**Ex vivo evaluation of the blood compatibility of mixed matrix haemodialysis membranes**

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**A B S T R A C T**

The patients with end stage kidney disease need haemodialysis therapies, using an artificial kidney. Nevertheless, the current therapies cannot remove a broad range of uremic toxins compared to the natural kidney. Adsorption therapies, using sorbent-based columns, can improve the clearance of uremic toxins, but the sorbent particles often require polymeric coatings to improve their haemocompatibility leading to mass transfer limitations and to lowering of their performance. Earlier, we have developed a dual layer Mixed Matrix fiber Membrane (MMM) based on polyether-sulfone/polyvinylpyrrolidone (PES/PVP) polymer blends. There, the sorbent activated carbon particles are embedded in the outer membrane layer for achieving higher removal whereas the inner blood contacting selective membrane layer should achieve optimal blood compatibility. In this work, we evaluate in detail the haemocompatibility of the MMM following the norm ISO 10993–4. We study two generations of MMM having different dimensions and transport characteristics; one with low flux and no albumin leakage and another with high flux but some albumin leakage. The results are compared to those of home-made PES/PVP single layer hollow fiber and to various control fibers already applied in the clinic. Our results show that the low flux MMM successfully avoids contact of blood with the activated carbon and has good haemocompatibility, comparable to membranes currently used in the clinic.

**Statement of Significance**

Haemodialysis is a life-sustaining extracorporeal treatment for renal disease, however a broad range of uremic toxins cannot still be removed. In our previous works we showed that a double layer Mixed Matrix Membrane (MMM) composed of polyethersulfone/polyvinylpyrrolidone and activated carbon can achieve higher removal of uremic toxins compared to commercial haemodialysers. In this work we evaluate the haemocompatibility profile of the MMM in order to facilitate its clinical implementation. The inner particle-free layer of the MMM successfully avoids the contact of blood with the poorly blood-compatible activated carbon. Moreover, thanks to the high amount of polyvinylpyrrolidone and to the smoothness of the lumen layer, the MMM has very good haemocompatibility, comparable to membranes currently used in the clinic.

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1. Introduction

A prominent loss of kidney function occurs in end-stage kidney disease (ESKD) patients. Currently, the only solution for patients’ survival are renal replacement therapies (RRT) and patients waiting for or not being suitable for kidney transplantation need to undergo dialysis therapy. During haemodialysis, a semi-permeable hollow fiber membrane is used for the transfer of uremic solutes from the blood compartment to the dialysis fluid compartment of the dialyser. However, the separation profile of haemodialysis membranes has to be improved in order to guarantee the removal of medium-sized and high molecular weight uremic toxins and protein-bound uremic toxins (PBUT) without the leakage of blood proteins through the membrane [1,2].

As an alternative to haemodialysis (HD), haemoperfusion (HP) is an efficient method to remove high quantities of various uremic...
toxins from blood [3]. Haemoperfusion has been proposed not only for the treatment of ESKD but also for the removal of toxins in poisonings and for the removal of cytokines in septic patients [4–8]. There, the blood can be purified by passing through a column filled with porous sorbent particles [9–11]. However, HP is not designed to correct the fluid balance and the use of small size adsorbent particles may result in high pressure drop across the column which could consequently lead to protein denaturation and/or blood cells damage. Moreover, unless additional filtration is applied, small fragments of the sorbent particles can be leached out of the column and end up in the patient's blood stream [12,13].

Activated carbon (AC) has a long lasting story of applications in blood purification via HP since it was firstly introduced in 1963 [9,13]. Nevertheless, its poor haemocompatibility is well known and haemocompatible coatings have to be applied to reduce the side effects of direct AC-blood contact. For example, Cai and co-workers developed a novel anti-foiling zwitterionic adsorbent to improve haemocompatibility of AC [14]. Sternkopf et al. studied the haemocompatibility of activated charcoal coated with polyvinylpyrrolidone for use in extracorporeal blood purification device [15]. Howell and collaborators investigated the haemocompatibility of dextran coated mesoporous AC [16]. These methods can improve the haemocompatibility profile of the AC, but often introduce mass transfer limitations which hamper AC adsorption capacity [16].

Recently, we developed the concept of double layer Mixed Matrix Membrane (MMM) based on polyethersul-fone/polyvinylpyrrolidone (PES/PVP) polymer blends [17–19]. This is characterized by an inner porous polymeric particle free layer and an outer layer composed of sorbent AC embedded in the polymeric matrix. The inner particle-free layer is responsible for the membrane selectivity and prevents contact of the blood with the AC. AC is selected as adsorptive particle because it adsorbs a wide range of solutes, including PPUT, and it has already widely studied in blood purification [9,13,20,21]. Thanks to the presence of the AC in the outer layer of the MMM, diffusion and adsorption can be combined in one step, thus improving the overall toxins removal [17–19]. In fact, due to the presence of AC in the adsorptive layer, the concentration gradient of the uremic toxins across the membrane is sharp leading to almost 2 times higher toxins removal, ex vivo, in comparison to industrial benchmark membranes [19]. Especially, the MMM is successful in removing PPUT which, although associated with cardiovascular disease, progression of ESKD and mortality [22–29], cannot be removed effectively by the current dialysis membranes [1,30]. Moreover, ex vivo removal of PPUT from blood plasma by the MMM can be combined with high removal of endotoxins from dialysate. Therefore, the MMM could also act as a safe barrier for reducing an inflammatory response within the patients at least in case of bacterial contamination of the dialysate system [31].

The objective of this work is a detailed study of the blood compatibility of the MMM in order to facilitate its clinical implementation. We perform ex vivo experiments, using freshly donated human blood, following the norm ISO 10993-4, as well as characterization of the membrane surface by means of x-ray photoelectron spectroscopy (XPS) and attenuated total reflectance-fourier transform infrared spectroscopy (ATR – FTIR). We investigate two types of MMMs, one low-flux and one high-flux membrane (indicated as LF-MMM and HF-MMM, respectively) and the results are compared to those of a single layer hollow fiber based on PES/PVP (home-made) and to two dialysers, Polysulfone® F60 and Cuprophan® F1. The Polysulfone® F60 membrane is used as negative control due to its excellent track record in clinic, while Cuprophan® F1 membrane is used as positive control because of its poor haemo-compatibility as a consequence of complement activation [32–36].

### 2. Materials and methods

#### 2.1. Membrane fabrication

We prepared dual layer high-flux MMM (HF-MMM) and low-flux MMM (LF-MMM) hollow fibers with AC particles and single layer PES/PVP hollow fiber control membrane (without particles) via dry-wet spinning technique, as described earlier [17,19]. Briefly, the polymer dope solutions were prepared by dissolving Ultrason E6020 PES (BASF, Ludwigshafen, Germany) and PVP K90 (molecular weight = 360 kDa, Sigma-Aldrich Chemie GmbH, München, Germany) in ultrapure N-methylpyrrolidone (NMP) (Acrós Organics, Geel, Belgium), Norit A Supra AC (Norit Netherlands BV, Amersfoort, The Netherlands) was used as sorbent material. It has a BET (Brunauer-Emmett-Teller) surface area of 1700 m²/g and it consists of micropores of approximately 0.5 nm (< 0.7 nm) and 0.9 nm and small mesopores of about 3 nm [37]. Prior to use, AC was sieved through 45 μm sieve and then it was added to the dope solution for preparing the outer layer of the MMMs. The concentrations of PES, PVP, and AC and the spinning parameters used in the study are specified in Table 1. All polymer solutions were mixed on a roller bench for at least 48 h, then they were transferred in stainless-steel syringes and left to degas for 24 h. Afterwards, the syringes were connected to high-pressure syringe pumps and to a double layer spinneret that allows simultaneous co-extrusion of two polymer layers [17,19]. Table 2 presents the dimensions of the two spinnerets used for the production of HF-MMM and LF-MMM. Ultrapure water was used as bore forming solution. The air-gap between the spinneret and the coagulation bath was adjusted (Table 1) and a take-up wheel was used for the collection of the produced fibers. The fabricated membranes were washed with demineralized-water and stored for further use. The single layer PES/PVP fiber was produced in the same way and with the same spinneret as the LF-MMM without the outer layer.

#### 2.2. Membrane characterization

##### 2.2.1. Scanning electron microscopy (SEM)

The morphology of the MMMs and of the PES/PVP membrane was analyzed by SEM (JEOL, Tokyo, Japan). For the imaging of the cross-sections, the membranes were dried in air and fractured in liquid nitrogen. Prior to SEM imaging, the samples were gold
Table 3  
Parameters of the membrane modules used in the study.

| Mini-modules materials | Reference name | Effective surface area (cm²) | Effective length (cm) | Number of fibers |
|------------------------|----------------|-----------------------------|----------------------|-----------------|
| High-flux MMM PES/PVP/AC | HF-MMM | 125 | 16 | 36 |
| Cuprophan® F1 Cellulose | F1 | 250 | 16 | 174 |
| Polysulfone® F60 | F60 | 250 | 14 | 284 |
| Single layer membrane PES/PVP PES/PVP | LF-MMM | 56 | 16 | 36 |
| Low-flux MMM PES/PVP/AC | LF-MMM | 58 | 16 | 36 |

sputtered using the Cressington 108 auto sputter (Cressington Scientific Instruments, Watford, UK).

2.2.2. Water transport experiments

The membranes were dried in air and membrane modules having known surface areas were used. Before water transport experiments, the membrane modules (HF-MMM n = 6, LF-MMM n = 3, PES/PVP n = 3) were pre-compacted with ultra-pure water at a trans-membrane pressure (TMP) of 2 Bar for at least 1 h. Afterwards, the amount of permeated water was measured over time at TMP of 0.5, 1, 1.5 and 2 Bar. The resulting water permeance was calculated as the slope of the linear fit of the flux (L/(m²·h)) versus the TMP (Bar).

2.2.3. Albumin leakage experiments and molecular weight cut-off (MWCO)

The protein leakage for the PES/PVP was studied in dialysis simulating experiments in vitro using human blood plasma (obtained by healthy donors in compliance with local ethical guidelines – Sanquin, Amsterdam, The Netherlands). Albumin leakage of the HF-MMM and LF-MMM were taken from literature [17,19]. The MWCO for all membranes were taken from literature [17,19,38,39], except for PES/PVP, for which the MWCO was not measured.

2.2.4. Attenuated total reflectance-fourier transform infrared spectroscopy (ATR – FTIR)

Analysis of the inner surface chemistry of the HF-MMM, LF-MMM and Polysulfone® F60 (Fresenius Medical Care, Bad Homburg, Germany) was performed by ATR-FTIR spectroscopy (Spectrum Two, PerkinElmer) and Spectrum Quant software. All scans were performed at room temperature in triplicate on various parts of the membrane surface at a resolution of 4 cm⁻¹ and were compared to FTIR scans of pure PES and pure PVP materials.

2.2.5. X-ray photoelectron spectroscopy (XPS)

XPS analysis of LF-MMM and Polysulfone® F60 membrane surface chemistry was performed using Quantera scanning XPS microprobe (Physical Electronics, Chanhassen, MN, USA) with Al Kα excitation radiation (hv = 1486.6 eV). The given elemental atomic percentages were averaged from 3 different samples. Data analysis was performed using Compass for XPS control, Multipak v 9.4.0.7.

2.3. Mini-modules preparation for haemocompatibility tests

A predefined surface area of the membranes was inserted into the modules’ housings and potted with polyurethane glue by MAT Adsorption Technologies GmbH (Obernburg, Germany). In total 5 different types of modules were prepared: 1. HF-MMM; 2. Cuprophan® F1 (3M Germany GmbH, Wuppertal, Germany); 3. Fresenius Polysulfone® F60; 4. Single layer PES/PVP membrane (PES/PVP); 5. LF-MMM. Module characteristics can be found in the Table 3.

2.4. Haemocompatibility tests

The haemocompatibility of the tested modules was assessed according to ISO 10993–4 and following standard operation protocols, developed by eXcorLab GmbH (Industrie Center Obernburg, Obernburg, Germany). All experiments were performed on a miniaturized set-up with freshly donated human blood. Blood flow rate was linearly downsized from a membrane surface area used clinically (1.5 m² and blood flow rate \( Q_b = 300 \) mL/min to 250 cm² of the respective Cuprophan® mini-module), resulting in an experimental blood flow rate of 5 mL/min. As a preparation step, all modules were rinsed with saline solution in single pass (30 min with flow rates of 5 mL/min). At the start of the experiment, saline was replaced by heparinized (5 IU/mL) human blood (20 mL), which was recirculated in each circuit for 180 min with intraluminal flow (5 mL/min). During the experiment, the modules were kept in a water bath at 37 °C, whereas the medium reservoirs were kept at room temperature on a shaker to avoid sedimentation of blood cells. Blood samples were collected at time 0, 30, 120 and 180 min. Total leukocytes (WBC), red blood cells (RBC) and platelets (PLT) counts were measured at each experimental time point. Thrombin-antithrombin III complex (TAT) (as indication of the activation of the coagulation pathway), C5a (as indication for complement activation), and haemolysis were measured at the start and at the end of the experiment (180 min). The pressures at the inlet and outlet of the modules were recorded with a multi-channel pressure transducer (DPT-6300, Codan pvb Medical GmbH, Forstinning, Germany) and using a laboratory data acquisition system (MSR-manager, HITEC Zang, Herzogenrath, Germany). WBC, RBC and PLT were counted in an ABX Pentra 60 counter (Axon Lab AG, Reichenbach an der Fils, Germany). C5a and TAT were determined by ELISA (DRG Diagnostics, Marburg, Germany; Roche, Mannheim, Germany). Haemolysis was measured by photometry of plasma samples using three wavelengths to correct for (mainly bilirubin) background (OD560nm, OD576nm, OD592nm, photometer UV1650PC, Shimadzu Deutschland GmbH). The following equation was used to calculate haemolysis (plasma free [Hb]) [40]:

\[
[Hb] = \frac{(2OD_{576} - (OD_{560nm} + OD_{592nm})) \times 99.82 + 0.36}{1000}
\]

Total [Hb] at the start of the experiment was measured after complete red cell lysis to express haemolysis of %. Each mini-module experiment was repeated three times with different donors.

2.5. Statistics

All the data are presented as mean ± SD (standard deviation). Statistical analyses were performed using GraphPad Prism version 5.02 (GraphPad Prism Software, La Jolla, CA, USA). Multiple comparisons between different groups were performed using one-way ANOVA with Bonferroni post-hoc test (p < 0.05) in order to determine statistical differences for the pilot experiment (performed to assess the effect of the wall shear stress on C5a and TAT generation) and for the haemocompatibility test.

3. Results and discussion

3.1. Membrane characterization

3.1.1. Membrane morphology

The morphology of the dual layer HF-MMM, LF-MMM and single layer PES/PVP hollow fiber was investigated using SEM imaging (Fig. 1). The LF-MMM [19,31] (Fig. 1B, E, H, K) is a double layer membrane composed by an inner particle-free PES/PVP layer and an outer layer where AC particles are embedded in the PES/PVP polymer matrix (some of the AC are highlighted with white arrows
in Fig. 1E). In order to guarantee fast diffusion and high adsorption, LF-MMM has a thinner inner layer compared to the outer layer. The two layers are well interconnected and AC particles are well dispersed in the outer layer without aggregation. The membrane has anisotropic morphology, with the selective layer at the inner lumen side of the membrane, in contact with blood, and with finger-like macrovoids throughout the membrane wall (Fig. 1E). The selective layer, responsible for transport selectivity via size exclusion, has lower porosity, with a thickness of approximately 1 μm (Fig. 1H) and no visible pores at the lumen surface (Fig. 1K). Given its unique morphology, LF-MMM can guarantee high adsorption of uremic toxins and, thanks to the inner selective layer, good transport properties without leakage of albumin [19] but also barrier properties to avoid transfer of pyrogens to the blood compartment in case of contamination of the dialysis fluid [31].

We produced PES/PVP single layer hollow fiber membrane (Fig. 1C, F, I, L) using the same spinning parameters as for the inner particle-free layer of the LF-MMM. PES/PVP hollow fiber presents similar morphology as the inner layer of the LF-MMM, having finger-like macrovoids (Fig. 1C and 1F), comparable selective layer (approximately 1 μm) at the blood-contacting side of the membrane (Fig. 1I) and no visible pores at the inner surface as LF-MMM (Fig. 1L).

The HF-MMM [17] (Fig. 1A, D, G, J), is a double-layer MMM having, as the LF-MMM, an inner particle-free PES/PVP layer in contact with blood and an outer layer with AC embedded in the polymer matrix (some of the AC are highlighted in Fig. 1D with white arrows). Its selective layer is also present at the lumen side of the fiber and the two layers are well interconnected (Fig. 1D and 1G). Nevertheless, important morphological differences can be observed in comparison to LF-MMM. The HF-MMM [17] has finger-like macrovoids at the outer layer while the inner layer has spongy structure and, since it was manufactured with a different spinneret (Table 2), it has larger dimensions (Figs. 1A and 1D) (Table 4). Another important difference to LF-MMM is that the selective layer of HF-MMM is very thin (Fig. 1G) with visible pores at the lumen surface (Fig. 1J).

The SEM images of Cuprophan® F1 and Polysulfone® F60 (kindly provided by Prof. Dr. J. Vienken) are presented in the Supplementary Information. The dimensions of all membranes are summarized in Table 4.

### 3.1.2. Membrane transport experiments

The ultrafiltration coefficients (KUF) of the produced LF-MMM and PES/PVP membranes were measured and compared to the KUF of HF-MMM, Cuprophan® F1 and Polysulfone® F60 control fibers. The KUF of LF-MMM and PES/PVP is $4 \pm 1 \text{ mL/(h-mmHg-m}^2\text{)}$ and

![Fig. 1. SEM images of the dual layer HF-MMM (A, D, G, J), LF-MMM (B, E, H, K) and PES/PVP membrane (C, F, I, L). Overall cross-sections: A, B, C; magnification of the wall: D, E, F; magnification of the inner selective layer: G, H, I; magnification of the inner surface: J, K, L. White arrows highlight some of the AC in Fig. 1D and 1E.](image-url)
membrane, pure PES and pure PVP. The peak at 1677 cm\(^{-1}\), corresponding to the carbonyl groups of PVP, has noticeably higher intensity for HF-MMM and LF-MMM in comparison to the Polysulfone\(^\circledR\) F60, indicating higher amount of PVP at the lumen surface of the two MMMs. PES/PVP blends were used for the production of HF-MMM and LF-MMM and therefore the characteristic FTIR peaks of the aromatic bonds of PES, at 1486 cm\(^{-1}\) and 1578 cm\(^{-1}\) [43, 44], can be identified in Fig. 2a. The F60 is a polysulfone-based membrane, as confirmed also by the peaks at 1488 cm\(^{-1}\) and 1586 cm\(^{-1}\) which are assigned to the aromatic bonds of polysulfone. Moreover, the weak bands at 1386 cm\(^{-1}\) and 1364 cm\(^{-1}\) are typical of methyl groups, exclusively present in the spectrum of polysulfone [43,44]. Fig. 2b shows that nitrogen elemental molar percentage (exclusively part of PVP) is higher at the inner surface of LF-MMM (5%) compared to Polysulfone\(^\circledR\) F60 (4%), consistent to the findings of ATR-FTIR. The carbon content is higher for the F60 compared to LF-MMM. This is due to the presence of the methyl groups in the backbone of polysulfone. Furthermore, as expected, the sulphur content is higher for LF-MMM because of the higher amount of sulfonoyl groups in the polymer chain of PES compared to polysulfone.

For the production of HF-MMM and LF-MMM, we used PES as membrane forming polymer which has thermal stability, mechanical strength, chemical inertness and it can withstand all sterilization techniques [36,45]. However, its hydrophobic nature favours proteins adhesion, which not only affects membrane performance but can also trigger a series of other reactions such as activation of the coagulation cascade, platelets and red blood cells adhesion with risk of blood clotting and complement and fibrinolysis reactions [36,45,46]. The incorporation of hydrophilic additives to the PES membrane guarantees, thanks to the formation of a hydration layer, a reduction of proteins adhesion [36,46,47]. Moreover, it is well known that membranes having a combination of hydrophilic and hydrophobic domains, rather than a uniformly hydrophilic or hydrophobic composition, have advantages with respect to biocompatibility [36,48]. In the membrane-forming dope

10 ± 1 mL/(h-mmHg-m\(^2\)), respectively, and therefore both can be classified as low-flux dialysis membranes [2]. Also, the stability of operation under transmembrane pressure up to 2 Bar was studied and revealed linear fit of the water flux versus pressure. The difference in the KUF of the two fibers could be due to the thinner selective layer and/or to higher surface porosity of the PES/PVP hollow fiber which cannot be appreciated by SEM imaging. It is important to highlight that in our previous work [31] we have shown that the water permeability of the LF-MMM can be improved by washing the membrane with pure ethanol, still maintaining retention properties of the selective layer against plasma proteins (only 0.6 ± 0.1% plasma proteins leaked to the dialysate compartment at 4 h during dialysis experiment compared to the initial amount of proteins in the blood plasma). Cuprophan\(^\circledR\) F1 has comparable KUF as the LF-MMM [41], whereas Polysulfone\(^\circledR\) F60 is a high-flux dialysis membrane [2,42]. The HF-MMM has a KUF equal to 76 ± 12 mL/(h-mmHg-m\(^2\)) and, differently from all the other fibers in this work, has albumin leakage [2,17]. Table 5 summarizes the membrane transport properties.

### 3.1.3. Surface characterization

The membrane lumen surface characterization was performed by means of ATR - FTIR and XPS measurements. Fig. 2a compares the ATR - FTIR spectra of LF-MMM, HF-MMM, Polysulfone\(^\circledR\) F60

| Ultrafiltration coefficient (KUF) | MWCO (KDa) | Albumin leakage |
|----------------------------------|------------|-----------------|
| HF-MMM                           | 76 ± 12 [17] | > 67 [17]       | YES [17]     |
| Cuprophan\(^\circledR\) F1        | 6 [41]     | 10 [38]         | NO [38]      |
| Polysulfone\(^\circledR\) F60     | 24 [42]    | 30 [39]         | NO [39]      |
| PES/PVP                          | 10 ± 1     | Not measured    | NO            |
| LF-MMM                           | 4 ± 1      | 12 [19]         | NO [19]      |

Fig. 2. a) ATR-FTIR results of LF-MMM and comparison with HF-MMM, Polysulfone\(^\circledR\) F60, pure PES and pure PVP material. b) XPS results of LF-MMM and comparison with Polysulfone\(^\circledR\) F60 commercial fiber.
Table 6

| Equation used for the calculations of the wall shear stress. |
|------------------------------------------------------------|
| \( \dot{v} = \frac{4}{\pi} \frac{Q_s}{d_i^n n} \) | \( w = \frac{8 \cdot v}{d_m} \) |
| \( Q_s = \) blood flow                                      |                              |
| \( d_i = \) inner diameter of the fiber                    |                              |
| \( n = \) number of fibers                                  |                              |

solutions of HF-MMM and LF-MMM, PES blends with PVP thanks to the strong donor-acceptor interaction between O=CN functional groups from PVP and O = S = O from the benzene ring of PES [45]. PVP is a non-ionic, highly polar, amphiphilic, physiologically inert water-soluble polymer which acts as hydrophilic agent [45], thus preventing adsorption and deposition of plasma proteins. Even though blending is the most widely used method in industry, many researchers believe that PVP does not form miscible blends with different grades of PES, and, as a consequence, PVP would be leached out during membrane formation and over time [45,46]. In this work, we used PVP K-90 for the production of both MMMs. PVP K-90 has been shown to be the most hydrophilic, highly negatively charged, with the lowest fouling behaviour and lowest tendency to leach out from the membrane compared to PVP with lower molecular weights [45]. The ATR-FTIR and XPS results (Fig. 2) show that, in our case, PVP is still present at the lumen surface of both MMMs and could contribute to low interaction with blood plasma proteins and low membrane fouling. Besides, during the haemocompatibility experiments (Paragraph 3.2.2) the pressure in the inlet and outlet was monitored and there was no indication of changes suggesting membrane fouling (data are provided in Supplementary Information). Moreover, in preliminary 4 h dialysis simulating experiments, we estimated that the amount of plasma proteins adsorbed on the LF-MMM and PES/PVP was very low (approximately 3% (n = 3) and 0.5% (n = 3) of the starting amount of plasma proteins, respectively). These data were obtained using a miniaturized set-up in diffusion mode (TMP = 0 Bar) and applying blood plasma and dialysate flow rates of 1 mL/min and 10 mL/min, respectively. These flow rates are much lower compared to those used in clinic, which are in the range of 300–500 mL/min for the blood flow rate and 500–800 mL/min for the dialysate flow rate [2,49]. The use of such low blood and dialysate experimental flow rates was dictated by the fact that they allowed to maintain the TMP equal to 0 Bar (diffusion mode) with our experimental set-up during the simulating haemodialysis experiment. It is possible that the protein amount missing from the system is due not only to proteins adsorption on the membrane surface but also to possible concentration polarization phenomena at the membrane surface. In fact, at low flow rates, retained blood proteins concentrate preferably at the lumen surface of the membrane instead of being well dispersed in the flowing blood and the measured concentration of the proteins in the blood can be therefore lower.

3.2. Haemocompatibility study

3.2.1. Mini-modules preparation and rheology

The geometries (inner diameter and wall thickness) of the fibers used in this work are very different from each other and, moreover, the mini-modules differ in effective membrane surface area, effective fibers length and number of fibers in the modules (see Table 3). As a result, the rheological parameters for the experimental and reference fibers differ from each other. Table 6 presents the equations used for the calculation of the wall shear stress, Table 7 presents the estimated values of the wall shear stress for all fibers; other rheological parameters are reported in the Supplementary Information. It is well known that geometrical differences influence flow conditions inside the mini-modules and, as a consequence, the haemocompatibility results. [32,45]. Especially, the wall shear stress (defined as the tangential force that is exerted by the flowing fluid on the surface of the hollow fiber) is an important parameter affecting the interaction of the blood cells with the membrane; high wall shear stress can, on one hand, reduce protein deposition, but on the other hand, it can induce rupture of red blood cells [50,51]. Since, in our study, the wall shear stress in Polysulfone® F60 and Cuprophan® F1 fibers is roughly 5 and 8 times, respectively, higher compared to HF-MMM and that in LF-MMM and PES/PVP is even higher (Table 7), we first performed a pilot experiment (Table 8) to evaluate the effect of wall shear stress on complement activation (C5a generation) and activation of coagulation pathway (TAT generation).

We used two different blood flow rates, i. e. 0.5 mL/min and 5 mL/min, to obtain wall shear stress values of ~ 60 s⁻¹ and ~ 600 s⁻¹, respectively. As expected, the generation of C5a was higher for Cuprophan® F1 mini-modules compared to Polysulfone® F60. Importantly, the difference in the wall shear stresses applied in this pilot experiment is not large enough to have an impact on the generation of the C5a. The generation of TAT was higher for Polysulfone® F60 compared to Cuprophan® F1, due to the more hydrophobic nature of the polysulfone-based membrane. As for C5a generation, when comparing TAT generation at different wall shear stress values, we could not observe statistically differences depending on the blood flow rate. This indicates that in our set-up differences in fibers geometry, mini-modules design and, in particular, in wall shear stress did not affect the C5a generation and generation of TAT (Table 8). Complement (via C5a generation) and coagulation pathway (via TAT-generation) were chosen to study the effect of the rheological parameters (represented by the wall shear stress) on the haemocompatibility profile [52]. And, therefore, we decided to proceed with the haemocompatibility experiments of all membranes.

3.2.2. Haemocompatibility tests

The red blood cells (RBC), white blood cells (WBC) and platelets counts, activation of coagulation, activation of the complement system and haemolysis are important parameters for the assessment of the haemocompatibility profile of medical devices [52]. Here, we investigated these parameters according to ISO 10993-4 after 3 h of blood contact with all membranes.

3.2.2.1. RBC count and haemolysis

The damage of erythrocytes can lead to reduced oxygen transport to tissues and organs in vivo and microvesicles derived from erythrocytes can promote thrombus formation [52]. The shear stress typical of circulatory assist devices affects the mechanical properties of RBC, subsequently impairing their deformability and leading to mechanical fragility [50]. In flowing blood, RBC are stretched to ellipsoids and further stretching imposed by high shear stress conditions would eventually lead to their rupture [51]. The roughness of the contact surface is also a key factor able to trigger their rupture [53]. In fact, there is a direct correlation of RBC rupture to surface roughness [53]. Haemolysis is the consequence of red blood cell lysis leading to haemoglobin release into the bloodstream, thus an increased concentration of free haemoglobin in the plasma is a direct indicator of erythrocytes destruction [51,52].

In our experiment we observed a significant drop of RBC at 3 h compared to 0 h only for the HF-MMM (Fig. 3a). Moreover, the level of induced haemolysis of HF-MMM (Fig. 3f) was higher compared to the other fibers, consistent with the RBC count results. Since the wall shear stress for HF-MMM is considerably lower compared to the other fibers (Table 7), the significant drop of RBC is not expected to be related to high shear stress conditions. Instead, it is probably due to the roughness of the lumen surface.
(Fig. 1) which entraps or damages the small RBC. Moreover, based on the FTIR results (Fig. 2a), there is no difference in the amount of PVP at the lumen surface between HF-MMM and LF-MMM, suggesting that the drop of RBC for the HF-MMM cannot be due to chemical adsorption/interaction of the RBC with the lumen surface. Importantly, Fig. 3f shows that some haemolysis was observed for all tested membranes at 3 h (significantly different compared to 0 h) but it was below 0.8% which compares well with the accepted limits for red cells concentrates stored for 42 days in blood banks in Europe (0.8%) [54] and in USA (1%) [55]. Moreover, haemolysis less than 5% is considered as no-toxic according to ASTM F-756-08 standard [56]. Comparable haemolysis results were obtained from our group for flat sheet membranes fabricated with the same blend of PES/PVP as for the PES/PVP hollow fiber and the inner selective layer of the LF-MMM [57]. Frank and co-workers measured the RBC count after two hours of ex-vivo blood test performed on LF-MMM, PES/PVP, HF-MMM, Cuprophan® F1 and Polysulfone® F60. a) Red blood cells count (RBC,%); b) White blood cells count (WBC,%); c) Platelets count (%); d) Thrombin-antithrombin III complex (TAT) generation (μg/L); e) C5a generation (μg/L); f) Haemolysis (%). "b" denotes significant difference versus HF-MMM (p < 0.05); “c” denotes significant difference versus Cuprophan® F1 (p < 0.05); “d” denotes significant difference versus Polysulfone® F60 (p < 0.05); “∗” denotes significant difference versus the same fiber at time 0 h.
recirculation with commercial membranes [58]. In accordance with our results, they did not observe statistical drop in the RBC count when using Polysulfone® and Cuprophan® dialysers [58]. Also, no significant effects on the RBC were observed by Luo et al. on 17 patients treated with polysulfone membranes [59]. Medina et al. reported destructive influence of HD on RBC and thus evidence of HD contributing to anaemia in patients receiving long-term HD with cellulose acetate membrane [60]. The latter results can be related to the poor biocompatibility of cellulose-based membranes, resulting in the production of free radicals and reactive oxidant species that can damage RBC and, ultimately, lead to microhaemolysis [59].

3.2.2.2. WBC count. A drop in WBC count (called leucopenia) is normally observed in clinical HD and indicates adhesion of leukocyte cells to the vessel walls of the lungs in patients and to the dialysis membrane [32,61]. HD-induced leucopenia is the result of many complex mechanisms cross-talking to each other. On one hand, leucopenia is induced by the activation of the complement system which induces the overexpression of the leukocyte receptors CD11b/CD18 and CD15s. As a consequence, WBC adhere and aggregate to the membrane surface [62]. On the other hand, the adsorption of plasma proteins on the membrane surface triggers the adhesion and aggregation of platelets, which activate the WBC [62]. The latter can result in the up-regulation of adhesive molecules on the cell surface. Moreover, activated cells are more prone to undergo apoptosis, which may ultimately lead to leucopenia [36]. As expected, a decrease of WBC was observed for all the fibers at 3 h, but statistical difference compared to the WBC at 0 h was obtained only for HF-MMM, Cuprophan® F1 and Polysulfone® F60 (Fig. 3b). For the LF-MMM and PES/PVP fibers, the smaller surface area, the shorter blood residence time in the fibers (Supplementary Information) and the high amount of PVP at the lumen surface (Fig. 2), may explain the lower adsorption of the WBC in comparison to the control fibers. The highest drop of WBC at 3 h was observed for HF-MMM and Polysulfone® F60 (Fig. 3b). Given the similar surface lumen chemistry of HF-MMM to LF-MMM (Fig. 2), it is unlikely that the WBC drop is due to the adsorption on the lumen surface; however, the roughness of the inner surface (Fig. 1) might be responsible for entrapment of the WBC, as for the RBC. Besides, the more hydrophilic lumen surface of the Polysulfone® F60 (Fig. 2) is expected to have higher protein adsorption and, consequently, higher WBC deposition. Frank et al. did not observe a significant drop of WBC count after recirculation of human blood in ex-vivo experiments using Polysulfone® and Cuprophan® membranes [58]. Other studies focusing on the inflammatory state of HD patients treated with polysulfone membranes showed that these hydrophobic membranes can cause neutrophil activation which can accelerate apoptosis [36,63].

3.2.2.3. Platelets count. Evaluation of the platelets count after contact with blood is a common parameter to investigate the potential of the material to trigger activation of the coagulation cascade [64,65]. High loss of platelets indicates adhesion of platelets on the membrane surface. The first event responsible for the adhesion and aggregation of platelets on the membrane surface is blood protein adhesion [52]. Fibrinogen has been shown to be the key protein in the adhesion process, while other proteins such as von Willebrand factor, immunoglobulins, vitronectin and fibronectin have only supporting effects that may be related to platelets activation [62]. After adhesion, activated platelets accelerate the formation of thrombin [62]. We observed a significant decrease of platelets count for all fibers at 3 h compared to the start of the experiment (Fig. 3c). As for the WBC, the highest drop of platelets occurred for HF-MMM and Polysulfone® F60 (Fig. 3c), probably due to the rough and porous lumen surface of the HF-MMM (Fig. 1, which could entrap platelets) and to the hydrophobic character of Polysulfone® F60 (Fig. 2, which represents favourable proteins and, consequently, platelets adsorption sites). It has actually been reported that for polysulfone membranes the platelets adhesion is associated and is proportional to proteins adsorption on the membrane surface [66,67] and that platelets count was lower for Polysulfone® compared to Cuprophan® membranes after blood recirculation [58]. Togo et al. [68] measured the platelets count after blood recirculation using two different types of polysulfone hollow fibers. They found that after 4 h the platelets count was approximately 40% lower compared to the start of the experiment. The results of these studies are consistent to our findings [68].

3.2.2.4. Thrombin generation. The interaction of plasma proteins with artificial surfaces triggers the coagulation pathway by contact activation which consists of a series of reactions leading to the conversion of factor X into factor IXa able to convert prothrombin to thrombin. Thrombin is the final enzyme in the coagulation cascade. It cleaves fibrinogen into fibrin monomers which can then polymerize and cross-link to form a fibrous mesh [62,65]. Antithrombin III inhibits thrombin by forming a thrombin-antithrombin III (TAT) complex. This complex reflects a functional state of the coagulation system and can be quantified to measure the amount of thrombin in the blood [52].

In this work, we found statistically higher amount of TAT only for HF-MMM at 3 h compared to 0 h (Fig. 3d). This is probably due to the interaction of blood plasma proteins with the AC particles since the selective layer of the HF-MMM has rather high porosity (Fig. 1) and albumin leakage. Proteins adsorb on the AC due to its hydrophobic nature resulting in the activation of the coagulation pathway, which leads to the conversion of prothrombin into thrombin (detected in this work via the measurement of TAT complex) [52]. The TAT generation by LF-MMM is not statistically different to the amount of TAT at the start of the experiment and to PES/PVP fiber at 3 h (Fig. 3d). This finding highlights that the lumen surface of the LF-MMM does not induce coagulation cascade activation itself and indicates that the inner layer of the LF-MMM could act as safe barrier to avoid the contact of blood.

| Membrane/Filter | Flow velocity in one fiber (cm/min) | Wall shear stress (s⁻¹) | CSa (µg/L) | TAT (µg/L) |
|-----------------|-----------------------------------|------------------------|-----------|-----------|
| Cuprophan® F1   | 0.5 mL/min                        | 60 s⁻¹                 | 77 ± 10   | 10 ± 4    |
| Cuprophan® F1   | 5 mL/min                          | 600 s⁻¹                | 73 ± 17   | 7 ± 1     |
| Polysulfone® F60| 0.5 mL/min                        | 60 s⁻¹                 | 6 ± 2     | 39 ± 24   |
| Polysulfone® F60| 5 mL/min                          | 600 s⁻¹                | 5 ± 2     | 25 ± 12   |
components with the AC present in the outer layer. Frank et al. observed that Cuprophan® and Polysulfone® membranes induce TAT generation in the same order of magnitude as the values obtained in our case for the commercial fibers, thus confirming the validity of our results [58].

3.2.2.5. Complement system activation. The complement system is a key component of the innate immune system, which can be activated in case of invading microorganisms and when blood contacts with artificial surfaces [52,69]. Complement activation during HD occurs via the alternative pathway [52,65,70]. The final step in the complement cascade is the formation of the membrane attack complex (MAC, C5b9) which leads to cell lysis [62]. Besides the formation of the MAC complex, the anaphylotoxins C3a and C5a are generated. C3a and C5a are potent agents capable of producing intense inflammation reactions as vascular smooth muscles contraction [65] and migration of activated neutrophils to the lungs [70]. This latter effect results in mild pulmonary dysfunction during haemodialysis, particularly if dialyse containing acetate is used [70].

Here, we evaluated the activation of the complement system by measuring the generation of C5a fragments in the blood [48,52] (Fig. 3e). We observed an increase of C5a generation at 3 h for all the fibers compared to 0 h; importantly, the LF-MMM induces low activation of the complement system similarly to that induced by Polysulfone®F60 and PES/PVP (Fig. 3e). As expected, the generation of C5a with Cuprophan® F1 is statistically higher compared to all other fibers (Fig. 3e). This well-known property of cellulose-based membranes to activate the complement system is due to the presence of free hydroxyl groups [36,65]. Similarly, in in vitro blood compatibility experiments using flat sheet membranes we observed an increased activation of the complement system with cellulose membranes compared to PES and PES/PVP membranes [57]. Moreover, Frank et al. reported that the C5a concentration during ex vivo recirculation with Cuprophan® was increased at a high extent, while only low level of C5a activation was detectable following recirculation with the Polysulfone® [58]. Also, Uhlenbush-Körwer observed higher C5a generation for Cuprophan® membranes compared to Polysulfone® membrane [71].

The generation of C5a for the HF-MMM at 3 h is significantly different to that at 0 h. Due to the high porosity of the selective layer of HF-MMM (MWCO > 66.5 KDa) [17], C5a (approximately 13 KDa) could reach the AC on the outer surface. We do not know if the amount of C5a measured for the HF-MMM derives only from C5a generation or if it is the result of generation and adsorption on the AC; further experiments should elucidate this point. In other studies it has been observed that the concentration of the low molecular weight protein C5a in the blood represents the net result of the simultaneous processes of generation and any dialytic removal (diffusion, convection, adsorption) that may occur [48,72,73]. For example, PAN (polyacrylonitrile) membranes have high adsorptive capacity for complement fragments. Thus, the net effect is only a modest elevation of these products systemically [48, 65]. In another work it was observed that the peak C3a concentration obtained with large pore cellulose acetate membrane was significantly less than that obtained with its smaller pore counterpart and was not significantly different from that of low-flux polysulfone [48].

3.2.2.6. Overall haemocompatibility assessment. In summary, LF-MMM has haemocompatibility profile similar to that of fibers currently used in clinics. The PVP-enriched lumen surface which prevents proteins adsorption, the smooth lumen surface and, especially, the lumen selective layer which protects the blood to contact the AC in the outer layer, are responsible for the good haemocompatibility of the LF-MMM. Moreover, the morphology of the LF-MMM guarantees high removal of uremic toxins, especially protein-blood toxins [19,31]. In the case of HF-MMM, there is activation of the coagulation pathway due to plasma protein leakage across the selective inner layer and the interaction with the AC. Furthermore, the reduction of red and white cells and platelets observed for the HF-MMM is attributable to the high surface roughness of its inner layer.

4. Conclusion and outlook

In this work, we studied the haemocompatibility profile of two Mixed Matrix Membranes (M3M), one with low flux properties and no albumin leakage (LF-MMM) and another characterized by high flux and albumin leakage (HF-MMM). The surface characterization of the lumen of the LF-MMM shows that the high amount of polyvinylpyrrolidone and the smoothness of the lumen blood-contacting surface limit adsorption of blood proteins and adsorption and/or entrapment of blood cells. Importantly, we consider that the good haemocompatibility performance of LF-MMM compared to the HF-MMM is also due to the protective selective layer of the LF-MMM which represents a safe barrier to prevent leakage of plasma proteins and contact of blood with activated carbon.

As a next step, we aim to upscale the production of the LF-MMM and test its performance in terms of toxins removal and haemocompatibility profile using full blood and dialysate flow rates usually applied in clinics.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Disclosure

The authors declare no conflict of interest.

Author contributions

I. G., D. P. and K. M. performed literature search, experimental design, data collection, data analysis and manuscript writing. M. R. performed part of the experiments and contributed to the experimental design. H. D. L. and D. S. contributed to experimental design, data interpretation and manuscript writing.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2020.05.016.

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