surface chemistry and base polymer materials to improve UPy-DOPA functionality in the future. This might be feasible in a high-throughput screening fashion.

Furthermore, the catechol surface can potentially be enriched with amines or guanidiniums, which both have been shown to improve catechol mediated adhesion.[35,36] Another alternative could be a polymeric catechol conjugated to a UPy-moiety, as polymeric L-DOPA addition showed positive effects.

The polymeric L-DOPA Col IV double coating allowed for monolayer formation on PCLdiUPy films and electrospun membranes. To assess function of these monolayer transporter activity of organic anion transporter 1 (OAT1) was assessed. The basolateral OAT1 transporter is crucial for the clearance of uremic toxins such as indoxyl sulfate and kynurenic acid which are associated with end-stage renal disease progression.[11,37,38] Fluorescein was used as model compound to assesses OAT1 function.[30] Fluorescein uptake by OAT1 was shown to be present in cells both on films as membranes, inhibition of OAT1 by probenecid resulted in a decrease in fluorescein uptake by cells indicating

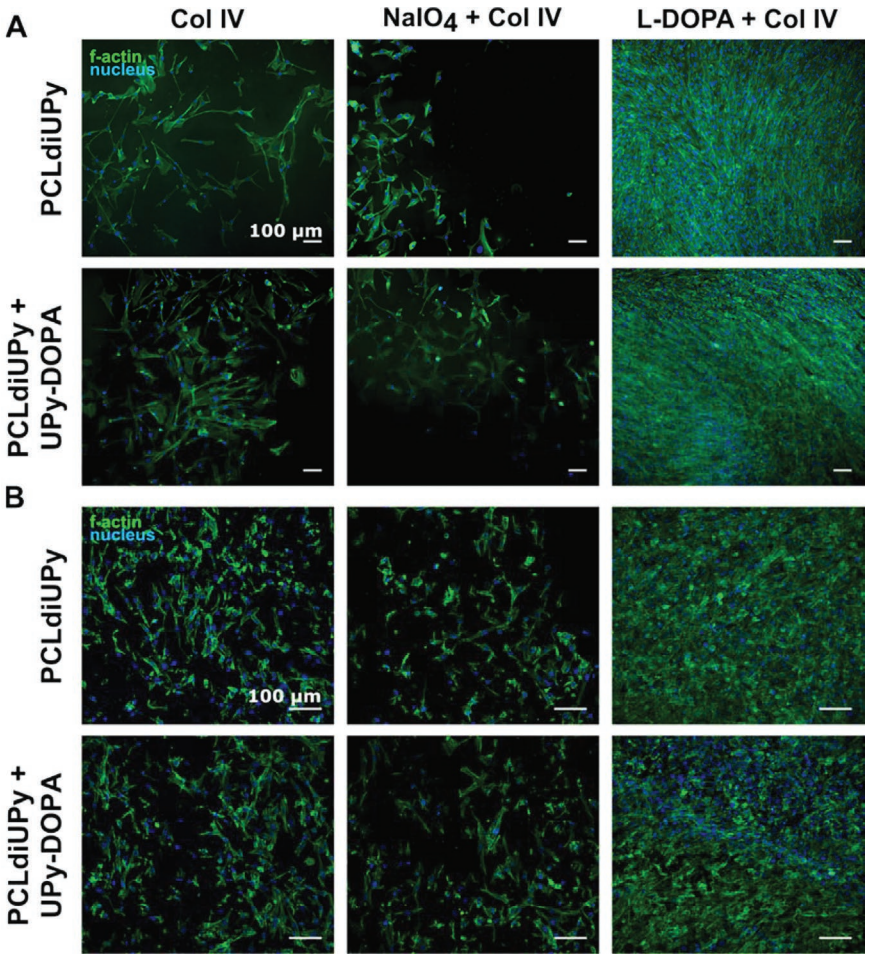

Figure 2. Double coating required for cell monolayer formation. Immunofluorescent staining of ciPTECs on PCLdiUPy (+UPy-DOPA) A) films and B) membranes coated with collagen type IV (Col IV), Col IV in the presence of oxidative sodium periodate ($NaIO_4$), or first coated with L-DOPA followed by Col IV cultured for 8d. Scale bar is 100 µm, f-actin (green), and nucleus (blue).

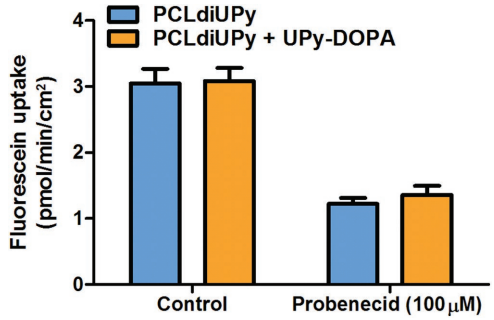

Figure 3. OAT-1 Transporter activity on double coated membranes. Fluorescein uptake by ciPTECs in the absence (control) and presence of the OAT-1 inhibitor probenecid on L-DOPA + collagen type IV-coated PLCdiUPy (blue), PCLdiUPy + UPy-DOPA (orange), mean ± SEM.

that uptake is mainly transporter mediated (**Figure 3**, Figure S3, Supporting Information). No significant difference in uptake was found between PCLdiUPy and PCLdiUPy functionalized with UPy-DOPA, indicating there is no effect on transporter function by UPy-DOPA. Membranes showed an almost four-fold increase in fluorescein uptake in the control samples and six times with probenecid inhibition (Figure 3). This increase can be contributed to i) fluorescein buffering by the membrane, which is released after cell lysis, and to ii) basolateral transporter accessibility. A membrane allows for fluorescein to reach the basolateral transporters, whilst a solid film does not. The slight transporter activity observed on films may be the result of imperfect polarization of the basolateral transporters. Renal epithelial cells exhibit basolateral transporters at the apical side near cell-cell contacts in the absence of flow.[39] In summary, ciPTECs retain transporter clearance potential when cultured on double coated supramolecular materials.

In this study the potential of monomeric catechol functionalization to induce a functional renal epithelial monolayer was compared to random polymeric catechol deposition on supramolecular biomaterials. Supramolecular monomeric catechol functionalization of PCLdiUPy films and membranes was achieved with UPy-DOPA. UPy-DOPA was shown to retain reactive capabilities after processing. Nevertheless, monomeric catechol functionalization was unable to improve PTEC monolayer formation over time, solely or in combination with Col IV coating. In contrast, random deposition of polymeric catechols combined with Col IV indeed induced a tight cell monolayer with OAT1 transporter activity. Mimicry of polymeric catechol coatings can therefore not be simply achieved by defined catechols as supramolecular additives, indicating further research is required to synthetically mimic natural catechol coatings. Overall these results help in future design of UPy-based bioactive membranes for the development of a bio-artificial kidney to aid end-stage renal disease patients.

Experimental Section

All materials were purchased at Sigma-Aldrich otherwise state.

Synthesis of UPy-DOPA: UPy-DOPA was synthesized by coupling dopamine via amide-bond formation to the carboxylic acid of a linear UPy-synthon. The carboxylic acid synthon, UPy-C_6-Urea-C_6-OEG_{12}-COOH, was synthesized in similar fashion as previously described by de Feijter et al. (Scheme S1, Supporting Information).[24] The mono-protected alkyl spacer *N*-Cbz-1,6-hexanediamine was coupled to monodisperse OEG_{12}-tBu propionate (PolyPure, Norway) via CDI-mediated coupling technique (1202 mg, 1.26 mmol, 88%). Then, the Cbz protecting group was removed by hydrogenation (755 mg, 0.92 mmol, 83%). The resulting amine-functionalized intermediate was reacted with UPy–hexyl isocyanate in the presence of base to yield the protected UPy-synthon (780 mg, 0.70 mmol, 96%). The carboxylic acid was deprotected in acid conditions to yield the linear UPy-COOH synthon as a white powder (345 mg, 0.33 mmol, 93%). Conjugation of the dopamine to the synthon was performed as reported by Spaans et al.[23] The reaction was performed using HATU as the coupling agent. A mild base, pyridine, was used, together with an Ar atmosphere to prevent oxidation of the catechol function. The product could be isolated after precipitation in water and several washing steps as a white fluffy solid (237 mg, 0.20 mmol, 71%). HPLC-MS (ESI) and $^{[1]}$H-NMR confirmed the successful conjugation of dopamine to the UPy-synthon. The catechol moiety has characteristic protons that are clearly distinguishable in the aromatic region (Figure S2, Supporting Information). A more detailed experimental procedure of the synthesis can be found in the Supporting Information.

Film and Membrane Fabrication: Polymer films were produced by evaporation induced phase separation. PCLdiUPy (Mn = 2.8 kg mol^{-1}; SyMO-Chem, The Netherlands) solutions were prepared with a concentration of 50 mg mL^{-1} in hexafluoroisopropanol (HFIP; Fluorochem, UK) with or without 10 mol% UPy-DOPA. A 45 µL droplet of the polymer solution was cast on a 14 Ø mm glass coverslip (VWR, USA), and the solvent was left to evaporate. Coated coverslips were place in vacuo overnight to remove potential residual solvent. Membranes were produced through electrospinning of polymer solutions. The solutions were prepared by dissolving 340 mg mL^{-1} PCLdiUPy with or without 10 mol% UPy-DOPA in HFIP, the solutions were left to stir overnight at room temperature. Electrospinning was performed on an IME Technologies EC-CLI (IME Technologies, The Netherlands), polymer fibers were collected on a cylindrical target (Ø = 31 mm) wrapped in aluminum foil while rotating at 100 rpm, chamber temperature was set to 23 °C and relative humidity to 30%. The polymer solution was fed through a nozzle (Ø = 1.0–0.8 mm) with a rate of 30 µL min^{-1}, while a voltage of 18 kV was applied. The nozzle tip was 12 cm removed from the target. Around 860 µL of polymer solution was effectively spun onto the target with a scanning distance of 75 mm.

Membrane Characterization: Incorporation and availability of UPy-DOPA in films and membranes were assessed through staining the catechol moieties and though determination of the hydrophobicity. To visualize catechol groups an adaptation of an Arnow's assay was performed. In short, 0.5 M HCl, 0.02 w/v% nitrite-molybdate in distilled water, and 1 M NaOH were sequentially, and in equal parts, added to the biomaterials and left to incubate for 10 min at room temperature (RT).[23,25] After aspiration of the reagents images were acquired with a digital camera (Canon Power Shot G15, Canon, Japan). The experiment was replicated three times. Hydrophobicity was assessed through WCA measurements. A 4 µL droplet of MilliQ was placed on a biomaterial surface with Contact angle system OCA (Dataphysics Intruments, Germany) the WCA was determined after 30 s. The experiment was performed on two different areas of each sample, with three replicates per condition.

To assess whether the addition of UPy-DOPA had an effect on electrospun fiber morphology scanning electron microscopy (SEM; Quanta 600; FEI, The Netherlands) micrographs were taken. Membranes were imaged with an applied voltage of 10 kV and a spot distance of 4 under low vacuum. Per condition three separately spun membranes were imaged, of which two distinct parts of the membrane, and four spots per part. The mean fiber diameter was determined from these micrographs through measurement of upper fibers with the help of imageJ (National Institutes of Health, USA). Furthermore pore size was determined through gas liquid porometry (Porolux 500; Porometer NV, Belgium) by submerging membranes in Porefil (Porometer NV) wetting

liquid. Pressure increased from 0 to 2 bar in 125 steps over 5 min. After obtaining the wet curve, a dry curve was acquired, both were analyzed with supplemented software to determine the max pore size, defined as the first bubble point, mean pore size and smallest pore size.

Cell Culture: ciPTECs expressing OAT1 were obtained from Radboudumc, Nijmegen, The Netherlands and developed as described previously.[29,30] Briefly, cells were retrieved from urine from a healthy volunteer in compliance with the guidelines of the Radboud Institutional Review Board and conditionally immortalized via transduction with the temperature-sensitive mutant of SV large T antigen (SV40T) and human telomerase reverse transcriptase (hTERT).[29] Transduction of OAT1 in ciPTEC was performed using lentiviral particles containing genes encoding for human OAT1.[30] Cells were cultured in Dulbecco's modified Eagle medium DMEM-HAM's F12 phenol-red free (Gibco, UK) supplemented with 10% v/v fetal bovine serum (Greiner Bio-one, The Netherlands), 5% v/v penicillin/streptomycin, 5 µ mL^{-1} insulin and transferrin, 5 ng mL^{-1} selenium, 10 ng mL^{-1} human epidermal growth factor 36 ng mL^{-1} hydrocortisone, and 40 pg mL^{-1} tri-iodothyronine at 33 °C. PCLdiUPy, or PCLdiUPy with 10 mol % UPy-DOPA membranes films were left uncoated or coated with either Col IV, Col IV with 10 mM sodium periodate ($NaIO_4$), or coated with polymeric L-DOPA followed by a Col IV coating, as previously reported in literature with minor adaptations.[7,8] In short, samples were clamped in a custom made ring and transwell insert to allow for compartmentalization of an inner apical compartment and an outer basolateral compartment. The constructs were UV-sterilized for 10 min on each side and afterward incubated with Hanks' Balanced Salt solution (HBSS) for 10 min at RT. Meanwhile 2 mg mL^{-1} L-DOPA was dissolved and left to polymerize in 10 mM Tris buffer (pH 8.5) for 1 h at 37 °C. On applicable samples the polymerized L-DOPA solution was incubated for 4 min at 37 °C, and subsequently washed with HBSS. To coat materials with Col IV a 25 µg mL^{-1} solution of the protein was incubated for 30 min at 37 °C. Proliferative ciPTECs were seeded with a density of 133 000 cells cm^{-2} on the films and membranes. Cells were allowed to adhere for 24 h at 33 °C afterward the ciPTEC quiescent phenotype was induced by culturing the cells at 37 °C, cells were kept at this temperature for 7 days.

Monolayer Morphology: To assess monolayer morphology, cells were washed twice with HBSS and fixated with 2% w/v paraformaldehyde and 2% w/v sucrose in HBSS for 5 min at RT. Cells were succeedingly permeabilized with 0.3% v/v Triton X-100 (Merck, Germany) in HBSS for 10 min, and afterwards washed with HBSS. The f-actin cytoskeleton and nucleus were respectively stained with 7 µM phalloidin-Atto 488 and 0.1 µg mL^{-1} 4′-6 diamidino-2-phenylindole (DAPI) for 30 min at RT, and finally mounted with mowiol. Films were imaged with Axiovert 200M (Zeiss, Germany), and z-stacks of the membranes were acquired with TCS SP5X confocal microscope (Leica, Germany) Z-stacks were compressed using maximum intensity projection in imageJ. The experiment was replicated three times in duplicate.

OAT 1Transporter Function: ciPTEC functionality was assessed through OAT1 transporter function, as described before by Nieskens et al.[30] with minor adaptations. In short, cells were incubated with Krebs buffer for 60 min at 37 °C to equilibrate cells. Two conditions for transporter function were assessed, either with or without inhibition of efflux and influx active transport by 100 µM probenecid in KH. Cells subjected to inhibition were pre-incubated with probenecid for 30 min at 37 °C. Afterward, both the basolateral as apical compartment were aspirated and replaced with 1 µM fluorescein in KH with or without probenecid for 10 min at 37 °C. Transport was arrested by washing the cells twice with ice-cold KH. All liquids were removed and cells were lysated with 0.3% v/v Triton X-100. Two times 100 µL was transferred to a 96 well plate and florescence of the lysate was measured with an excitation of 492 nm and emission of 518 nm on a Fluoroskan Ascent (Thermo Scientific, The Netherlands). The experiment was replicated three times in duplicate.

Statistical Analysis: Data regarding WCA and fluorescein uptake were subjected to a Mann-Whitney test to compare PCLdiUPy with PCLdiUPy UPy-DOPA mixture. Tests were performed with the use of Prism 5 (GraphPad Software Inc., USA). Probabilities of $p < 0.05$ were considered as significantly different.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

bio-artificial kidney, catechols, renal epithelial cells, supramolecular biomaterials, ureido-pyrimidinones

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