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Mixed matrix hollow fiber membranes for removal of protein-bound toxins from human plasma

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ABSTRACT

In end stage renal disease (ESRD) waste solutes accumulate in body fluid. Removal of protein bound solutes using conventional renal replacement therapies is currently very poor while their accumulation is associated with adverse outcomes in ESRD. Here we investigate the application of a hollow fiber mixed matrix membrane (MMM) for removal of these toxins. The MMM hollow fiber consists of porous macrovoid free polymeric inner membrane layer well attached to the activated carbon containing outer MMM layer. The new membranes have permeation properties in the ultrafiltration range. Under static conditions, they adsorb 57% p-cresylsulfate, 82% indoxyl sulfate and 94% of hippuric acid from spiked human plasma in 4 h. Under dynamic conditions, they adsorb on average 2.27 mg PCS/g membrane and 3.58 mg IS/g membrane in 4 h in diffusion experiments and 2.68 mg/g membrane PCS and 12.85 mg/g membrane IS in convection experiments. Based on the dynamic experiments we estimate that our membranes would suffice to remove the daily production of these protein bound solutes.

1. Introduction

Prevalence of chronic kidney disease (CKD) increases globally [1,2] due to the aging population and increasing incidence of risk factors such as diabetes mellitus [3]. Despite considerable amounts of healthcare budgets spent on renal replacement therapy [3,4], mortality of dialysis patients remains high [5,6] and their overall health related quality of life low [7]. In fact, the accumulation of uremic retention solutes plays an important role in CKD related morbidity and mortality [8–11]. Small water-soluble molecules can be removed by dialysis, but middle molecules and protein bound toxins are difficult to remove with conventional renal replacement therapies. Protein bound toxins are involved in generation of reactive oxygen species and associated with cardiovascular disease, progression of CKD and mortality [9,11–17]. Although improved uremic toxin removal has been achieved by extending the duration of dialysis, and this is associated with lower mortality rates [18], the removal of protein bound toxins is not improved by extended treatment if total blood and dialyzate fluid crossing the dialyzer is kept constant per session [19] or high-flux dialysis [20]. Convective therapies such as hemodiafiltration can improve removal of the middle molecule β2-microglobulin [6]. Post dilution online hemodiafiltration has shown to significantly lower total pre-dialysis concentrations of p-cresylsulfate (PCS) and 3-carboxyl-4-methyl-5-propyl-2-furanpropionic acid (CMPF), two protein bound toxins with high protein binding. However, the effect on their total concentration was only moderate [21].

To improve protein bound toxin removal, the concentration of free toxin on the dialyzate side should be low, so that there is a continuous driving force for the free fraction in the blood to diffuse to the dialyzate side over the whole hemodialyzer length [22,23]. Indeed, Dinh et al. showed that adding powdered activated carbon into the dialyzate compartment, improved the clearance of protein bound solutes

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by continuous binding of the diffused free fraction [24]. Furthermore, raising the dialyzate flow can have a similar effect [22] and Sirich et al. showed *in vivo* that removal of protein bound solutes increased by raising the dialyzate flow rates [25]. Another way of maintaining a high concentration gradient over the entire length of a hemodialyzer membrane, thereby probably enhancing protein bound toxin removal, could be incorporation of adsorptive particles in the membrane itself. In fact, more than 30 years ago, so called sorbent membranes were developed, in which adsorptive particles were embedded between two cuprophan membrane layers, or within a cuprophan matrix, to combine both filtering and adsorbing capacity for uremic toxins [26]. However, removal of protein bound toxins was not addressed. After a clinical trial with sorbent membranes, patients rated the treatment low and complained about increased lethargy [27]. This might be due to lack of adsorbents with high purity [28]. In addition, manufacturing difficulties and rapid saturation caused these membranes to be removed from the market [29,30].

Previously, we showed the concept of a membrane with embedded adsorptive particles, a so-called porous mixed matrix membrane (MMM) [31]. These flat sheet MMMs consisted of a porous particle free layer attached to the mixed matrix membrane layer with embedded particles and showed high adsorption capacity of creatinine and could combine diffusion and adsorption of creatinine in one step [31]. Here, we develop a dual layer hollow fiber MMM to remove protein bound uremic toxins. Ultimately this membrane might maintain a concentration difference and thereby a diffusion driving force over the entire membrane length, leading to improved protein bound toxin removal. Polyethersulfone (PES) is used as a membrane forming material, blended with the hydrophilic additive polyvinylpyrrolidone (PVP). This polymer blend is often used for hemodialysis membranes [32,33]. Activated carbon is selected as adsorptive particle because it adsorbs a broad range of solutes, including protein bound toxins, and it has a long track record in blood purification [24,34]. A special triple layer spinneret is designed for the spinning of a polymeric inner layer and a thicker outer MMM layer. The influence of spinning parameters such as bore liquid composition and pumping speeds is investigated. Fabricated fibers are characterized in terms of adsorptive capacities and transport properties. Creatinine, a small molecular weight uremic retention solute, often used as a marker of kidney function, is used as a model for water soluble solute. Hippuric acid (HA), indoxyl sulfate (IS) and p-cresylsulfate (PCS), often used as representatives for the protein bound uremic toxins and associated with adverse/toxic effects [12–15], are used as model for the protein bound uremic toxins. Static adsorption experiments as well as experiments under flow conditions are performed to estimate the transport properties of the new hollow fiber MMMs.

2. Materials and methods

2.1. Materials

Ultrason E 6020 PES, obtained from BASF (Ludwigshafen, Germany), and PVP K90 (360,000 g/M) (Fluka, Sigma–Aldrich Chemie Gmbh Munich, Germany) were used as membrane forming materials. N-methylpyrrolidone (NMP) (Acros Organics, Geel, Belgium) was used as solvent. Ultra-pure water was used as non-solvent in the bore liquid and distilled water was used as non-solvent in the coagulation bath. Norit A Supra EUR (European pharmacopoeia grade) (Norit Netherlands BV, Amersfoort, The Netherlands) was sieved in a 45 μm sieve (Fritsch GmbH, Idar-Oberstein, Germany) to a median particle size of 27 μm and was used as activated carbon (AC) particles for incorporation in the MMM and tested separately as pure particles.

The chemicals needed for Tyrode's buffer and the dialyzate solution were obtained from Fluka, Sigma–Aldrich: 5.4 mM KCl, 137 mM NaCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 11.9 mM $NaHCO_3$ and 5.5 mM glucose were dissolved in ultra-pure water to obtain Tyrode's buffer (pH 7.4). For the dialyzate solution, 2 mM KCL, 140 mM NaCl, 1.5 mM $CaCl_2$, 0.25 mM $MgCl_2$, 35 mM $NaHCO_3$ and 5.5 mM glucose were dissolved in ultra-pure water. Creatinine, IS and HA were purchased from Sigma–Aldrich whereas PCS is not commercially available and was synthesized as described before [35].

Polyethylene tubes (Rubber BV, Hilversum, The Netherlands) were used for module fabrication and two-component glue (Bison kombi snel rapide, Eriks, Almelo, The Netherlands) was used for potting of the modules. Single fiber modules were applied for the creatinine diffusion experiment, while four fiber modules were used for the plasma diffusion and plasma convection experiments.

Uremic human plasma was obtained from six patients who received a plasma exchange treatment because of acute kidney injury and human blood plasma was obtained from six healthy donors from Sanquin (Amsterdam, The Netherlands) in compliance with local ethical guidelines.

Table 1
Spinneret dimensions.

	Spinneret 1	Spinneret 2
Inner diameter needle (mm)	1.2	0.26
Outer diameter needle (mm)	1.5	0.46
Inner diameter first orifice (mm)	2.2	0.66
Outer diameter first orifice (mm)		0.96
Inner diameter second orifice (mm)		1.66

2.2. Hollow fiber MMM fabrication

For the adsorptive layer, a dope solution (MMM1) was prepared containing 14 wt% PES and 1.4 wt% PVP K90 dissolved in NMP. The AC particles were added to this dope and after mixing on a roller bank for at least 48 h; a homogenous dope was obtained with a loading of 60 wt% activated carbon particles in relation to the amount of PES. This solution was degassed for at least 48 h. The polymer dope of the particle free inner layer (IL1) consisted of 15 wt% PES and 10 wt% PVP dissolved in NMP. After 24 h mixing on a roller bank the solution was filtered using a Bekipor ST AL3 15 μm filter (Bekaert, Kortrijk, Belgium) and allowed to degas for at least 24 h. The following bore liquids were prepared by 24 h mixing: B1) 5 wt% PVP in ultra-pure water, B2) 5 wt% PVP and 60 wt% NMP in ultra-pure water and allowed to degas for at least 24 h. For the single layer MMM spinneret 1 was used and for the dual layer HF MMM, spinneret 2 was designed (see Table 1). The dimensions of the spinnerets were chosen because of practical reasons so that dual layer hollow fibers could be spun for the proof of concept of this study.

All hollow fiber membranes were fabricated by dry–wet spinning via immersion precipitation. The MMM dope was pressurized, while the particle free inner layer dope and the bore liquid were pumped through the spinneret. After a 3 cm air gap, the nascent hollow fiber was immersed into a water coagulation bath at room temperature and the hollow fiber was formed by phase separation.

During hollow fiber spinning, several parameters were varied. Table 2 describes the spinning conditions. For the single layer hollow fiber MMM (SL), the pulling wheel was not constantly used to collect the hollow fiber, for the other fibers the applicable pulling wheel speeds are presented in Table 2. The pulling wheel speed was adjusted so that the wheel continuously picked up the fiber. The pressurized MMM dope speed was aimed to be the same in DL1, DL2, and DL3. But because of practical difficulties with the pressure regulator the pressurized MMM dope speeds were a little bit lower for DL3. Dual layer HF MMMs were collected during at least three succeeding periods of 5 min of spinning. The collected fibers were washed in ultra-pure water to remove any remaining solvent, and stored in ultra-pure water upon further use. The AC proportion in the membrane was estimated using the content of AC particles, PES and PVP in the dopes, density and pumping speeds of the dopes similar as described before [31].

Table 2
Spinning conditions.

	SL	DL1	DL2	DL3
Bore liquid	B1	B1	B1	B2
Inner layer polymer dope	–	IL1	IL1	IL1
Mixed matrix membrane layer dope	MMM1	MMM1	MMM1	MMM1
Bore liquid pumping speed (mL/min)	2.7	2.7	2.7	2.7
Inner layer dope pumping speed (mL/min)	–	2.03	0.9	0.9
Pressurized mixed matrix membrane dope speed (mL/min)	8.4	3.9	3.9	3.2
Pulling wheel speed (m/min)	–	4.4	4.4	7
Spinneret	1	2	2	2

Bore liquid B1 contains 5 wt% PVP in ultra pure water, bore liquid B2 contains 5 wt% PVP and 60 wt% NMP in ultra pure water. Inner layer polymer dope IL 1 contains 15 wt% PES and 10 wt% PVP in NMP. Mixed matrix membrane layer dope MMM1 contains 14 wt% PES, 1.4 wt% PVP in NMP and 60 wt% AC in relation to the amount of PES.

2.3. Membrane characterization

2.3.1. Scanning electron microscopy (SEM)

To investigate their surface, the fibers were cut open to expose the inner surface and glued on a sample holder. To examine cross sections, the hollow fiber membranes were dried in air followed by fracturing in liquid nitrogen and were clamped in a cross section sample holder. Then, the samples were dried under vacuum at 30 °C and subsequently gold coated using a BalzersUnion SCD 040 sputter coater (Oerlikon Balzers, Balzers, Liechtenstein) and examined using a JEOL JSM-5600LV Scanning Electron Microscope (JEOL, Tokyo, Japan). Cross sections of fibers DL1, DL2 and DL3 collected in succeeding periods of 5 min during the spinning were examined. Inner diameter (I.D.), outer diameter (O.D.) and membrane layer thicknesses were determined using SEM pictures originating from all collecting periods.

2.3.2. Clean water permeance

Hollow fiber membranes were dried in air and single fiber modules were prepared by gluing the fiber in a tube with a Festo T-connection (Eriks, The Netherlands). After potting, both ends were cut open. The modules were equilibrated in ultra-pure water for at least seven days. Before testing, modules were prepressurized at 2 bar for 1 h, then transmembrane pressures of 0.5, 1.0, 1.5 and 2.0 bar were applied and the amount of permeated ultra-pure water was measured over time. The clean water permeance (L_p) (L/m^2/h/bar) was determined by calculating the slope of a linear fit of the flux versus pressure graph. Fibers from each succeeding 5-min period during the spinning were tested for clean water permeance.

2.3.3. Adsorption isotherms

Air dried hollow fiber membranes were cut in pieces of 4 cm length and were incubated in 5 mL solutions containing different concentrations of creatinine, IS or HA in Tyrode's buffer in a shaking water bath at 37 °C. The range of creatinine concentrations was close to the creatinine levels present in the normal (0.012 mg/mL) and uremic state (0.136 mg/mL) [36]. The range of IS and HA concentrations was also close to the average uremic concentrations 0.053 mg/mL and 0.247 mg/mL and highest reported uremic concentrations 0.236 mg/mL and 0.471 mg/mL, respectively [36], although recent data suggests lower concentrations [8].

After a 72, 48 or 24 h incubation period, the creatinine, IS and HA concentrations were measured by photo spectrometric analysis at 230, 278 and 228 nm at 25 °C in a 2, 2 and 10 mm quartz cuvette respectively. The adsorptive capacity for each uremic retention solute is expressed in mg adsorbed per gram of embedded activated carbon. For the isotherms, the equilibrium adsorption capacity (q) is plotted against the equilibrium concentration of the component (C). The exact particle proportion in DL3 is necessary for this and is calculated to be 0.47. A Langmuir curve fit was obtained as described before [31] and the maximum adsorption capacity (q_m) and dissociation constant (K_d) were estimated.

2.3.4. Static adsorption from human blood plasma

Uremic plasma was used for measurements of creatinine, osmolarity, pH, and total protein. Frozen human plasma from three healthy donors was thawed and spiked with PCS, IS and HA to obtain either similar total concentrations as in uremic patients [8] or higher total concentrations. The spiked plasma solutions were allowed to mix for 10 min prior to the start of the experiment. This plasma was tested for concentrations of PCS, IS, HA, creatinine, urea, total protein, Na^+, K^+, Ca^{2+} and osmolarity and pH.

A PES/PVP particle free flat sheet membrane was used as reference (made as described before as a single particle free membrane [31]). Approximately 25 mg activated carbon particles, DL3 containing approximately 25 mg activated carbon and approximately 25 mg flat sheet home-made PES/PVP particle free membrane were incubated in approximately 4 mL of different plasma samples. The amount of added plasma was adjusted to the amount of incubated material so that the material–plasma relation was always similar to 25 mg material in 4 mL plasma. All tubes were incubated on a roller bank. After the incubation time, tubes were centrifuged at 3500 rpm for 10 min, and supernatant was collected and one part was stored at 4 °C and another part was directly frozen and stored in −80 °C. Osmolarity, pH, Na^+, K^+, Ca^{2+}, total protein, urea and creatinine concentrations are measured using the techniques described in Table 3. Furthermore, free and total PCS, IS and HA concentrations were analyzed as described before [37,38]. The absolute value of a sample was always related to the value of the control plasma at that time point as follows:

$$\text{Relative concentration} = \frac{C_t}{C_0}$$

where C_t is the concentration in the plasma incubated with a sample (AC, DL3 or PES/PVP membranes) at that time point, and C_0 is the concentration in the blank (plasma without sorbents or membranes). Relative osmolarities and pH were calculated in a similar way. Statistical differences were determined using a one-way ANOVA and a post-hoc Tukey test for PCS, IS, HA, osmolarity, pH, and Na^+, K^+, Ca^{2+}, total protein, urea and creatinine concentrations.

2.3.5. Cross flow measurements

Fig. 1a and b shows the schematic representations of the experimental set up used for the diffusion and convection cross flow experiments respectively.

Table 3
Test methods for analysis of blood plasma.

Parameter	Kit/device
Osmolarity	Advanced instruments osmometer model 3320
pH	Radiometer Copenhagen PHM lab pH meter
Sodium	Corning 480 Flame Photometer
Potassium	Corning 480 Flame Photometer
Calcium	Bio-Rad Microplate reader Benchmark 16-channel photometer DiaSys Calcium CPC FS (1 1121 99 10 021)
Total protein	Bio-Rad Microplate reader Benchmark 16-channel photometer Bio-Rad Laboratories GmbH Protein Assay (cat# 500-0006)
Urea	Starrcol standard SC-60-S photometer DiaSys Urea CT FS (1 3115 99 10 026)
Creatinine	Bio-Rad Microplate reader Benchmark 16-channel photometer DiaSys Creatinine PAP FS (1 1759 99 10 026)

Membrane modules containing one or four hollow fibers were prepared from 8 mm tubes and two Kartell T connections (VWR, Amsterdam, The Netherlands) for the experiments with model solutions and spiked human plasma respectively. Both ends were cut open after potting. Modules were equilibrated in water for at least seven days. Before the start of the experiment with human plasma, clean water was pressurized through the membranes at 1 bar for at least 1 h to check if all hollow fiber membranes were open. The modules for the diffusion and convection experiments with human plasma contained on average 96.1 ± 4.1 mg DL3. For the model solution diffusion experiment, the feed consisted of 50 mL 0.1 mg/mL creatinine in

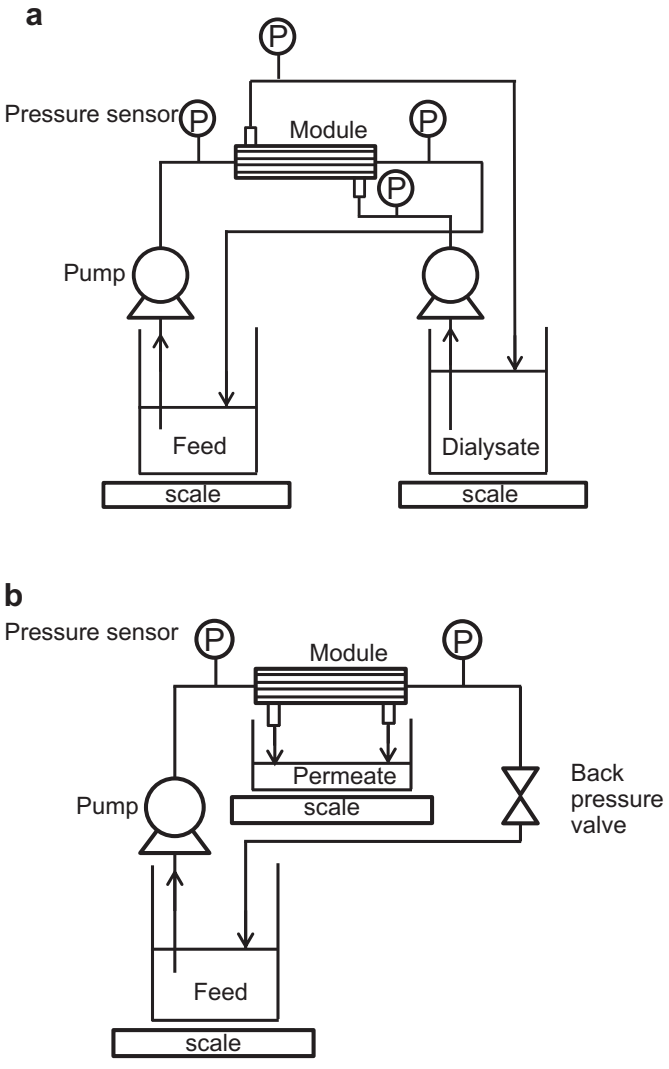

Fig. 1. Schematic representations of experimental set up for a) diffusion experiments, b) convection experiments.

ultra-pure water solution (close to mean uremic creatinine concentration [36]) and dialyzate was 100 mL ultra-pure water, while for the spiked plasma diffusion experiment the feed consisted of 50 mL spiked human plasma (spiked as described in Section 2.3.4.) and the dialyzate was 100 mL dialyzate buffer. For the diffusion experiments, the feed was pumped through the lumen of the hollow fiber, while the dialyzate was pumped around the fibers in the counter current direction. The feed and dialyzate solutions were pumped at 5 mL/min and 31.4 mL/min respectively. Using these flow rates no transmembrane pressure could be detected.

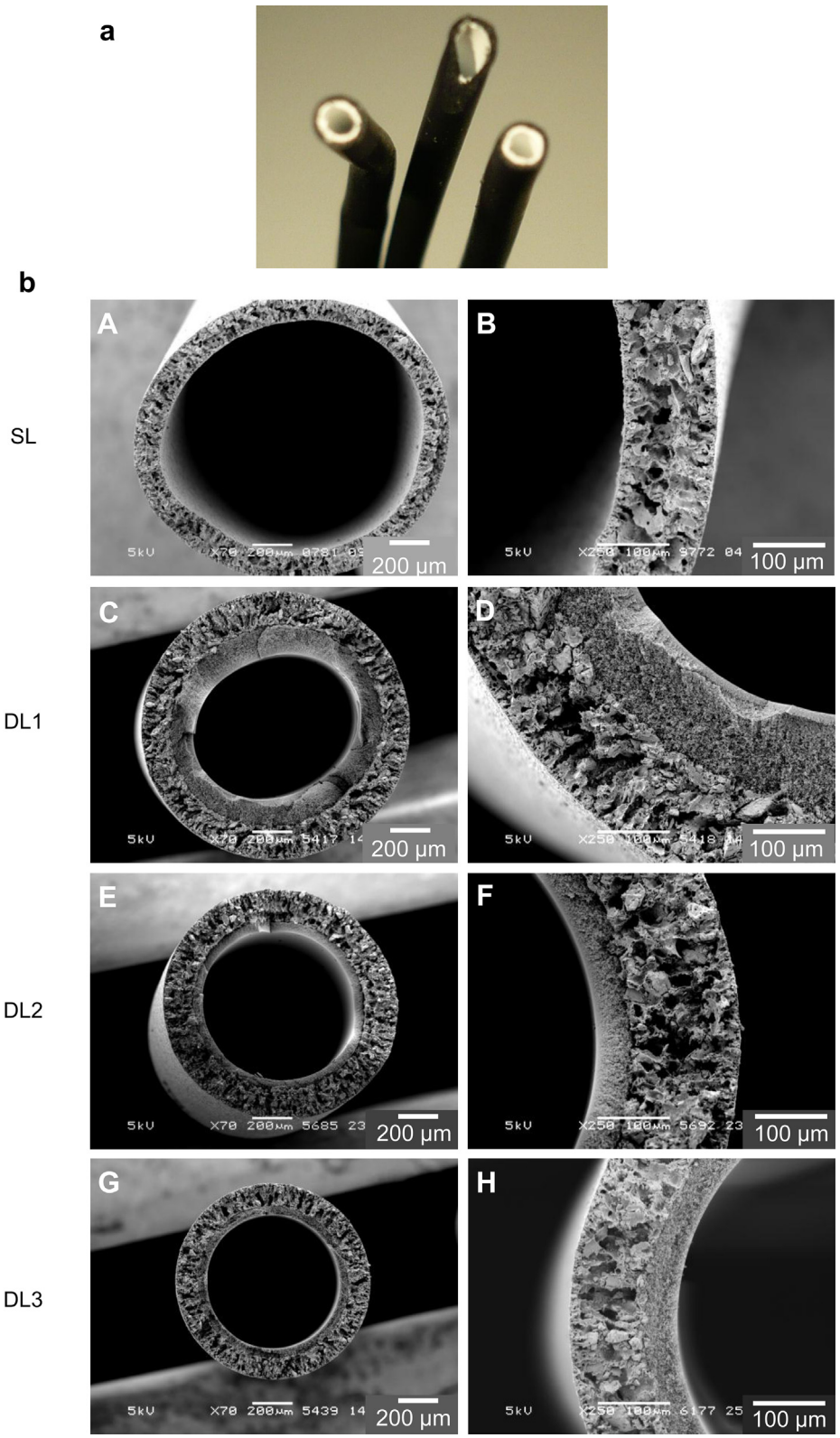

Fig. 2. **a**) Photograph of dual layer hollow fiber MMMs DL1. **b**) Scanning electron microscopy images of single layer hollow fiber MMM SL (A, B) and dual layer hollow fiber MMMs DL1 (C, D), DL2 (E, F), and DL3 (G, H). **c**) Scanning electron microscopy images of fiber DL2 (A, B, C) and DL3 (D, E, F) of cross section (A, D), inner surface (B, E) and outside surface (C, F) with magnifications of 1500× (A, B, D, E) and 1000× (C, F).

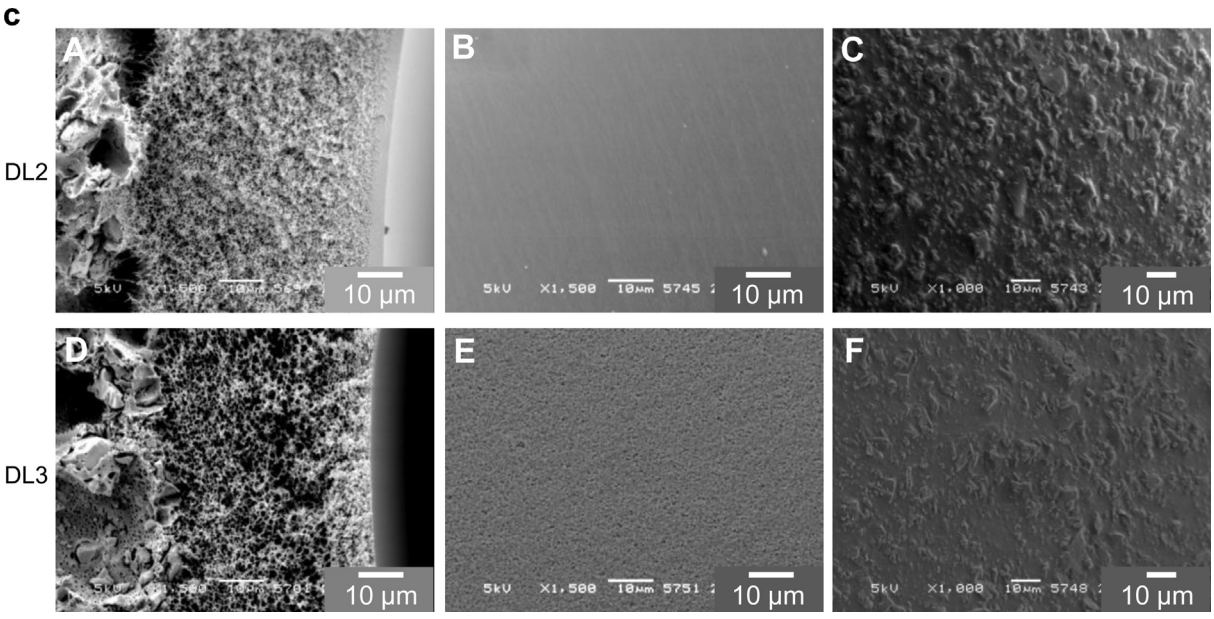

Fig. 2. (*continued*).

For the spiked human plasma convection experiment the Spectrumlabs Kross Flo Research Ili system and a backpressure valve (SpectrumLabs automatic backpressure valve, JM separations, Tilburg, The Netherlands) was used in order to obtain constant transmembrane pressures during the whole experiment. The 50 mL spiked human plasma (see Section 2.3.4.) was pumped through the hollow fiber membranes at 15 mL/min and the transmembrane pressure was set at 0.25 bar.

For all cross flow experiments, at the indicated time points the pressures and the weight of the feed and dialyzate or permeate compartments were measured and 2 mL samples were taken from both compartments. After the model solution diffusion experiment, creatinine concentrations were measured using photo spectrometric analysis as described in Section 2.3.3. The amount of creatinine removed from the feed solution was defined as total removal. The amount of creatinine that appeared in the dialyzate solution was considered as creatinine, which was diffused from the feed solution. The creatinine deficiency in the mass balance was considered to be adsorbed onto the MMM. These amounts were related to the dry membrane weight, which was estimated based on the measured active fiber length in the modules. Plasma samples were immediately frozen in liquid nitrogen and kept at −80 °C until analysis. Total protein concentration, PCS, IS and HA were analyzed as described in Section 2.3.4. The relative total protein concentrations were calculated as described in Section 2.3.4. In this case, the value of plasma at the start of the experiment was taken as blank (C_0).

3. Results and discussion

3.1. Hollow fiber membrane fabrication and morphology

Fig. 2a shows a photograph of membrane DL1. The two membrane layers can clearly be distinguished because of the black and white colors of the MMM layer with embedded black activated carbon particles and the white particle free porous polymeric inner layer, respectively.

Fig. 2b shows SEM images of several fabricated hollow fiber mixed matrix membranes. In the round single layer hollow fiber MMM (SL) the AC particles are well distributed in the porous membrane matrix, no cluster formation is observed. Relatively big pores are present in the middle of the membrane structure, while close to the lumen and close to the outside surface, smaller pores are visible. No macro-voids through the complete cross section of the membrane wall are found.

Using 5% PVP in the bore liquid and a high bore liquid pumping speed ensured formation of a fiber with a circular bore. Water as bore liquid and/or lower pumping speeds often resulted in the formation of an irregular shape of the inner contour of the fiber (data not shown). The amounts of PES and PVP in the dopes and composition of coagulation fluids were based on the literature [39] and previous experience in our lab with flat sheet membranes [31] and hollow fiber membranes (data not shown). PVP K90 is often used for hemodialysis membranes. Because of its relatively high molecular weight the viscosity of the dope solution was high, promoting formation of macro-void free membranes. Moreover this type of PVP has shown to give macro-void free membranes with high solute permeability [40,41].

Fig. 2b also presents SEM pictures of DL1, a dual layer hollow fiber MMM. The two membrane layers can be clearly distinguished, and are well attached to each other. The inner layer is a porous sponge like particle free membrane layer and seems to have a dense skin layer on the inside, whereas pores become bigger toward the outside. The outside MMM layer has a similar structure as the SL membrane. The particle free membrane layer and the MMM layer have almost the same thickness of around 140 ± 20 μm and 155 ± 20 μm (Table 4). To avoid mass transfer limitations, a thinner particle free membrane layer is desirable. Therefore, the inner layer polymer dope pumping speed was decreased and DL2 was obtained (Fig. 2b) having a much thinner inner membrane layer of 52 ± 3 μm. The MMM layer thickness and membrane structure is similar as in DL1. However, still a rather dense skin layer is observed on the lumen of the hollow fiber membrane. In general, by using bore liquids with high amounts of solvent, slower phase separation can be obtained leading to bigger pore sizes [42]. Therefore we changed the composition of the bore liquid from solvent free to a 60% solvent containing bore liquid, also based on previous experiences in flat sheet membranes [31,39]. Dual layer hollow fiber DL3 was obtained (Fig. 2b), and the inside layer seems to have a thinner skin

Table 4
Average ± SD dimensions of hollow fiber mixed matrix membranes.

	SL	DL1	DL2	DL3
Outer diameter (O.D.) (μm)	1487 ± 38	1339 ± 14	1186 ± 37	984 ± 11
Inner diameter (I.D.) (μm)	1247 ± 21	776 ± 80	774 ± 29	669 ± 9
Inner layer thickness (μm)	–	140 ± 20	52 ± 3	49 ± 5
Mixed matrix membrane layer thickness (μm)	112 ± 4	155 ± 20	154 ± 8	111 ± 4

layer than DL2. This can also be seen in Fig. 2c, no pores are visible at the inner surface of DL2 while on the inner surface of DL3 pores can be observed. The MMM layer structure of DL3 is similar as in the other dual layer hollow fiber MMM, however, the two layers seem to be better connected in DL3. Probably due to solvent containing bore liquid, slower phase separation occurs, allowing more time for the separate layers to connect. The pulling wheel speed was higher for this fiber than for DL1 and DL2. Probably, due to the slower phase separation, the nascent fiber became more extended by the pulling. This might explain the smaller dimensions of DL3 compared to DL2, see Table 4. Besides, the little decrease in pumping speed of the MMM dope may have played a role in this. The outer surfaces in Fig. 2c show that the particles are well distributed in the MMM matrix.

3.2. Clean water permeance

Fig. 3 presents a linear clean water flux vs. pressure relationship for DL3 with a clean water permeance of 58.4 ± 9.3 L/m^2/h/bar. Lower as well as higher clean water permeances have been described for polyethersulfone based hollow fibers used for hemodialysis [43,44]. Neither particle loss during the experiment nor delamination of the two membrane layers was observed. DL1 and DL2 show a clean water permeance of less than 3 L/m^2/h/bar. For all hollow fibers DL 1, 2 and 3 the clean water permeance was measured for fiber samples from different collection periods and show constant clean water permeances in all cases. Because of higher water permeance, DL3 was selected for further characterization in this paper.

3.3. Static adsorption

3.3.1. Adsorption isotherms

DL3 adsorbs creatinine, IS and HA, which is illustrated by the adsorption isotherms in Fig. 4. Langmuir isotherm curve fits have been performed, even though for the tested concentration range a plateau was not reached yet in all cases. For creatinine, IS and HA, $q_m = 3064$ mg/g AC and $K_d = 1.433$ mg/mL, $q_m = 350$ mg/g AC and $K_d = 0.023$ mg/mL, $q_m = 134$ mg/g AC and $K_d = 0.0195$ mg/mL were obtained respectively. DL3 has much higher adsorption capacity for creatinine in comparison to flat sheet dual layer MMMs developed earlier [31]. For example, at a creatinine equilibrium concentration

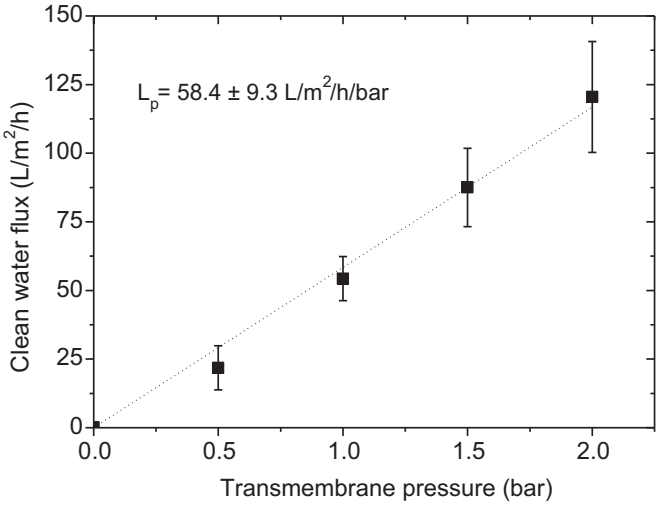

Fig. 3. Average clean water flux versus transmembrane pressure for DL3 ($n = 6$). Error bars indicate standard deviations.

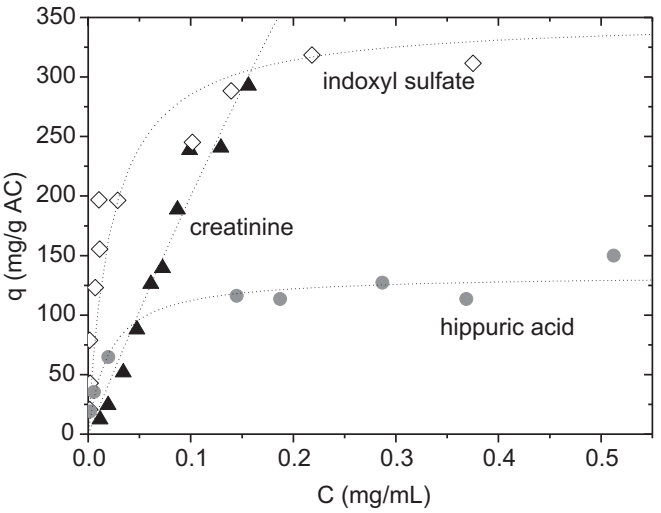

Fig. 4. Adsorption isotherms at 37 °C for DL3. The equilibrium adsorption capacity (q) expressed in mg adsorbed creatinine (▲), indoxyl sulfate (◇) or hippuric acid (●) per gram of activated carbon (AC) embedded in DL3 is plotted against the equilibrium concentration (C) of creatinine, indoxyl sulfate and hippuric acid. The dotted lines represent the calculated Langmuir isotherm fits.

of 0.05 mg/mL the flat sheet dual layer MMM adsorbed approximately 29 mg creatinine per gram of activated carbon while the DL3 adsorbed around 100 mg/g AC. It is rather difficult to directly compare our results to other studies because of different experimental conditions and limited availability of *in vitro* adsorption data. In any case, we will discuss here some examples where comparison is possible. For example for creatinine adsorption, other publications with different approaches but using activated carbon as sorbent, report lower adsorption values at similar equilibrium concentrations [45–47]. Furthermore, granular and powdered carbons have been tested for adsorption of several compounds including IS. Using a sorbent/volume ratio of 2 mg/mL and an initial concentration of 0.03 mg/mL IS in a protein free solution, removal of more than 90% of the free IS was obtained after 24 h. For our isotherm experiment we also used a protein free solution and free IS. Using a lower sorbent/volume ratio of 1.2 mg/mL but a longer incubation time (48 h) we obtained removal of 95% at a similar initial concentration. Thus, IS adsorption by DL3 is in the same range as a commercial activated carbon [24].

3.3.2. Adsorption from human blood plasma

Fig. 5 shows the average relative creatinine concentrations after incubation in uremic plasma. The DL3 significantly removes creatinine compared to the particle free PES/PVP membrane up to 83 ± 4% is adsorbed after 4 h. This approaches the creatinine removal by activated carbon particles alone, which suggests a good creatinine accessibility of the particles embedded in DL3. Relative plasma pH and osmolarity did not change significantly. The total protein concentration is unchanged indicating that undesired protein adsorption is also limited (data not shown).

The percentage of PCS, IS and HA bound to protein was on average 98%, 97% and 53%, respectively, and this was constant for the duration of the experiments (data not shown). Fig. 6 shows the average relative PCS, IS and HA concentrations after 1 h and 4 h incubation with a PES/PVP membrane, DL3 or AC particles for both initial spiked concentrations. The DL3 and AC both remove PCS, IS and HA significantly compared to the PES/PVP membrane. The DL3 and AC lower the relative PCS concentration to an average of 0.43 and 0.16, respectively, after 4 h. The IS is also removed by DL3 and AC and after 4 h average relative IS concentrations of 0.17 and 0.03

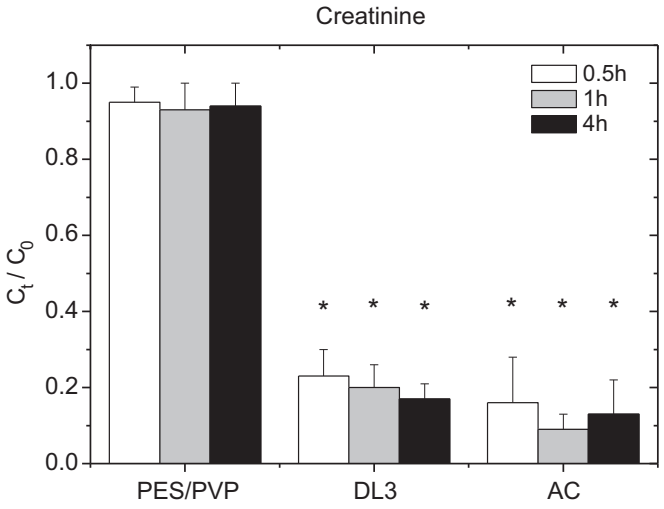

Fig. 5. Average relative creatinine concentrations (C_t/C_0) after incubation of a PES/PVP membrane, dual layer hollow fiber mixed matrix membrane DL3 or pure activated carbon (AC) in plasma ($n = 6$) for 0.5, 1 or 4 h. * Indicates a significant difference compared to particle free PES/PVP membrane ($p < 0.05$). Error bars indicate standard deviations.

are obtained, respectively. For HA, the DL3 membrane and AC decrease the average relative concentrations after 4 h to 0.05 and 0.01, respectively. The PES/PVP membrane does not adsorb PCS, IS and HA. Thus, even in these experimental conditions with a relatively low sorbent–plasma ratio 6.25 mg/mL (compared to ~100 mg/mL in hemoperfusion) the majority of PCS, IS and HA can be removed from the plasma within 4 h by DL3. However, the activated carbon particles lowered the relative concentrations significantly more ($p < 0.05$) than DL3 in all cases, which may be due to some diffusion limitations, or reduction in adsorption capacity introduced by the membrane matrix. Despite their higher performance, the small particles used here (median size 27 μm) probably cannot be applied in adsorption columns, which commonly have much larger particles, for removal of solutes from blood or plasma. Small particles in columns could introduce high pressure drop into the system, as shown in Ref. [48], with the risks of protein denaturation and leakage of particle fragments into the patient's blood circulation. In contrast to adsorptive columns, small particles can be used in MMMs without high pressure drop and channeling since particles are well distributed into the polymeric matrix [48]. In the future, we plan to develop improved dual layer membranes with improved pore connectivity and higher particle loading.

Table 5 presents average relative plasma values after incubation in plasma. Low levels of creatinine (compared to uremic situation) are present in this plasma from healthy donors, and DL3 and AC completely remove creatinine within 4 h ($p < 0.05$), indicating the good adsorptive power even at low solute concentrations. There is no effect on pH or concentrations of Na^+, K^+, Ca^{2+}, total protein and urea (see Table 5) by DL3 or AC. DL3 lowered osmolarity ($p < 0.05$ versus PES/PVP membrane), although this was not observed in the previous experiment using plasma exchange plasma.

3.4. Cross flow measurements

3.4.1. Creatinine diffusion cross flow measurements

As we showed before [31], dual layer MMMs can combine diffusion and adsorption in one step. Fig. 7 shows that a lot of creatinine is adsorbed in the beginning. After 1 h, DL3 becomes more or less saturated whereas the diffusion of creatinine continues during the whole experiment. After 4 h, which is a common duration of a hemodialysis treatment, both diffusion and adsorption equally contributed to the total creatinine removal. Almost 40 mg creatinine per gram of membrane was removed after

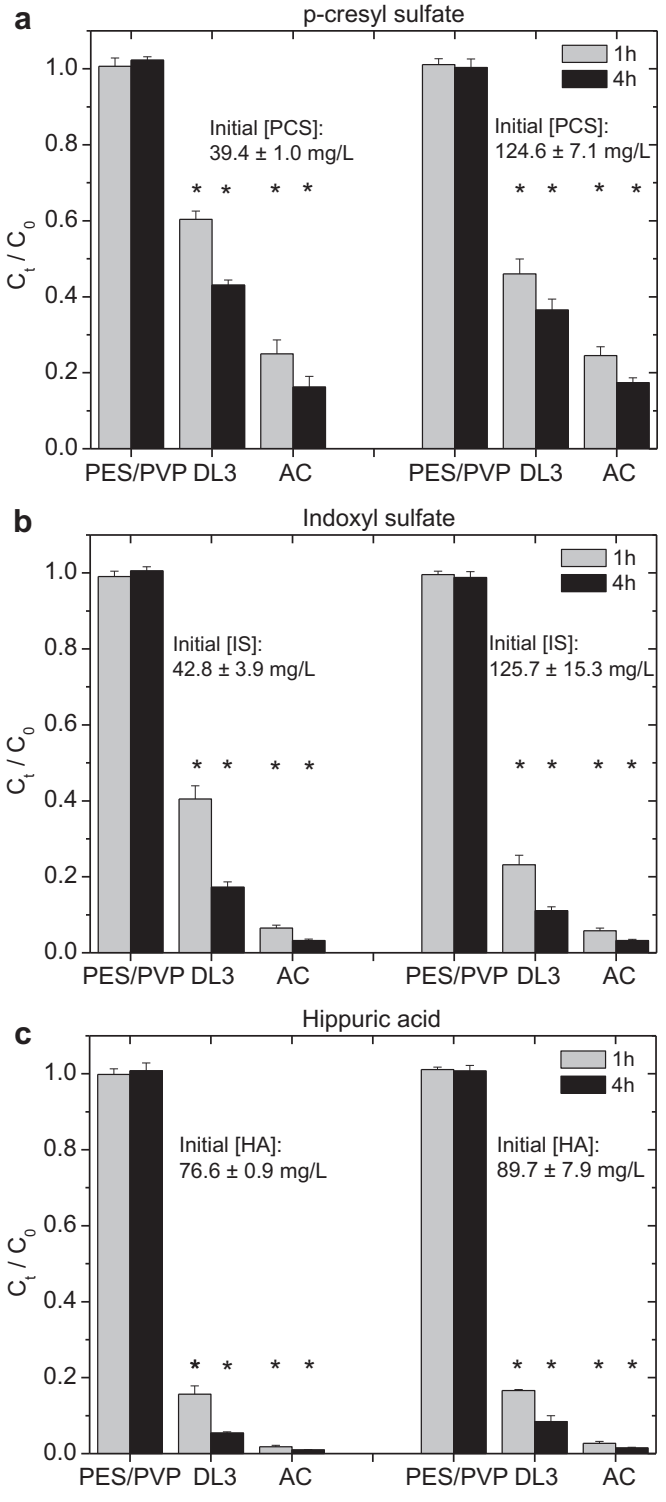

Fig. 6. Relative concentrations after incubation of a particle free PES/PVP membrane, dual layer hollow fiber MMM DL3 and pure activated carbon (AC) particles in spiked plasma with 2 different initial concentrations for 1 or 4 h. a) p-Cresylsulfate. * Indicates a significant difference compared to the particle free PES/PVP membrane ($p < 0.05$). b) Indoxyl sulfate, **c**) hippuric acid. * Indicates a significant difference compared to the particle free PES/PVP membrane ($p < 0.05$).

Table 5
Average relative plasma values ± SD after incubation with a PES/PVP membrane, dual layer hollow fiber MMM (DL3) or pure activated carbon (AC) particles in human blood plasma of three healthy donors for 4 h.

	PES/PVP	DL3	AC
Relative osmolarity	1.008 ± 0.01	0.963 ± 0.01[a]	0.984 ± 0.01
Relative pH	1.004 ± 0.01	1.041 ± 0.07	1.046 ± 0.06
Relative Na^+ concentration	0.965 ± 0.07	0.952 ± 0.07	1.003 ± 0.02
Relative K^+ concentration	0.959 ± 0.08	0.935 ± 0.07	0.983 ± 0.01
Relative Ca^{2+} concentration	1.008 ± 0.04	1.040 ± 0.10	0.981 ± 0.07
Relative total protein concentration	1.002 ± 0.03	0.982 ± 0.05	0.996 ± 0.02
Relative urea concentration	0.986 ± 0.03	0.959 ± 0.06	0.957 ± 0.02
Relative creatinine concentration	0.946 ± 0.02	0.000 ± 0.00[a]	0.000 ± 0.00[a]

[a] Indicates a significant difference compared with PES/PVP ($p < 0.05$).

4 h. Although this data cannot be directly extrapolated to the *in vivo* situation, a crude estimation assuming similar *in vivo* removal shows that approximately 45 g (or 0.6 m^2) MMM would be required to remove the daily creatinine production (~1800 mg), which seems to be in a realistic range.

3.4.2. Plasma cross flow measurements

For testing the removal of protein bound toxins PCS, IS and HA from spiked plasma, cross flow experiments were performed either in diffusion (Fig. 1a) or convection mode (Fig. 1b). The amount of PCS, IS and HA bound to protein was on average 90.0%, 86.6% and 38.1% respectively at the start of the experiments. In the diffusion experiments, the membrane retains albumin (relative total protein concentration in plasma after 6 h: 1.000 ± 0.02) equivalent to membranes in hemodialysis. In the convection experiments, albumin partially passes through the membrane (together with a lot of fluid) with a relative total protein concentration of 2.224 ± 0.49 in the feed and 0.306 ± 0.18 in the permeate at the end of the experiment, respectively.

Fig. 8a presents the results of toxin removal in the diffusion experiments. The removal presented there corresponds to the amount of toxin depleted from the feed plasma in time. In most cases the toxin concentration in the dialyzate was very low or below the HPLC detection limit. Therefore we can reasonably assume that the toxin removal here is mostly due to adsorption on the MMM.

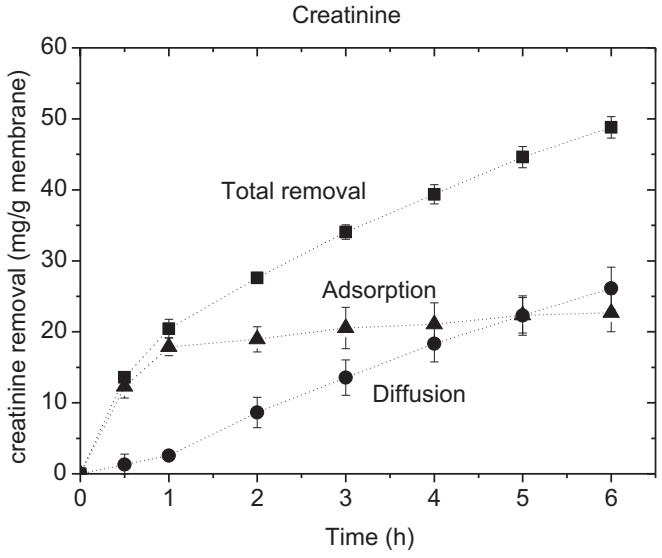

Fig. 7. Average creatinine total removal (■), diffusion (●) and adsorption (▲) plotted vs. time ($n = 3$). Total removal is the amount of creatinine removed from the feed solution. This removal is mediated by diffusion and adsorption of creatinine. Error bars indicate standard deviations. The dotted lines are plotted to guide the eye.

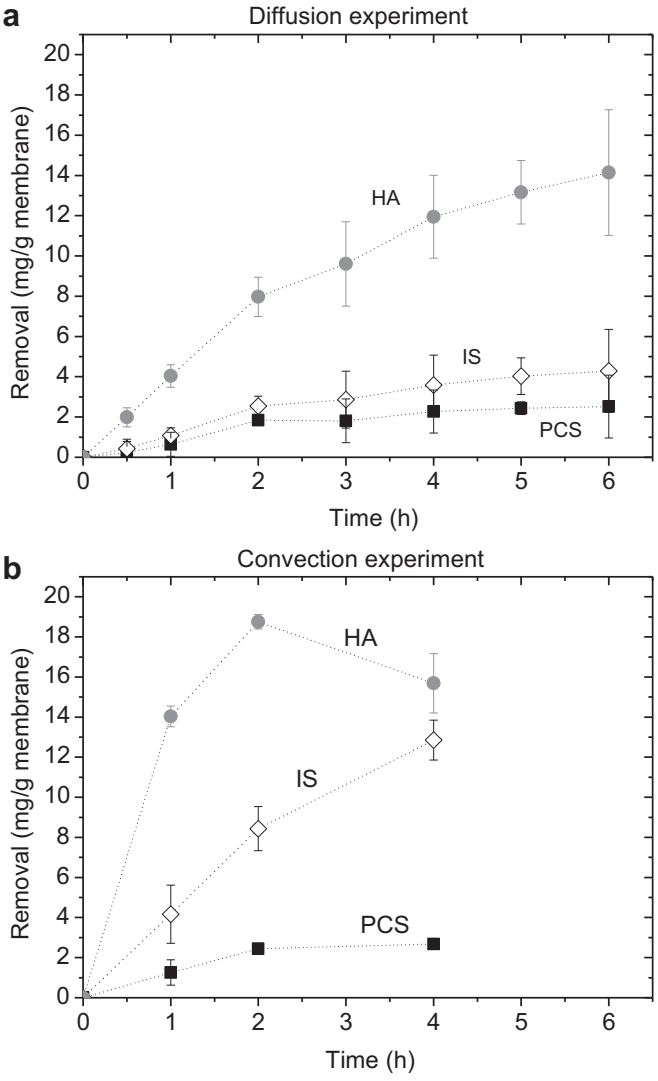

Fig. 8. Removal of p-cresylsulfate (PCS) (■), indoxyl sulfate (IS) (◇) and hippuric acid (HA) (●) plotted over the time. **a**) Diffusion experiment ($n = 3$). **b**) Convection experiment ($n = 3$). Dotted lines are plotted to guide the eye.

Fig. 8b presents the results of toxin adsorption onto the hollow fiber MMMs during the convection experiments. This was estimated based on the amount of toxin depleted from the feed plasma and from the collected permeate, based on the mass balance. In both cases, the cumulative toxin adsorption on the MMMs increases in time. The amount adsorbed in the convection experiments is higher than in the diffusion experiments, probably due to higher transport of the toxins due to the pressure difference.

The removal of these hard to remove protein bound toxins by the DL3 in diffusion and convection cross flow experiments with spiked human plasma shows the great potential of our membranes. However, direct comparisons of our work with other studies are rather difficult because of different experimental conditions. Nonetheless, a crude extrapolation can be made. Our membranes remove on average 2.27 mg PCS/g membrane and 3.58 mg IS/g membrane in 4 h, in the diffusion experiment and 2.68 mg/g membrane PCS and 12.85 mg/g membrane IS, in the convection experiment. Assuming similar removal in the *in vivo* situation, one

would need 5–35 g (0.07–0.5 m^2) MMM for daily removal of these toxins (healthy subjects excrete 78 mg PCS and 69 mg IS in their urine in 24 h [49]). Comparing to hemoperfusion columns containing around 300 g of particles, or hemodialyzers containing 1–2 m^2 membrane, these values show the good potential of our membrane.

Future research should focus on further membrane optimization: The inner layer thickness can be reduced to minimize mass transport limitations; porosity can be optimized to achieve optimal particle accessibility and to obtain a sharp cut off to avoid albumin loss; the overall particle proportion of the membrane can further increased. Besides, larger modules of dual layer hollow fiber membranes, for example comparable to hemodialysis modules in size or comparable in terms of activated carbon content to commercially available adsorptive columns, should be developed and tested for the removal of uremic retention solutes.

4. Conclusions

In this work we fabricated a dual layer hollow fiber MMM with a porous macro-void free inner membrane layer that was well attached to the MMM outer layer containing AC particles. This MMM adequately adsorbs both creatinine and difficult-to-remove protein bound toxins from human plasma solutions. Based on the dynamic experiments we estimate that our membranes would suffice to remove the daily production of these protein bound solutes.

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