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Development and characterization of modified polysulfone hemodialysis membranes by means of immobilized neutrophil elastase inhibitors.

Diploma thesis

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Hradec Králové, 2021 ……...

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ABSTRACT

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Title of diploma thesis: **Development and characterization of modified polysulfone hemodialysis membranes by means of immobilized neutrophil elastase inhibitors.**

Chronic kidney disease (CKD) is a major health and financial burden, mainly because of the costly renal replacement therapy and treatment associated. The last stage, end-stage renal disease, is associated with high morbidity and mortality rate, generally due to cardiovascular complications. Chronic inflammation is frequently present in CKD patients, which is enhanced by the long term intra-dialytic recurrent contact between blood and hemodialysis (HD) membrane and further contributes to development of atherosclerosis. Contact with the artificial material of HD membranes leads to oxidative stress and neutrophil activation with release of neutrophil serine proteases such as human neutrophil elastase. Patients on HD often present increased level of free neutrophil elastase, decreased intracellular level, and decreased amount of endogenous inhibitor. Development of modified HD membranes by adsorption or incorporation of exogenous synthetic human neutrophil elastase inhibitors (HNEIs) is an approach that might be used to reduce free elastase in blood through contact with the membrane during the HD session. In this thesis we proved that it is possible to produce HNEI-modified polysulfone membranes by coating (adsorption) method with sufficient inhibitory activity to reduce elastase activity in plasma. These biomaterials' biocompatibility showed to be comparable to non-modified HD membranes which is required for applicability as medical devices.

keywords: chronic kidney disease, hemodialysis membranes, neutrophil elastase inhibitors

ABSTRAKT

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Název diplomové práce: **Vývoj a charakterizace polysulfonových hemodialyzačních membrán modifikovaných inhibitory neutrofilní elastázy.**

Chronické onemocnění ledvin představuje velkou zdravotní a finanční zátěž, hlavně z důvodu nákladné renální substituční terapie a přidružené léčby. Jeho poslední stádium je spojeno s vysokou morbiditou a mortalitou, což je obvykle zapříčiněno kardiovaskulárními komplikacemi. U pacientů s chronickým onemocněním ledvin je často přítomen chronický zánět, který je zesílen dlouhodobým opakujícím se intra-dialytickým kontaktem mezi krví a hemodialyzační (HD) membránou a dále přispívá k rozvoji aterosklerózy. Kontakt s umělým materiálem HD membrán vede k oxidačnímu stresu a aktivaci neutrofilů uvolňujících neutrofilní serinové proteázy, jako například lidskou neutrofilní elastázu. Pacienti na hemodialýze často vykazují zvýšenou hladinu volné neutrofilní elastázy, sníženou intracelulární hladinu a snížené množství endogenního inhibitoru. Vývoj modifikovaných HD membrán adsorpcí nebo inkorporací exogenních syntetických inhibitorů lidské neutrofilní elastázy je přístup, který by mohl být využit ke snížení volné elastázy v krvi kontaktem s membránou během HD sezení. V této práci jsme dokázali, že je možné vyrobit polysulfonové membrány modifikované inhibitory lidské neutrofilní elastázy metodou potahováním (adsorpcí), s dostatečnou inhibiční aktivitou na snížení aktivity elastázy v plazmě. Biokompatibilita těchto materiálů je srovnatelná s nemodifikovanými HD membránami, což je vyžadováno pro jejich použitelnost jako zdravotnické prostředky.

klíčová slova: chronické onemocnění ledvin, hemodializační membrány, inhibitory neutrofilní elastázy

List of abbreviations

AAT	α1-antitrypsin
APC	allophycocyanin
APD	automated peritoneal dialysis
CAPD	continuous ambulatory peritoneal dialysis
CG	cathepsin G
CKD	chronic kidney disease
CRP	C-reactive protein
CVDs	cardiovascular diseases
DMSO	dimethyl sulfoxide
EAA	elastase activity assay
ELANE	elastase, neutrophil-expressed
EPO	erythropoietin
ESA	erythropoiesis stimulating agent
ESRD	end-stage renal disease
GFR	glomerular filtration rate
Hb	hemoglobin
HD	hemodialysis
HNE	human neutrophil elastase
HNEI	human neutrophil elastase inhibitor
IC50	half maximal inhibitory concentration
IL	interleukin
ISO	the International Organization for Standardization
KoA	mass transfer-area coefficient
KUF	ultrafiltration coefficient
LDL	low-density lipoprotein
mRNA	messenger ribonucleic acid
MW	molecular weight
MWCO	molecular weight cut-off
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
NMP	*N*-methyl-2-pyrrolidone
NO	nitric oxide
NSP	neutrophil serine protease
PBS	phosphate-buffered saline
PE	phycoerythrin
PES	polyethersulfone
PD	peritoneal dialysis
PLT	platelet
PMA	phorbol-12-myristate-13-acetate
PR3	proteinase 3
PSf	polysulfone
PTH	parathyroid hormone
PVP	polyvinylpyrrolidone
RBCs	red blood cells
rpm	revolutions per minute
ROS	reactive oxygen species
RRT	renal replacement therapy
SEM	scanning electron microscopy
SEM	standard error of the mean
SIV	sivelestat
uH_2O	ultrapure water
VEM	vitamin E-coated membrane

1. Introduction

The experimental part of this thesis was executed as a part of a project called "Dial4Life: Design of dialysis membranes targeting neutrophil elastase to reduce inflammation/oxidative stress in end-stage renal disease." co-financed by FCT/MCTES (PTDC/MEC-CAR/31322/2017) and FEDER/COMPETE 2020 (POCI-01-0145-FEDER-031322), of which prof. Conceição Branco, Ph.D. is the principal investigator. Development of new types of membranes was carried out in Applied Chemistry Laboratory and Biochemistry Laboratory, Faculty of Pharmacy, University of Porto. A new neutrophil elastase inhibitor from their library of 4-oxo-β-lactam molecules was designed and synthetized by the group of Medicinal Chemistry, Faculty of Pharmacy, University of Lisbon for this purpose.

End-stage renal disease, as a last stage of chronic kidney disease (CKD), requires treatment in terms of renal replacement therapy, most commonly hemodialysis. Long term intra-dialytic recurrent contact of hemodialysis membrane with blood leads to production of reactive oxygen species and degranulation of neutrophils with release of neutrophil serine proteases, such as neutrophil elastase, into extracellular space. This enhances the chronic inflammation typical in CKD, further contributing to atherosclerosis, being the main cause of morbidity and mortality in these patients.

Based on the successful reduction of oxidative stress by antioxidant-modified membranes and clinical use of neutrophil elastase inhibitors in respiratory diseases associated with elevated free elastase we assumed, that modification of hemodialysis membranes by immobilization of synthetic neutrophil elastase inhibitors could reduce the level of circulating elastase in blood at the blood/membrane contact site during the hemodialysis procedure. This might result in suppressing inflammatory response and consequently prevent hemodialysis associated cardiovascular complications. Therefore, the focus of this work was to develop membranes with sufficient inhibition activity towards human neutrophil elastase and favorable safety profile. Their biocompatibility is a requirement for application as medical devices, as described in the *International Organization for Standardization (ISO) 10993-4: 2017 Biological evaluation of medical devices*.

2. Theoretical part

2.1 Chronic kidney disease

Chronic kidney disease (CKD) is a general term used to describe a slow, progressive change in kidney function and/or structure. It can often be asymptomatic and possibly reversible in early stages; however, advanced stages are irreversible and associated with high risk of complications and eventually death, especially due to cardiovascular events. [1, 2]

CKD is defined as structural or functional abnormalities of the kidney, present for period greater than 3 months. The diagnosis includes either decrease of glomerular filtration rate (GFR) < 60 mL/min/1.73 m^2 or markers of kidney damage including abnormalities in composition of blood or urine (e.g., albuminuria, urine sediment, electrolytes), abnormalities in imaging tests and histology, or history of previous kidney transplantation. [3, 4]

In 2012, *Kidney Disease: Improving Global Outcomes* [4] established new clinical practice guidelines for better classification, evaluation, and management of CKD. It recommends to classify CKD according to cause, GFR (into 5 stages), and albuminuria (into 3 stages) values differentiating from normal young adult values (Tables 1 and 2). According to these categories, it is possible to estimate coexisting complications and predict future outcomes.

***Table 1**: Classification of CKD according to GFR. [4]*

CATEGORY	***GFR (mL/min/1.73 m^2)***	***TERMS***
G1	≥90	Normal or high
G2	60–89	Mildly decreased
G3a	45–59	Mildly to moderately decreased
G3b	30–44	Moderately to severely decreased
G4	15–29	Severely decreased
G5	<15	ESRD or kidney failure

CKD: chronic kidney disease; GFR: glomerular filtration rate; ESRD: end-stage renal disease

***Table 2**: Classification of CKD according to albuminuria. [4]*

CATEGORY	*AER (mg/24h) or ACR (mg/g)*	*TERMS*
A1	<30	Normal to mildly increased
A2	30–300	Moderately increased
A3	>300	Severely increased

CKD: chronic kidney disease; AER: albumin excretion rate; ACR: albumin-to-creatinine ratio

The main risk factors participating in the development of this disease differ among populations. Diabetes and hypertension are prevailing in developed countries, which is associated with the rising prevalence of obesity worldwide as well as increasing age. On the contrary, infectious diseases, poor sanitation, drugs, and toxins are leading contributors in developing countries. [1, 5]

CKD is considered as a major public health burden associated with high morbidity and mortality. Costs of the treatment, especially in patients with higher stages requiring renal replacement therapy, together with prevention and treatment of comorbidities, are very high, making the therapeutic access unaffordable for the majority of the affected. The Global Burden of Disease study estimated prevalence of CKD as 9.1 % worldwide, with stage 5 prevalence of 0.07 %. Mortality globally resulted in 1.2 million deaths ranking as 12th leading cause of death in 2017. [6, 7]

2.1.1 Complications of CKD

Complications in CKD patients reflect the loss of renal functions in excretion of waste products and in production of hormones. [4]

Endocrine function of the kidneys includes production of hormones such as erythropoietin (EPO), 1,25-dihydroxycholecalciferol (calcitriol), and renin. They also synthesize prostaglandins and contribute to degradation of insulin and parathyroid hormone (PTH). Malfunction in this function may result in anemia, changes in bone mineral metabolism, mineral imbalance, and cardiovascular complications. [8]

As the renal damage progresses, the kidneys are no longer able to preserve their function in elimination of uremic solutes, resulting in their accumulation in the blood. Some of these uremic solutes are proven toxins, whose accumulation results in uremia

and other complications. Other non-toxic substances can be used as markers of retention. [9]

The uremic toxins are classified into three groups according to their molecular weight (MW) and protein-binding capacity: a) low-MW water-soluble molecules with MW < 500 Da (e.g., urea, creatinine), b) middle-MW molecules with MW > 500 Da (e.g., β_2-microglobulin, PTH, leptin), and c) low-MW protein-bound molecules (e.g., homocysteine, cytokines, advanced glycation end products), that present the biggest challenge for dialysis removal. [10] Table 3 shows examples of clinical problems related with accumulation of these uremic toxins.

***Table 3**: Complications associated with retention of certain uremic toxins. [9, 10, 11, 12]*

Uremic toxins	**Complication due to retention**
Middle molecules:	
β_2-microglobulin	dialysis-related amyloidosis, carpal tunnel syndrome
PTH, pro-inflammatory cytokines	cardiovascular complications, osteodystrophy
leptin, pro-inflammatory cytokines	malnutrition
endothelin, uridine adenosine tetraphosphate	vasoconstriction
hepcidin, erythropoiesis inhibitors	anemia
advanced glycation end products	cardiovascular complications, inflammation, oxidative stress
Protein-bound molecules:	
homocystein	cardiovascular complications
indoxyl-sulfate, p-cresol	endothelial dysfunction, inflammation

PTH: parathyroid hormone

Although probability of occurrence of these complications increases with severity of CKD, not all individuals within the same category must develop them at the same rate or to the same degree. [4]

2.1.1.1 Anemia

Anemia is a common complication in CKD patients although with variability in time of occurrence and its severity. With appropriate treatment, it is potentially reversible and thus should be evaluated independently of CKD stage to identify any possibility of being a reversible process. [4] If untreated, it is a serious contributor to cardiovascular mortality, worsening left ventricular hypertrophy and heart failure. [13] Anemia is diagnosed when hemoglobin (Hb) concentration level drops below the cut-off value established by the World Health Organization, which is dependent on gender and age. The cut-off Hb values are 11.0 g/dL for pregnant women and children aged 0.5 to 5 years, 12.0 g/dL in non-pregnant women and 13.0 g/dL in men. [4, 14]

The mechanism of anemia in CKD is multifactorial, including EPO deficiency due to loss of kidney endocrine function, uremic-induced inhibition of erythropoiesis, shortened red blood cells (RBCs) lifespan, increased iron loss, and impaired dietary iron absorption. The iron homeostasis is potentially impaired by increased levels of hepcidin, the hormone responsible for iron regulation, presumably due to its reduced renal clearance and upregulation by inflammatory cytokines. [15]

The treatment includes oral or intravenous iron supplementation, erythropoiesis stimulating agents (ESAs), and RBCs transfusion. Higher doses of ESAs due to relative resistance to it are connected with serious adverse effects, such as, increased risk of cardiovascular events, stroke, and progression of malignancy in cancer patients, therefore it is recommended using ESA therapy with great caution. The choice for the treatment depends on the balance between benefits and harms, which varies among patients. [14, 15]

2.1.1.2 CKD-mineral and bone disorder

Hyperphosphatemia is a result of reduced phosphate excretion and failed hydroxylation of calcifediol into calcitriol, the active form of vitamin D, by kidneys. This causes serum calcium levels to fall, inducing secretion of PTH which subsequently increases calcium levels by bone resorption. Secondary hyperparathyroidism is commonly observed in these patients distorting their bone architecture even before first changes in serum phosphorus are detected. [13]

CKD-mineral and bone disorder is a term involving renal osteodystrophy, a histological manifestation of changes in bone architecture, and vascular calcification,

both related to abnormal bone and mineral metabolism. The treatment usually involves dietary restrictions and intake of phosphate binding agents. [1, 4, 13]

Hyperparathyroidism increases the risk of bone fracture, while vitamin D deficiency contributes to changes in immune response and insulin resistance. However, both are risk factors for cardiovascular diseases (CVDs). [1, 13]

2.1.1.3 Cardiovascular risk

It is well known that CKD and CVDs are interconnected. As it was previously mentioned, CVDs, such as hypertension, belong to the major causes behind progressive loss of kidney function and, on the other hand, CKD takes part in development and enhancement of CVDs. Apart from influencing each other, frequent co-existence of these diseases can be explained by them sharing a number of traditional risk factors such as diabetes, hypertension, hyperlipidemia, obesity, smoking or advanced age. [7, 16]

Even mild to moderate reduction in GFR, and microalbuminuria itself are associated with an increase in cardiovascular complications risk. CKD patients are more prone to experience cardiovascular events than to progress to the last stage, furthermore, CVDs are the leading cause of death in dialysis patients. [4, 13, 16] Apart from traditional risk factors, CKD contributes to development of CVDs with non-traditional risk factors, which are associated with decrease of renal physiological functions. These include the previously mentioned anemia, mineral and bone disorder, but also proteinuria, hypertension, and chronic inflammation.

It was confirmed that proteinuria increases CVDs rate independently of GFR, hypertension or diabetes. Albuminuria is associated with hypertension, adverse lipid profile, and abnormalities in coagulation and is additionally linked to increased risk of myocardial infarction, stroke, and cardiovascular death. [4, 7, 13, 16]

CKD is a cause and consequence of hypertension. Worsening of hypertension due to reduced renal function is multifactorial. Impaired salt and water retention increases extracellular fluid volume, decreased perfusion of blood through kidneys activates renin-angiotensin-aldosterone system which causes vasoconstriction and further activation of sympathetic nervous system, and finally increased production of endothelin together with nitric oxide (NO) deficiency and imbalance in prostaglandins and kinins results in increased vascular resistance. [2, 17] Due to the renal protective effect of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, patients with

diabetic nephropathy and proteinuric nondiabetic kidney disease benefit from these drugs and they are used as first-line agents. [13, 17]

Chronic inflammation, oxidative stress, and dyslipidemia present in CKD patients contribute to endothelial dysfunction and progression of atherosclerosis. Markers of inflammation such as C-reactive protein (CRP), interleukin (IL)-1, IL-6, IL-10, tumor necrosis factor-α and fibrinogen are often elevated, suggesting that polymorphonuclear leukocytes are the key mediators and at the same time they act as predictors of cardiovascular risk. [16, 18, 19] Chronic inflammation is associated with uremia, oxidative stress, and metabolic acidosis. [18] CRP is responsible for reduced NO production and stimulation of endothelin-1 formation; additionally, oxidative stress reduces NO availability, which all together results in changed vascular permeability allowing entrance of low density lipoprotein (LDL) cholesterol into the intima, where it is oxidized. Increased levels of this highly atherogenic oxidized LDL and lipid hydroperoxide were confirmed in these patients. Presence of oxidative stress is common in renal dysfunction and is proved by elevated markers like plasma F2-isoprostanes, advanced oxidation protein products and malondialdehyde. [19] Besides atherosclerosis, chronic inflammation in CKD patients is associated with mineral and bone disease, malnutrition, anemia, and EPO resistance. [18]

2.1.1.4 Others

Metabolic acidosis often occurs when GFR drops below 30 mL/min/1.73 m^2. Serum bicarbonate level lower than 22 mmol/L increases risk of CKD progression. Additionally, chronic metabolic acidosis was associated with increased protein metabolism, uremic bone disease, muscle wasting, chronic inflammation, and impaired glucose homeostasis. Supplementation with bicarbonate showed reduced progression to the final stages and improved nutritional status. [1, 4]

Uremic malnutrition is another prevalent complication. Despite adequate protein and carbohydrate intake, nutritional requirements in CKD patients are altered, affecting metabolism of proteins, water, salt, potassium, and phosphorus resulting in inadequate energy generation. Therefore, serum albumin, creatinine (possibility of muscle wasting due to poor nutrition), and cholesterol should be monitored. [13]

2.2 End-stage renal disease

CKD can potentially progress into irreversible loss of kidney function known as end-stage renal disease (ESRD), characterized by GFR lower than 15 mL/min/1.73 m^2, requiring renal replacement therapy (RRT) by means of dialysis or renal transplantation. Without this treatments, hyperkalemia or pulmonary edema would be fatal complications for ESRD patients within days or weeks. Besides CKD, ESRD may also result from acute kidney injury. [20]

RRT includes renal transplantation, which should be the treatment of choice in stage 5 CKD patients predicted to have improved life expectancy and quality of life post-transplantation. Access to renal transplantation requires ideally a living donor, assessment of suitability of patient, and his absolute and relative contraindications, such as:

- risk factors of cardiac mortality after transplantation;
- obesity causing technical difficulties and increased risk of peri-operative complications;
- viral infections (e.g., HIV, hepatitis B and C, Epstein-Barr virus);
- malignancy (waiting time after tumor treatment/remission should be at least 2 years);
- potential risk of recurrence of the original renal disease. [21]

Patients not eligible or waiting for transplantation usually undergo dialysis treatment unless the life expectancy is lower than a few months. [20] If the patient does not wish to initiate dialysis, conservative care is received. This non-dialytic method includes treatment of anemia, hypertension, correction of acidosis and hyperkalemia, fluid balance, and monitoring of calcium and phosphorus metabolism. [22]

2.3 Dialysis

Dialysis is a form of RRT using a semipermeable membrane for removal of uremic toxins and excess water from the blood. The basic removal mechanisms occurring during dialysis are summarized in Table 4. The two main dialysis modalities are peritoneal dialysis (PD) and hemodialysis (HD). [23]

***Table 4**: Basic mechanisms occurring during dialysis. [23]*

Removal of solutes (uremic toxins)	***Removal of fluids***
Diffusion	**Ultrafiltration**
○ movement of solutes from area of higher concentration to area of lower concentration ○ dependent on concentration gradient, permeability of the membrane, and surface area of the membrane ○ removal of small MW solutes (e.g., urea or creatinine)	○ water flow driven by hydrostatic or osmotic gradient
Convection	
○ passive entraining of solutes by water during ultrafiltration ○ independent of solute concentration gradient, determined by porosity of the membrane and ultrafiltrate rate ○ removal of small and middle MW solutes	

MW: molecular weight

2.3.1 Peritoneal dialysis

PD is a transport of solutes and water through the peritoneum (serosal membrane lining the peritoneal cavity) between two compartments: a) the blood in the peritoneal capillaries containing uremic toxins and waste products, and b) a hyperosmolar dialysis solution usually containing sodium, calcium, magnesium, chloride, lactate or bicarbonate as buffer solutions, and glucose as osmotic agent. PD can be done in a form of continuous ambulatory peritoneal dialysis (CAPD) or as an automated peritoneal dialysis (APD). In CAPD, the dialysis solution is packaged in clear, flexible plastic bags connected to the peritoneal catheter by a transfer set and infused into peritoneal cavity by gravity. The exchange of solution is performed manually 4 to 5 times a day. APD uses cyclers, to which a patient is hooked up at night, exchanging solution in the abdomen by hydraulic pumps. [24]

Compared to HD, it appears that the survival rate on PD is better in the first few years of dialysis, considering the decrease in infection-related complications. Other advantages include lower cost and opportunity of home therapy with flexible schedule. On the other hand, risk of peritonitis, ultrafiltration failure caused by changes in the membrane by glucose, and catheter malfunction as well as catheter-related infections are some of the reasons for transfer to HD. [25]

2.3.2 Hemodialysis

HD is the most common modality of RRT. Unlike PD, using the peritoneum as the membrane for waste products removal, HD is an extracorporeal blood cleansing technique using an artificial semipermeable membrane produced from polymers. Conventional HD therapy is usually conducted in clinics 3 times per week with 3-5 hours duration per session. Although other more intensified modalities exist, such as home HD, short daily HD, and long nocturnal HD, showing better results in uremic toxins clearance, better control of blood pressure and anemia, improved nutrition, and lower mortality rates, conventional HD still remains prevailing among the others. The reasons for this include logistic problems, increased expenses, training of caregivers for home HD, and conservatism of physicians. [12, 24] Characteristics of these regimes are summarized in Table 5.

***Table 5**: Summary of different regimes of HD. [12, 24, 26]*

Type of HD	Advantages	Disadvantages
Conventional HD - performed in centers 3 times per week for 3 to 5 hours	Hospital/center staff does all the work, shorter time spent weekly on dialysis.	Traveling to center, inflexible schedule.
Home HD - performed 3 to 6 times a week, either during the day or at night	Home environment, more positive attitude, caregiver/family support. Better control of blood pressure and serum phosphate, better quality of life. May reduce left ventricular hypertrophy.	Requires caregiver, HD machine, water purification system, waste disposal.
Short daily HD - the conventional time of HD (3–5 h) broken up into 5 to 6 sessions per week; done usually at home, rarely in centers	Better control of blood pressure, anemia, serum phosphate and improved nutrition. Reduction in left ventricular hypertrophy.	Includes disadvantages of home HD. Performing dialysis almost every day of the week.
Nocturnal HD - performed in centers 3 times per week for 7 to 9 hours	Increased weekly dialysis time, dialysis spent sleeping. Better control of blood pressure, serum phosphate, and anemia.	Traveling to center, inflexible schedule. Needs schedule for water regeneration between day and night shifts in centers. Adaptation of patients to sleep in center.

HD: hemodialysis

2.3.2.1 HD apparatus

The HD apparatus consists of two circuits, blood and dialysate, separated by a semipermeable membrane, and a complex of detectors, controllers, monitors, and safety devices. [27]

The blood circuit begins at the vascular access, circulates through inflow (arterial) blood line to the dialyzer and returns via outflow (venous) blood line. The preferred access placement is arteriovenous fistula. Side ports are attached to the blood lines for saline or heparin infusion, pressure measurement, and air entry detection to prevent air embolus. The pressure is measured in two segments: prepump (arterial pressure monitor) where the pressure between the access and the blood pump is negative, and postdialyzer (venous pressure monitor) where the pressure is positive. If the pressure goes out of range or the entrance of air is detected, these side port detectors activate the alarms and stop the blood pump. The blood moves through the blood tubing made of biocompatible and nontoxic material by a spring-loaded roller pump at a rate of 200 to 600 mL/min. [24, 27]

The dialysate circuit contains several components that include water purification system, heating and degassing of purified water, dialysate proportioning system, monitors, alarms, and ultrafiltration control. Before mixing with the dialysate concentrate, the purified water is heated to 35–38 °C and degassed by subjecting to negative pressure. Cold dialysate (below 35 °C) might cause shivering while too warm dialysate (over 42 °C) causes blood protein denaturation and hemolysis. The water is then mixed with dialysate concentrates containing Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, bicarbonate or acetate buffer to obtain dialysate of proper osmolality, pH, and conductivity. The conductivity should be in the range of 12 to 16 mS/cm to ensure proper water/concentrate ratio and the recommended pH range is 6.8 to 7.6. [24, 27]

To avoid the concentration equilibrium between blood and dialysate, fresh dialysis solution is continuously refilled, and the two compartments run in counter-current flow to maximize the concentration gradient in all parts of the dialyzer. [12, 24] The scheme of the two circuits is shown in Figure 1.

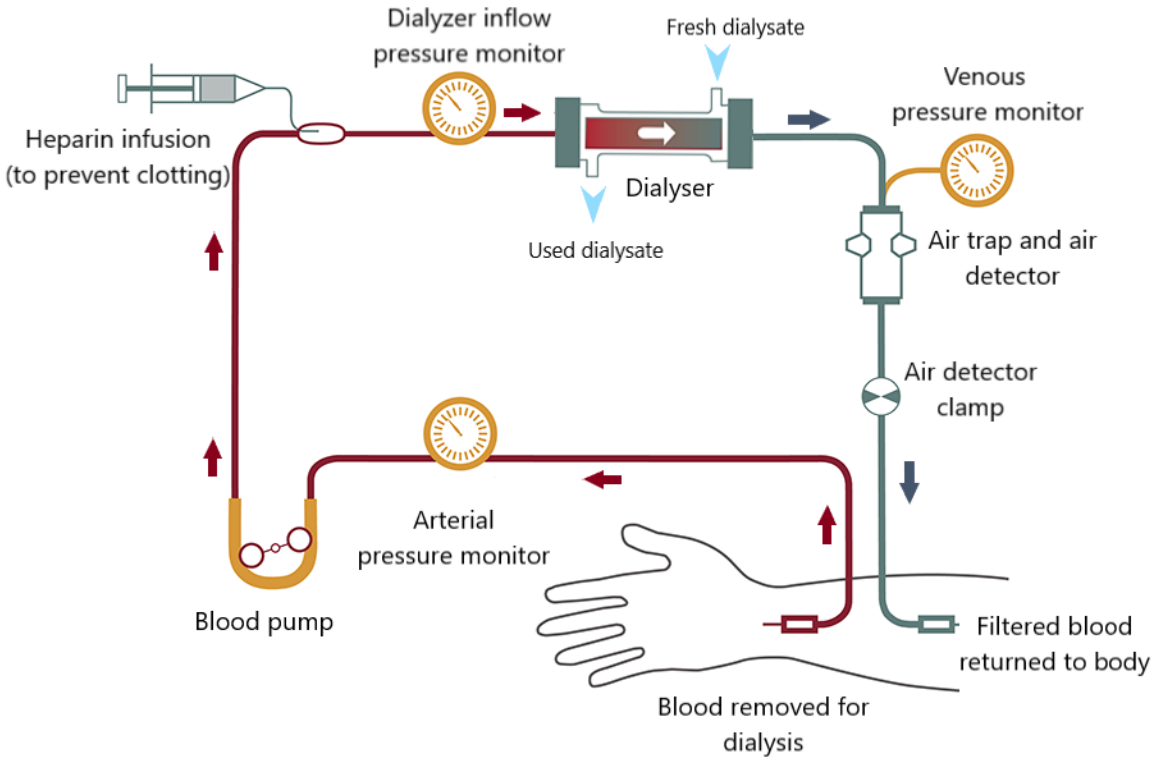


***Figure 1**: Scheme of hemodialysis circuits. Modified from [28].*

2.4 HD membranes

HD membranes are semipermeable membranes used for removal of uremic toxins and excess water accumulating in a ESRD patient's body. [29] An optimal membrane should be able to remove large molecules up to 55000 Da but at the same time prevent loss of albumin (MW of 66000 Da) over clinically acceptable level. [30]

HD membranes are characterized based on various parameters. Efficiency of the membrane depends on the clearance of small solutes by diffusion and is described by KoA (the mass transfer coefficient normalized to the membrane surface area). [29] The KoA of urea is provided by manufacturers. [11] High-efficiency dialysers have a KoA > 800–1000 mL/min. [30] The ultrafiltration coefficient (KUF; mL/h/mmHg/m^2) describes the permeability of the membrane to water. It is a ratio of the ultrafiltration rate (Q_f; mL/h/m^2) to the transmembrane pressure (mmHg). [29] According to the pore size, KUF value, and the ability to clear β_2-microglobulin, the HD membranes are divided into low-flux membranes (KUF < 15 mL/h/mmHg and β_2-microglobulin clearance < 10 mL/min) unable to remove larger sized uremic toxins and protein-bound molecules and high-flux membranes (KUF > 15 mL/h/mmHg and β_2-microglobulin clearance > 20

mL/min) capable of removing higher amounts of these toxins, reducing their accumulation in circulation and thus lowering cardiac and cerebrovascular mortality rates. [12]

As an attempt to increase clearance of protein-bound molecules, two new membrane classes were introduced, the medium cut-off and the high cut-off, used in super high-flux dialysis. In both of these membrane types, molecular weight cut-off (MWCO) was increased allowing bigger molecules to pass through the membrane. MWCO represents the solute molecular weight with sieving coefficient of 0.1, connected with mean pore size of the membrane. High cut-off membranes enabled removal of excess myoglobin in trauma patients, inflammatory cytokines, or free light chain proteins in multiple myeloma, however, loss of albumin was too high for long-term use. Better results were provided by medium cut-off membranes with a narrow interval between MWCO and molecular weight retention onset (solute molecular weight with sieving coefficient 0.9) achieved by tight pore size distribution. This allows higher removal of middle and protein-bound molecules while decreasing the albumin loss to the clinically accepted range. [11, 29] The performance characteristics of low-flux, high-flux, high cut-off, and medium cut-off membranes are shown in Figure 2.

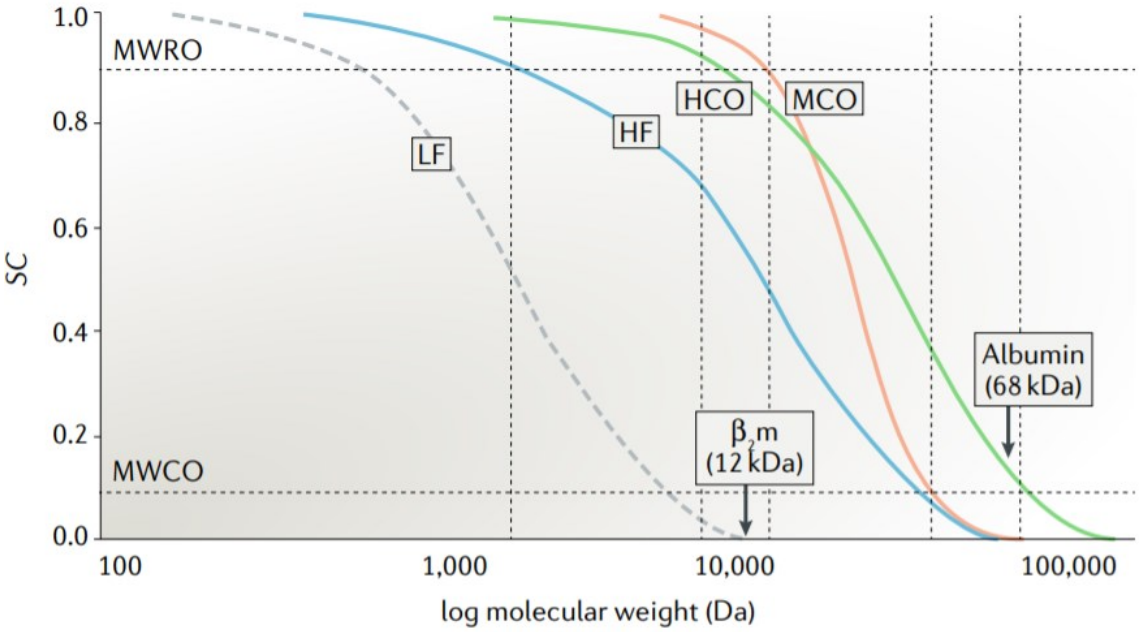


***Figure 2**: Performance characteristics of hemodialysis membrane types. [29]*

SC: sieving coefficient; MWRO: molecular weight retention onset; MWCO: molecular weight cut-off; LF: low-flux; HF: high-flux; HCO: high cut-off; MCO: medium cut-off; β_2m: β_2-microglobulin

Another aspect according to which HD membranes are characterized is their chemical composition. Membranes originally used for HD were cellulose membranes, also known as cuprophan membranes, that due to exposed hydroxyl groups activate complement and therefore are relatively bio-incompatible. [11] In order to decrease complement activation by the membranes, a group of modified cellulose membranes was developed by replacing the hydroxyl groups with other moieties. Example of such membranes are cellulose triacetate membranes, where a high percentage of hydroxyl groups is substituted with triacetate, or Hemophan® membranes, where a small percentage of the hydroxyl groups is substituted with a bulky chemical group (in this case a tertiary amine) sterically preventing complement activation. Apart from increased biocompatibility, the cellulose membrane modification also resulted in increased pore size providing higher water permeability and wider solute removal spectrum. [31] The third group of HD membranes is a group of diverse synthetic membranes (Table 6). They are characterized by better biocompatibility and possess significantly larger pore size and higher hydraulic permeability than cellulose-based membranes. [11, 31]

Cellulose membranes have a symmetrical structure with same pore size across all layers. They also have a relatively thin wall being an advantage in respect to diffusive transport but making them unable to withstand high ultrafiltration rates. [11, 31]

On the other hand, synthetic membranes have thicker wall with either symmetrical structure (e.g., polymethyl methacrylate) or asymmetrical structure with a thin, selective inner layer acting as size-discriminating element in size removal and a thicker outer supportive layer providing mechanical stability. The outer layer is macroporous, forming a sponge-like or finger-type structure, while the structure of the inner layer is compact. Due to their mostly hydrophobic structure they tend to adsorb proteins and activate platelets. For this reason, they are often combined with a hydrophilic additive (e.g., polyvinylpyrrolidone, polyethylene glycol) to increase membrane hydrophilicity, improve wettability, and decrease platelet activation. [11, 29, 31]

***Table 6**: The most common dialysis membrane materials. [11, 30, 32]*

Material	***Advantages***	***Disadvantages***
cellulose triacetate (CTA)	• high diffusion efficiency • structural homogeneity • low thrombogenic potential	• lower biocompatibility compared to synthetic membranes • narrow solute removal spectrum
polysulfone (PSf)	• mechanical strength, chemical and thermal resistance • high biocompatibility • high permeability for low molecular weight proteins • high retention of endotoxins • can withstand all sterilization techniques	• protein accumulation on surface • causes neutrophil activation and pro-inflammatory cytokine production
polyethersulfone (PES)	• mechanical strength, chemical, thermal, and oxidative resistance • high permeability for low molecular weight proteins	• hydrophobic nature causing membrane fouling and complement activation
polyacrylonitrile (PAN)	• good mechanical strength • good biocompatibility • high permeability and specificity for low and medium molecular weight proteins	• higher risk of anaphylactic reaction and bradykinin production
polymethyl methacrylate (PMMA)	• high permeability • high biocompatibility • ability to remove proteins through absorption • reduces inflammation by removing pro-inflammatory cytokines	• slight complement activation
polyester polymer alloy (PEPA)	• ability to filter endotoxins • good β_2-microglobulin and low albumin removal	• slight complement activation
ethylene vinyl alcohol co-polymer (EVOH)	• reduction in neutrophil activation and oxidative stress • hydrophilic structure	• not described (possibly not sufficient mechanical strength)

2.4.1 Membrane fabrication methods

Phase inversion is the most common method for production of synthetic polymeric membranes used for ultrafiltration. [33] It is a process of controlled transformation of polymer solution from liquid state into a solid state. This includes several techniques such as thermally induced, evaporation-induced, and vapor-induced phase separation, but the majority of commercially available membranes are prepared by immersion precipitation. [33, 34]

Immersion precipitation is a phase inversion technique in which a polymer solution is casted as a thin film on a mechanical support and immersed into a coagulation bath containing non-solvent (usually water). For production of porous membranes, it is necessary for solvent and non-solvent to be miscible. [33, 34] An exchange of solvent and non-solvent takes place until the solution becomes thermodynamically unstable and demixing takes place. Liquid-liquid demixing transforms polymer solution into a polymer-rich phase (forming membrane matrix) and polymer-lean phase (forming membrane pores). Two types of demixing process may occur differentiating in membranes` morphology. If the liquid-liquid demixing is instantaneous, a membrane with relatively porous top layer is obtained (microfiltration/ultrafiltration type) whereas, if it is delayed, a dense top layer membrane is formed (gas separation type). In the end, a solid polymeric film with asymmetric structure is produced. [33, 34, 35]

Thermally induced phase separation uses the phenomenon of decreased solvent quality with decrease of the temperature. This induces demixing and the solvent is removed by extraction, evaporation or freeze drying. [33] Microfiltration membranes are frequently prepared by this technique. [34]

In an evaporation-induced phase separation, the polymer solution consists of solvent and non-solvent mixture. The more volatile solvent evaporates, shifting the composition to a higher non-solvent content, which leads to a precipitation of polymer. This results in the formation of skinned membranes. [33]

Vapor-induced phase separation occurs after exposure of polymer solution to a vapor atmosphere containing non-solvent saturated with the solvent in order to prevent its evaporation. Penetration of non-solvent into the cast film creates a porous membrane without a top layer. [33]

2.4.2 Geometry of polymeric membranes

Polymeric membranes are usually available in two geometries, hollow fiber and flat-sheet. The majority of the membranes available on the market are hollow fibers, however their fabrication requires a more complicated set-up. On the other hand, flat-sheet membranes are simple and affordable option, therefore it is advantageous to use them at laboratory scale.

2.4.2.1 Flat-sheet membranes

Flat-sheet polymeric membranes are mainly prepared by casting technique followed by phase inversion process. Casting solution is placed on a suitable plate (a belt, a glass or silicon wafer), uniformly spread and adjusted to the desired thickness using casting knife or spin coating method. [36, 37]

Spin coating is a process of applying a solution onto the center of a horizontal rotating disc and spinning it at high speed which results in formation of uniform thin films. Centrifugal force in combination with the adhesive force at the solution/support interface causes spreading of solution on and off the disc in a radial flow. Ejection of polymer solution from the edges of rotating disc with subsequent evaporation of solvent thins the polymer layer. [38, 39] A schematic representation of the spin coating process is shown in Figure 3. After spin coating, the formation of the membrane is usually completed by immersion precipitation. For flat-sheet membranes, the phase inversion starts form the top surface upon immersion in a coagulation bath. [35] The final thickness and properties of created membranes depend on the polymer solution (viscosity and concentration) and parameters of spin coating process (rotational speed, acceleration, temperature, and humidity). These need to be held constant in order to produce uniform, reproducible membranes. Advantages of spin coating method include high reproducibility, high structural uniformity, easy handling, and low cost. [38, 39]

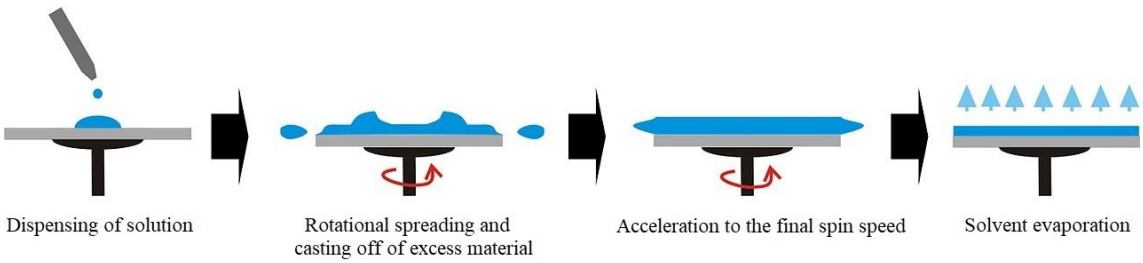


***Figure 3**: Spin coating process. Modified from [40].*

2.4.2.2 Hollow fiber membranes

Compared to flat-sheet membranes, hollow fiber geometry offers larger effective membrane area, however, their fabrication with the desirable morphology and separation performance is more challenging. [35]

Hollow fiber fabrication requires internal coagulant controlling inner skin morphology and external coagulant controlling outer skin morphology. After polymer solution and bore liquid (internal coagulant) are filtered and degassed, they are pumped and spun simultaneously through a hot spinneret with a short air-gap region (a spinning shaft), where a moisture-induced early phase separation (external coagulation) occurs at the outer surface of the membrane. The spinneret contains two concentric openings, where the polymer solution enters through an outer ring slit while the bore solution is injected through the inner tube. The hollow fiber is stretched by gravity and motor-driven roller from top to bottom. Polymer solution and bore liquid meet at the exit of the spinneret, inducing internal coagulation and forming the inner void of the hollow fiber. The hollow fiber formation scheme can be seen in Figure 4. Control of temperature and humidity inside the spinning shaft are crucial. Phase inversion process of hollow fiber is completed by external coagulation bath. The process of membrane fabrication is finished by final washing, drying, undulation, additional surface treatments, and winding of the membrane bundles. [35, 41]

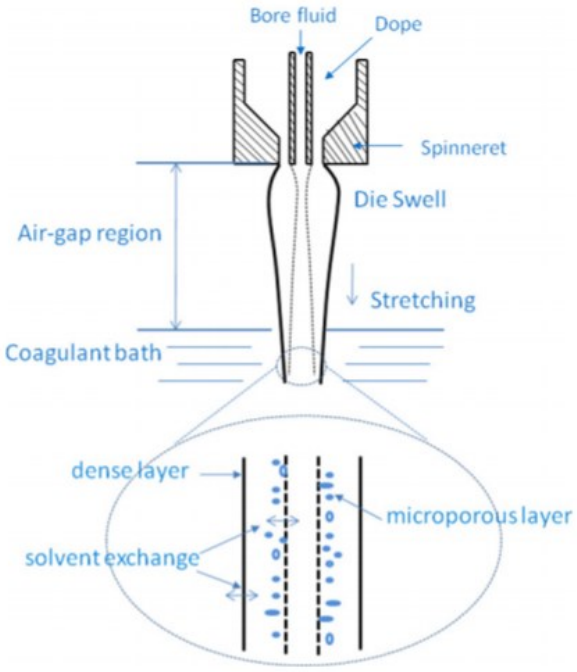


***Figure 4**: Hollow fiber formation. [35]*

2.5 Human neutrophil elastase in CKD

As mentioned above, the most common case for mortality in ESRD patients on HD is CVDs. These are, among the other factors, caused by chronic inflammation associated with oxidative stress and neutrophil activation with release of neutrophil serine proteases (NSPs). [42]

Neutrophils are the most abundant type of leukocytes and play an important role in an acute phase of inflammation. [43] They are granulocytes developed from pluripotent stem cells in bone marrow, containing a segmented nucleus and four subsets of granules. These granules are divided into primary (azurophilic) granules containing NSPs and myeloperoxidase; secondary (specific) granules containing lactoferrin and cathelicidin; tertiary granules with gelatianse and lysozyme; and secretory granules with complement and chemotaxis receptors. After activation of neutrophils by pathogens, they migrate to the inflammatory site and act in four ways: 1) phagocytosis of pathogen and its destruction in the phagolysosome, 2) degranulation into extracellular space, 3) production of reactive oxygen species (ROS), and 4) formation of neutrophil extracellular traps. [42, 44]

Human neutrophil elastase (HNE) is one of the NSPs from the chymotrypsin family, among proteinase 3 (PR3), cathepsin G (CG), and neutrophil serine protease 4, stored in azurophilic granules acting in intracellular and extracellular pathogen destruction in the nonoxidative pathway. [42, 44] Apart from neutrophils, it can be also found in mast cells, monocytes, eosinophils, keratinocytes, and fibroblasts. As the name implies, HNE degrades elastin, but also other extracellular matrix and plasma proteins such as collagen, fibronectin, laminin, and proteoglycans. [42, 45] NSPs are responsible for pathogen digestion within phagolysosomes together with myeloperoxidase and ROS produced by NADPH oxidase system. [44] They also play an important role in proteolysis activity of neutrophil extracellular traps, tissue homeostasis, chemotaxis, and migration of neutrophils, they activate other proteases such as metalloproteinases and are able to degrade components of coagulation. HNE is activated by dipeptidyl peptidase 1, a lysosomal cysteine protease, after N-terminal modification and inhibited by endogenous protease inhibitors. The active site of the enzyme is composed by catalytic triad Ser195-Asp102-His57, where Ser195 is responsible for the nucleophilic attack on the carbonyl carbon of the substrate. [42, 45] The S1 pocket of HNE is hemispherical and hydrophobic with Val190 and Val216 playing an important role in defining the substrate

specificity. [44] Upon degranulation, HNE is released from azurophilic granules into the extracellular space, although some remains bound to the outer cell membrane. Gene mutations, excessive secretion, or protease-antiprotease imbalance are associated with several pathologies such as congenital neutropenia, autoimmune diseases, chronic lung diseases (chronic obstructive pulmonary disease, cystic fibrosis, acute lung injury), tumors, and cardiovascular complications. [42, 45]

CKD is associated with chronic inflammation also in the pre-dialysis period, but is even more enhanced after the long-term intradialytic contact of blood with artificial materials during HD. It was proven that in ESRD patients treated with HD, the level and activity of extracellular and cell surface bound HNE is elevated, while the level of intracellular HNE is lowered, without difference in its mRNA expression. Additionally, the amount of endogenous inhibitor, α1-antitrypsin (AAT), is also decreased, leading to enzyme-inhibitor imbalance. Elevated elastase and CG levels are responsible for cleavage of vascular endothelial cadherin leading to endothelial dysfunction followed by chronic inflammation, atherosclerosis, and cardiovascular complications, which are the number one case of CKD mortality. [42, 43]

2.5.1 Neutrophil elastase inhibitors

In order to prevent degradation of connective tissue, regulate blood coagulation and inflammation, and restore protease-antiprotease balance, the activity of NSPs is regulated by protease inhibitors. The tree main families of serine protease inhibitors include serpins (α1-proteinase inhibitor also called AAT, monocyte/neutrophil protease inhibitor), chelonianins (elafin, secretory leukocytes protease inhibitor), and macroglobulins. [44, 45] AAT is the major circulating serpin having HNE as the main target. Production of ROS in ESRD patients oxidizes and inactivates AAT leading to degradation of extracellular matrix components, tissue damage, and endothelial dysfunction enhancing inflammation and release of inflammatory cytokines, creating a vicious cycle. [42, 43]

As the imbalance between HNE and its endogenous inhibitors is connected with many disorders such as chronic lung diseases, autoimmune diseases or chronic kidney disease, a group of exogenous inhibitors was developed in order to decrease level of active NSPs and inflammation. [42, 45] An ideal therapeutic NSP inhibitor should: 1) be able to inhibit HNE, PR3, and CG with similar efficiency, 2) be resistant to oxidation and

proteolysis, 3) be of small size, therefore accessing bound and intracellular NSPs, 4) resist aerosolization and *in vivo* degradation. [44]

Therapeutic human neutrophil elastase inhibitors (HNEIs) are currently classified into 5 generations, where the 1st generation consists of biological inhibitors with peptide structure while the other generations include low molecular weight synthetic, non-peptide inhibitors (Table 7). [42, 45] The only two inhibitors available on the market are Prolastin® (purified AAT) used in AAT deficiency, and sivelestat (ONO-5046, 2nd generation), inhibiting released and membrane-bound elastase, used for treatment of acute lung injury and acute respiratory distress syndrome associated with systemic inflammatory response syndrome. Compared to peptide inhibitors, synthetic ones offer decreased immunogenic response, enhanced enzyme selectivity, decreased proteolytic inactivation, and easier structure modification optimizing pharmacokinetic properties. [45]

***Table** 7: Exogenous therapeutic human neutrophil elastase inhibitors. [42, 45]*

Classification	Description	Examples
1st generation	- biological, peptide structure - some present low stability	AAT (Prolastin®, Aralast®, Glassia®, Zemaria®), elafin (Tiprelestat®)
2nd generation	- small synthetic inhibitors - irreversible, suicide inhibition	ONO-5046 (sivelestat), ONO-6818 (freselestat)
3rd and 4th generation	- modern synthetic inhibitors derived from pyridine and dihydropyrimidinone lead structure - reversible inhibition with high selectivity and target specificity	AZD9668 (alvelestat, 3rd) BAY-678 (4th)
5th generation	- pre-adaptive pharmacophores derived from 4th generation - improvement in potency and inhibitory capacity	BAY 85-8501

2.5.1.1 4-oxo-β-lactams

A group of molecules containing the 4-oxo-β-lactam scaffold (Figure 5), originally described as porcine pancreatic elastase inhibitors, were proved to be selective acylating irreversible inhibitors of HNE, which lead to development of new structures. Their potency as HNEIs is strongly dependent on C-3 substituents responsible for molecular recognition. A small hydrophobic substituent at C-3, such as diethyl group, is preferred for optimal interaction with the S1 pocket where it interacts with Val-190, Phe-192 and Val-216. Enzyme inhibition is significantly increased in *N*-aryl derivatives compared to *N*-alkyl ones, with additional increase in molecular recognition by adding aryl/heterocyclic moieties in *para* position. The potency is negatively affected by *ortho* substitution, while the effect of electronic properties of substituents in the aromatic moiety is not significant. Their advantage lies in additional inhibition of PR3, on the other side, the β-lactam ring is rapidly hydrolyzed by plasma and liver enzymes, showing low stability in human plasma with half-lives between 0.2 to 2 hours. [46] Their use is currently considered for ELANE-associated neutropenia and as a promising diagnostic tools as small-molecule activity-based probes. [42, 47]

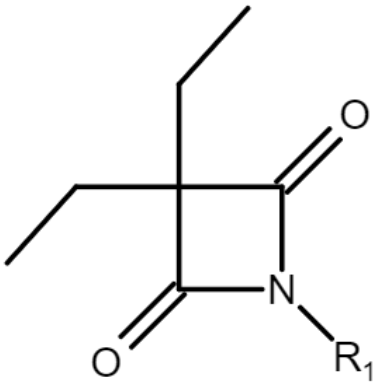


Figure 5*: Core molecular structure of 4-oxo-β-lactam inhibitors. Diethyl substituent at C3 is optimal for S1 subsite recognition while N-substituents vary between inhibitors in order to increase molecular recognition and inhibitory potency.*

2.6 Modification of hemodialysis membranes

In order to improve biocompatibility of membranes and reduce complications connected with dialysis and CKD itself, various bioactive membranes started to be designed and developed by membrane surface modification with bioactive substances.

2.6.1 Modification methods

Two main approaches exist for HD membrane modification: blending and surface coating. [30] Both of these techniques belong to physical modification methods; however, these are changing chemical composition of the membrane surface unlike other methods in this group. An advantage of both methods is that they can be used for modification of flat-sheet membranes as well as hollow fiber membranes. [48]

Blending is the simplest and most practical method. Membrane surface is modified by incorporating additives during membrane formation and no pre- or post-treatment is needed. [30, 48] On the other hand, this method requires blending additive to have close resemblance with the polymers' chemical properties and similar polarity with the polymers' solvent. For example, heparin mimicking polyurethane PES membranes were prepared by blending to prevent blood clotting occurring during HD. [30]

Coating is another simple modification method, wherein a material is noncovalently adhered to the membrane surface, forming a thin layer. A disadvantage of this method lies in its low stability; however, novel methods have been developed to keep the two layers intact. [30, 48] Example of such prepared membranes includes vitamin-E coated PSf membrane for improvement of oxidative stress in HD patients. [30]

Chemical modification includes techniques of membrane surface modification through covalent bonding. This includes chemical post-treatment, where the membrane is treated with modifying agents to introduce various chemical groups on its surface, and chemical grafting, using free radicals production for initiation of graft polymerization on the membrane surface. Unfortunately, these methods involve great quantities of organic solvent and chemicals consumption, leading to environmental pollution. [48]

2.6.2 Modified membranes with focus on oxidative stress

Oral supplements of antioxidants such as vitamin E, C, lipoic acid, and glutathione have been used to alleviate oxidative stress present in CKD patients. In order to reduce ROS production directly at the site of contact between the HD membrane and blood, a new type of modified membranes was developed. A cellulose-based vitamin E-coated membrane (VEM) was first introduced in the late 90s. Its use demonstrated decrease in lipid peroxidation, increase in total antioxidant capacity, and even significant decrease in markers of inflammation. After biocompatibility of PSf was confirmed, a PSf-based VEM was developed and nowadays a number of cellulose/PSf/PES-based vitamin E coated membranes are commercially available. Other attempts to design even more biocompatible membranes with antioxidant activity led to development of chemically immobilized lipoic acid PSf membrane and a PSf membrane with incorporated vitamin E in combination with lipoic acid by blending method. Both of these membranes proved reduction in oxidative stress *in vitro*, while not affecting selective separation ability. However, more experiments need to be carried out to prove their efficacy *in vivo*. [32, 49]

2.6.3 Modified membranes with focus on inflammation

As chronic inflammation in CKD patients is connected not only with oxidative stress but also with elevated HNE, this led to the development of new membranes addressing such an issue. Grano V. *et al* proposed and demonstrated, that the concentration of active free elastase can be reduced by modification of HD membranes by means of chemically immobilized HNE inhibitors. This was tested on PES and Nylon membranes using peptide exogenous inhibitors showing promising results *in vitro*. [50, 51] However, no more experiments have been carried out testing their activity *in vivo* or designing membranes with more biocompatible and selective synthetic inhibitors, creating new opportunities for development of such membranes.

3. Aims

As it was previously mentioned, neutrophil activation with release of neutrophil serine proteases arising from and promoting chronic inflammation is a common complication in CKD patients, further enhancing atherosclerosis and cardiovascular complications. This is specially intensified in ESRD. Development and effectiveness of antioxidant-enriched dialysis membranes reducing oxidative stress right at blood/membrane contact site in these patients brought a new perspective to design HNEI-enriched dialysis membranes with the purpose of inactivating active free elastase in blood during HD procedure.

The main objective of this work was to develop modified PSf HD membranes by adsorbing (coating) or incorporating (blending) potent and selective HNEIs. Their characterization was also the scope of this work by assessing properties such as bioactivity (inhibitory capacity) and biocompatibility (platelet activation, hemolysis, red blood cell morphology) to test their applicability as medical devices. Two types of membranes, using sivelestat, a commercially available 2nd generation inhibitor, and a newly *in-house* synthetized inhibitor chosen from the library of potent and selective 4-oxo-β-lactam based HNEIs were developed, characterized, and compared.

4. Experimental part

4.1 Chemicals and instrumentation

Chemicals:

polysulfone (PSf) pellets (average MW 35 000 g/mol, Sigma-Aldrich Co.),
polyvinylpyrrolidone (PVP) (average MW 40 000 g/mol, Sigma-Aldrich Co.),
N-methyl-2-pyrrolidone (NMP) (MW = 99.13 g/mol, Sigma-Aldrich Co.),
ultrapure water (uH_2O) (18.2 MΩ.cm, Milli-Q, Millipore purification system, France),
human neutrophil elastase, (specific activity >20.0 U/mgP, Merck KgaA, Germany),
fluorogenic elastase substrate V (MW 627.7 g/mol, Merck KgaA, Germany),
sivelestat (Abcam, Cambridge, UK),
dimethyl sulfoxide (DMSO) (MW = 78.13 g/mol, VWR Chemicals BDH®),
4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES buffer) (0.1 M, pH 7.4, prepared *in-house*),
phosphate buffered saline (PBS) (pH 7.4, prepared *in-house*),
Triton X-100 (Sigma-Aldrich Co.),
phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich Co.)
CD42a Monoclonal Antibody, phycoerythrin (PE) (Invitrogen)
CD62P (P-Selectin) Monoclonal Antibody, allophycocyanin (APC) (Invitrogen)

Instrumentation:

laboratory glass, single channel and multichannel pipettes, eppendorf tubes, 96-well plates, 12-well plates, analytical balance, magnetic stirrer, drying oven,
ultrasonic cleaner (Thermo Fisher Scientific, MA, USA),
N-type silicon wafer (Sigma-Aldrich Co.),
spin coater (model WS-650HZB-23NPPB, Laurell Technologies Co., USA),
VLS laser platform (LaserMaq, Portugal),
water-jacketed CO_2 incubator (model 3111, Forma Scientific, Inc., USA; set to 25 °C),
microplate reader (Synergy HT, BioTek Instruments, USA),
bacteriological incubator (model 2001247, J.P. Selecta, s.a., Spain; set to 37°C),
centrifuge (1730R, GYROZEN), centrifuge (Heraeus Fresco 21, Thermo Scientific),
flow cytometer (BD Accuri C6, BD Pharmingen),
optical microscope (Nikon Eclipse Ci).

4.2 Procedures and methods

4.2.1 Production of PSf membranes

Composition and preparation of membranes used in following experiments was optimized by Kohlová, M., *et al.* in order to improve membrane properties such as porosity and biocompatibility by optimal polymer/hydrophilic additive/solvent ratio. [52]

Before weighting, the PSf pellets were dried at 100 °C for at least 1 hour. After cooling down to room temperature, all components, PSf pellets, PVP as hydrophilic additive, and NMP as solvent, were weighted into a glass vessel in standardized mass fraction 15, 2.5 and 82.5 wt%, respectively. The mixture was left to dissolve overnight under constant stirring. After complete dissolution, air bubbles were removed using an ultrasonic bath for 10 minutes. Flat-sheet membranes were produced using the spin-coating technique (Figure 6) followed by immersion precipitation. Temperature and humidity inside the spin coater were set to 22 °C and 32 %, respectively. 1 mL of solution was casted onto the silicon wafer from the opening on the top of spin-coater and spun for 20 s with spinning speed of 600 rpm. Immediately afterwards, the silicon wafer with the thin film of membrane solution was immersed into ultrapure water (non-solvent) coagulation bath adjusted to 15 °C for the phase inversion process to take place. After the process was finished, the membrane peeled off the silicon wafer and was put into fresh ultrapure water bath to be washed in order to remove residual solvent and additive (approximately 24 hours). At the end, the membranes were air-dried (Figure 6) and stored at 4 ºC.

Samples used in the following assays were cut from prepared membranes using a laser cutter and further stored at 4 ºC with silica to prevent membrane wetting. In Table 8 are depicted size and weight parameters of the membranes used in this work.

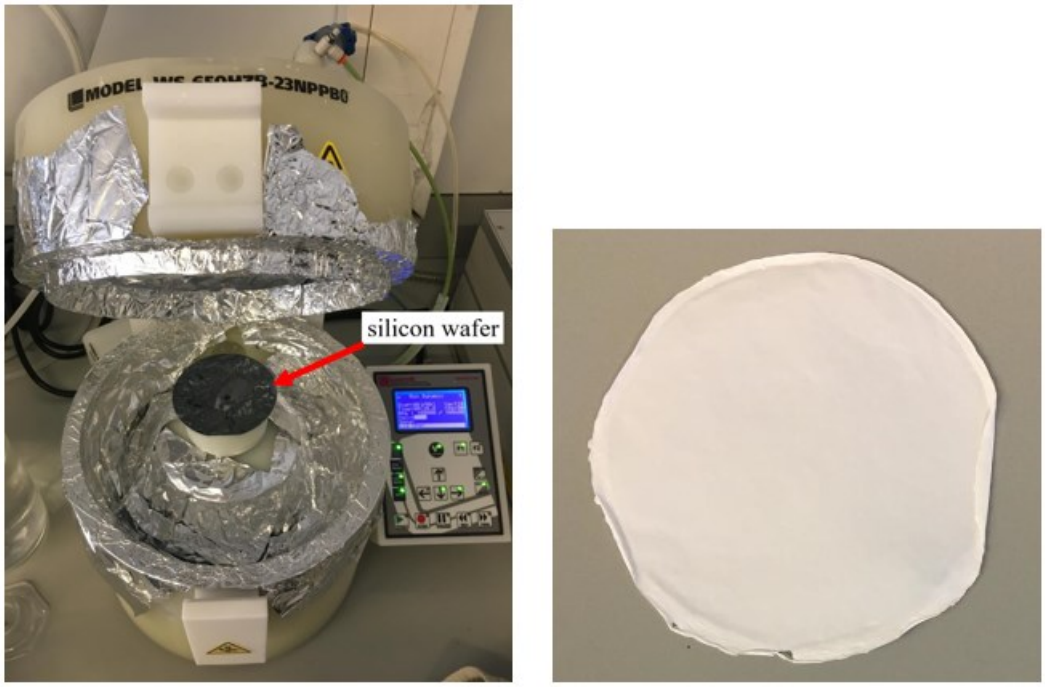


Figure 6: *Image of the spin coater with silicon wafer (left) and a dry polysulfone membrane (right).*

Table 8: *Parameters of membrane samples used in the assays.*

	Samples used for incubation in 12-well plate	**Samples used for incubation in 96-well plate**
Diameter Ø (cm)	2	0.6
Area (cm^2)	3.14	0.28
Weight (mg)	3.77 ± 0.25	0.30 ± 0.02

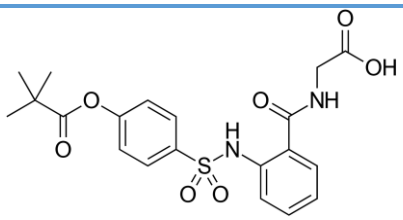
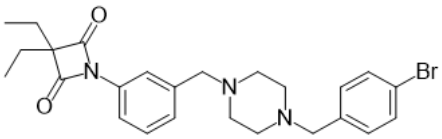
4.2.2 Neutrophil elastase inhibitors

For the following experiments focused on the development of modified PSf membranes with HNEIs, a newly *in-house* synthetized inhibitor identified as LUSA-84 containing the 4-oxo-β-lactam scaffold was provided from the group of Medicinal Chemistry, Faculty of Pharmacy, University of Lisbon, headed by Prof. Rui Moreira and compared with the commercially available HNEI, sivelestat (SIV).

To be used in the experiments, both inhibitors were dissolved in DMSO to obtain concentration of 1 mg/mL, aliquoted and stored at -20 °C. Characteristics comparing the two inhibitors, namely molecular weight, polar surface area, partition coefficient, and aqueous solubility, as well as their molecular structure are shown in Table 9.

The determined half-life of LUSA-84 (4-oxo-β-lactam inhibitor) in HEPES 0.1 M buffer solution, pH 7.4, was 12 h. Its relatively low stability in aqueous solutions is the main obstacle for preparing of the membranes by blending method (24 hours washing period).

***Table 9**: Chemical characteristics of the two tested inhibitors: SIV and LUSA-84*

	SIV	**LUSA-84**
Molecular structure		
MW (g/mol)	434.46	484.40
tPSA (Å²)	147.00	43.86
LogP	2.68	5.59
LogS	-4.50	-5.77

MW: molecular weight; tPSA: topological poral surface area; LogP: partition coefficient; LogS: aqueous solubility

4.2.3 Elastase activity assay

For determination of the inhibitory activity of HNEIs and membranes modified with inhibitors, an elastase activity assay (EAA) of HNE was performed using an enzymatic kinetic assay. The HNE activity was quantified by fluorimetry, using the fluorogenic elastase substrate V, which is hydrolyzed by HNE, releasing intensely fluorescent 7-amino-4-methylcoumarin. [46]

Before the experiment, aliquots of HNE and elastase substrate V stock solutions were prepared and stored at -20 °C to be later used for the assay. HNE was reconstituted in 1 mL of 0.05 M acetate buffer solution, pH 5.5, to obtain 3.39 µM stock solution. Prior to use, an aliquot was diluted with HEPES 0.1 M buffer, pH 7.4, to get the final concentration of 0.2 µM. The elastase substrate V was reconstituted in DMSO, to obtain stock solution of 8.0 mM. At the time of use, an aliquot was diluted 1:4 in HEPES buffer, obtaining final concentration of 2.0 mM. The fluorogenic substrate had to be protected from light during storage as well as throughout handling.

The assay was carried out in a 96-well plate with a total volume of 180 µL per well, in triplicates. The first two triplicates were always used as positive control (160 µL of HEPES buffer with 20 µL of elastase 0.2 µM) and background correction (180 µL of HEPES buffer), respectively. To determine the inhibition by HNEIs in solution, each well contained 5 µL of HNEIs solution in DMSO (well concentration of 2.5 %), 155 µL of HEPES buffer and 20 µL of elastase 0.2 µM. In the case of assessing elastase activity inhibition of modified membranes, 160 µL of HEPES buffer plus the biomaterials were used (Figure 7). In all assays the appropriate HNEIs vehicle (DMSO 2.5% or PSf membranes incubated with DMSO 2.5%) was run together with the doped membrane samples. The plate was then put to incubate at 25 °C for 30 min. After incubation, 90 µL of reaction mixture was added into a black 96-well plate containing 10 µL of substrate V 2.0 mM in each well and the elastase activity assay was carried-out. The fluorescence was measured using a microplate reader with excitation at 360 nm and monitoring of emission at 460 nm, at 25 °C for 30 min recording the fluorescence at 1 minute intervals.

The percentage of inhibition was then calculated from elastase activity (EA) as follows:

$$Inhibition\ (\%) = 1 - \left(\frac{mean\ of\ EA\ of\ positive\ control}{mean\ of\ EA\ of\ sample}\right) \times 100$$

Obtained data will be presented as percentage of inhibition of HNEIs/membranes modified with HNEIs standardized to the inhibition of vehicle/membranes incubated with vehicle sample (reference; inhibition = 0 %).

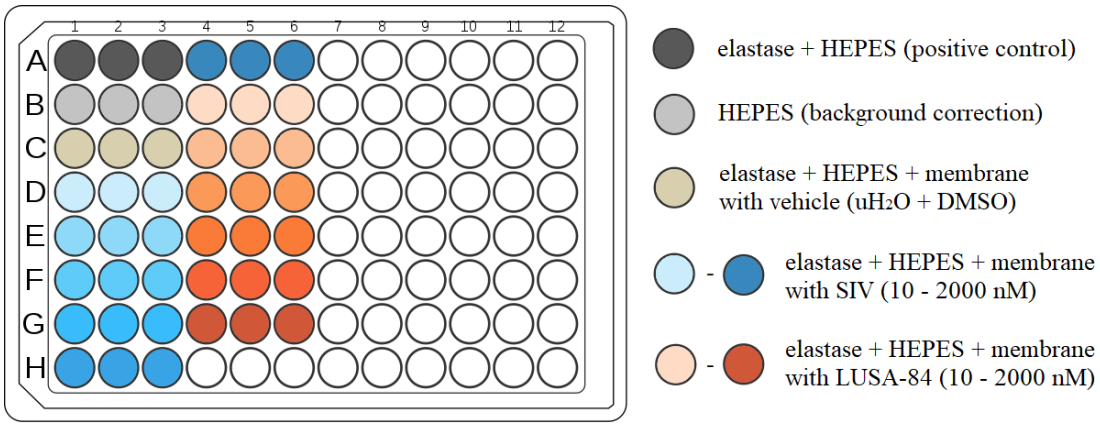


***Figure 7**: Template used in the elastase activity assays: elastase 0.2 µM, HEPES 0.1 M buffer, pH 7.4, and HNEI-modified PSf membrane samples with 0.6 cm Ø were placed into the appropriate wells of the 96-well plate for incubation at 25 ºC during 30 min.*

4.2.3.1 Inhibitory potential of HNEIs

To perform the assay, different concentrations of HNEIs were prepared to obtain the range of concentrations representing 0 to 100 % of HNE inhibition. The solutions were prepared in pure DMSO at a 40x higher concentration than the final concentration in the well in order to obtain DMSO 2.5%. The highest concentration of solutions was prepared from 1 mg/mL stock solutions in DMSO and further diluted with DMSO. Range of final concentrations can be found in Table 10. The EAA was performed as described above. DMSO 2.5% was used as reference sample. The half maximal inhibitory concentration (IC50) of each HNEI was determined using GraphPad Prism 8 software.

***Table 10**: Concentrations of HNEIs solutions used for determination of inhibitory potential (IC50).*

concentration of SIV (nM)	**concentration of LUSA-84 (nM)**
1	2
2	5
5	10
10	20
20	40
30	60
40	80
80	120
160	160
300	300
450	450
600	600
900	

4.2.3.2 Bioactivity assay of membranes modified by coating

For the bioactivity assay of modified PSf membranes with HNEIs, solutions of both inhibitors, SIV and LUSA-84, in DMSO, were prepared in 6 concentrations (10, 50, 100, 500, 1000 and 2000 nM) and further diluted 1:40 with ultrapure water (uH_2O). 2 cm Ø circular membrane samples were placed into a 12-well plate (Figure 8). 1 mL of HNEIs solution of each concentration was added into the well with membrane in triplicate, covered, and incubated for 3 hours at 25 °C, under constant agitation (60 rpm). One triplicate containing 1 mL of vehicle (DMSO 2.5%) was prepared to be used as a reference sample. After the incubation, membranes were dried on tissue paper and 0.6 cm Ø samples were cut from them to perform EAA.

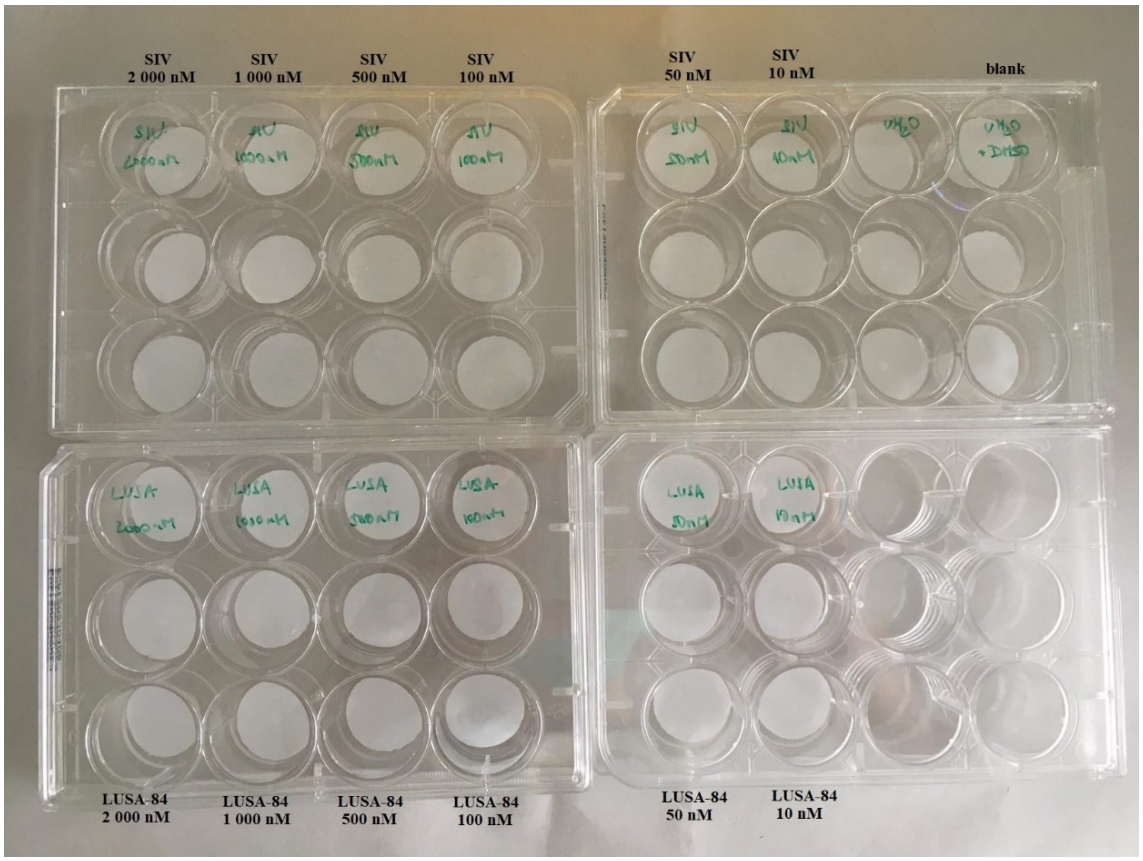


Figure 8*: Typical layout used in the 12-well plates for adsorption process: triplicates of 2 cm Ø PSf membrane samples with 1 mL of HNEIs solution into each well.*

4.2.3.3 Stability test (change in bioactivity with storage time)

In order to detect possible degradation of HNEIs or decreased elastase inhibition by the modified membranes, their stability over one month time period was tested.

PSf membranes modified by coating were monitored at 3 time points (24 hours, 1 week and 1 month) and compared with their initial activity right after adsorption. The membranes were prepared as described in 4.2.3.2 in triplicates per concentration. After drying on tissue paper, one 0.6 cm Ø sample was cut out from each membrane, placed into 96-well plate and the EAA was performed (4.2.3). The remaining membranes were stored at 4 °C until the EAA assays at different time points.

4.2.4 Biocompatibility assay

Since HD membranes are classified as medical devices in direct and long-term contact with circulating blood, it is required for them to meet the conditions for biocompatibility described in ISO 10993-4: *2017 Biological evaluation of medical devices* [53]. In PSf membranes modified with HNEIs by coating method some of the recommended assays were performed: material-induced hemolysis, platelet (PLT) activation, and evaluation of RBC morphology.

All experiments that involved utilization of whole-blood, requiring blood collection from volunteers, were approved by the Ethic Committee of Faculty of Pharmacy, University of Porto, and performed in compliance with the Declaration of Helsinki.

Three independent assays were carried out on different days using blood from different healthy individuals (2 women, 1 man; 1 volunteer per assay). Informed consent was obtained from all volunteers participating in the study. The blood was collected by venipuncture into sodium citrate collection tubes.

For biocompatibility assay, modified PSf membranes were prepared by adsorption (coating). 2 cm Ø membrane samples were incubated with HNEIs solutions of several concentrations (10, 50, 100, 500, 1000 and 2000 nM), vehicle (DMSO 2.5%), and uH_2O for 3 hours at 25 °C, in duplicates. The membranes incubated in uH_2O were used to ascertain if the vehicle by itself had an effect on biocompatibility. After incubation, the membranes were dried and stored at room temperature overnight. The next day, biocompatibility assay was performed using whole-blood diluted with sterile PBS, pH 7.4, to obtain Hb concentration 10 g/dL (t_0). The modified membranes were placed into a 12-well plate and 1 mL of whole-blood (10 g/dL Hb) was added to each well. Two extra wells contained 1 mL of blood alone (reference) and another two wells contained triton 0.01% (v/v) and blood, as the positive control for hemolysis (Figure 9). The plates were then incubated for 3 hours at 37 °C. From the initial tube (t_0), 800 µL of blood was centrifuged at 1500 g, 10 min, 4 °C, to obtain PLT poor plasma and 200 µL was centrifuged at 250 g, 15 min, 4 °C, to obtain PLT rich plasma. Both plasmas were kept at 4 °C until the end of the incubation to be used in the following assays. At the end of incubation, blood (t_1) from the wells was transferred into eppendorf tubes and homogenized for 5 min. PLT poor plasma and PLT rich plasma were obtained as

described above. PLT poor plasma was used to assess the hemolytic capacity, while PLT rich plasma was used for assessment of PLT activation.

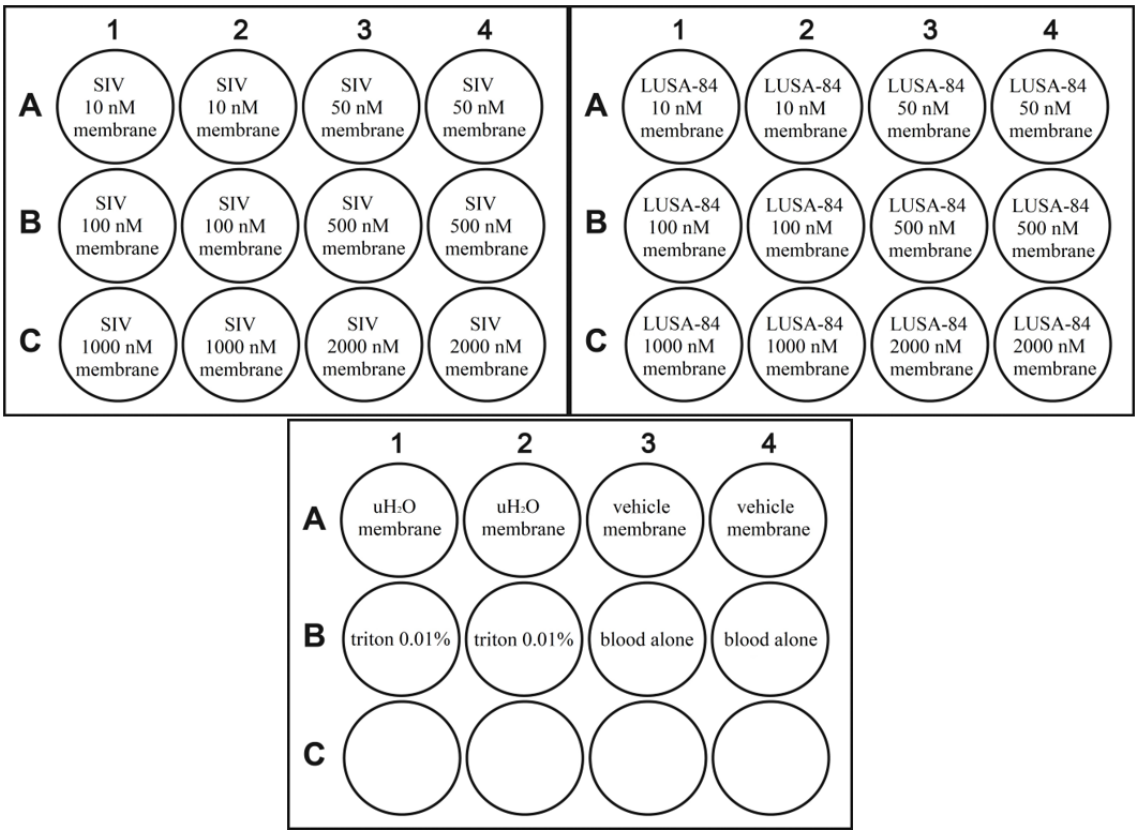


***Figure 9**: Template used in biocompatibility assay.*

4.2.4.1 Hemolysis assay

A protocol recommended by ISO 10993-4:2017 was used for determination of material-induced hemolysis of the modified membranes. This suggests the cut-off level of free Hb in plasma to be below 10 mg/dL. A material meeting this cut-off value is considered as non-hemolytic with low risk of concern. However, even higher levels may be acceptable if the benefits outweigh the risk, which needs to be thoroughly assessed. [53]

For the hemolysis assay, 50 µL of Hb standards (200, 160, 115, 75, 40, 20, 10, 4, 1 and 0 mg/dL) and PLT poor plasma samples obtained as described above (4.2.4) were added into 96-well plate, in triplicates, and the absorbance was measured spectrophotometrically at 540 nm (reference 690 nm). The plasma Hb concentration was then interpolated from the standard curve and normalized to Hb value of blood incubated without membrane.

4.2.4.2 PLT activation assay

For the PLT activation assay, the number of PLTs/μL was determined in PLT rich plasma t_0 diluted 1:4 with Leukoplate diluent solution and left to react for 10 min. Afterwards, the number of PLTs was assessed using a Neubauer counting chamber. The volume of plasma containing about 125 000 PLTs was double-stained for 15-20 min in dark with monoclonal antibody mix containing CD42a, PE (2 μL) which stains all PLTs and diluted CD62, APC (1 μL of 1:4 dilution with PBS) staining only activated PLTs. Thereafter, prepared samples were kept on ice and acquired by flow cytometer. In addition, one sample with PLT rich plasma t_0 without antibody mix and one positive control of the method (PLT incubated with PMA 1 μM, an activator of PLTs), were prepared in order to determine quadrant gates in cytometry graphs software separating different populations. PLT-specific events were analyzed using FL2 (585 nm; PE) and FL4 (675 nm; APC) filters. PLT activation was calculated as the percentage of double-positive events ($CD42^+/CD62^+$) within all $CD42^+$ events.

4.2.4.3 RBC morphology

As the HD membrane is an artificial material, its surface could cause mechanical stress to RBC, resulting in irregularities in cell's shape or even rupture of cell membrane followed by hemolysis. For this reason, blood smears were examined and evaluated for any morphological changes of RBC.

After incubation of modified membranes with blood, Wright-stained blood smears [54] were prepared from each sample and observed under optical microscope. The structure of RBCs in blood incubated with membranes was compared to blood incubated alone.

4.2.5 Test of efficacy in plasma and leaching of HNEIs from modified membranes

To prove the concept of the presented work, an assay testing efficacy of modified membranes with HNEIs in plasma was carried out. We hypothesized that human plasma contains HNE that, when incubated with the modified membranes, will irreversibly bind to HNEIs, creating an enzyme-inhibitor complex which will make them unavailable for reacting with the later added elastase in the EAA. As it was unknown whether HNE in plasma will bind to HNEIs on membrane surface or the HNEIs leached into plasma causing inhibition there, both elastase activity inhibition of membranes after incubation and plasma itself were assessed.

Solutions of both inhibitors, SIV and LUSA-84, in DMSO 2.5% were prepared in concentrations ranging from 10 to 2000 nM as described in 4.2.3.2. Membranes incubated with vehicle were used as reference. 0.6 cm Ø membrane samples were cut and placed into a 96-well plate with 90 µL of HNEIs solutions (9 membranes per NEI concentration) and incubated for 3 hours at 25 °C under constant agitation (60 rpm). After the incubation, membranes were dried and stored in eppendorf tubes overnight for further assessment. A triplicate of modified membranes was used for EAA (4.2.3) to assess their baseline inhibition. Another triplicate was incubated with 120 µL of diluted plasma (1:2 with PBS, pH 7.4) and the last triplicate with 120 µL of 0.1M HEPES buffer, pH 7.4, for 4 hours at 37 °C, to mimic the time period and temperature to which the membrane is exposed to during HD procedure. Elastase level of the diluted plasma before incubation was 17.58 ± 0.04 ng/mL. After the incubation, 64 µL of plasma/HEPES was transferred to another 96-well plate with 8 µL of elastase (0.2 µM) and incubated for 30 minutes at 25 °C. 45 µL of this mixture was added into black 96-well plate with 5 µL of substrate V (2.0 mM) and fluorescence kinetic reaction was performed to measure HNE activity. This allowed to ascertain the presence of HNEIs in plasma/HEPES in case of leaching from the membranes. In parallel, the incubated membranes were gently wiped on tissue paper and put into another 96-well plate to perform EAA after incubation.

4.2.6 Statistical data analysis

Statistical data analysis was performed using GraphPad Prism 8. Data are presented as mean ± SEM (standard error of the mean). One-way and two-way ANOVA tests with Bonferonni's multiple comparisons test were carried out, as appropriate, for analysis between groups of different concentrations within each inhibitor as well as to evaluate differences between the two inhibitors. A p-value < 0.05 was considered as statistically significant.

5. Results and discussion

5.1 Inhibitory potential of HNEIs

The first step in the characterization of the two HNEIs on study, commercial and novel, was to compare their inhibitory potential towards HNE by determining their half maximal inhibitory concentration (IC50) in solution. Regarding SIV, the supplier of the product stated that its IC50 should be between the values of 19 to 49 nM, while IC50 of LUSA-84 was unknown.

Calculation of IC50 values was done by GraphPad Prism 8 using logarithmical concentration of HNE and inhibition of HNE activity obtained from EAA. The IC50 of SIV and LUSA-84 in solution were determined to be 30.9 and 87.6 nM, respectively (Figures 10 and 11). Although the value for SIV is lower than for LUSA-84, meaning that SIV is a more potent inhibitor, we were not able to obtain 100 % inhibition by SIV even in much higher concentrations in comparison with LUSA-84, which was able to cause 100 % inhibition in concentration 600 nM. On the other hand, results for LUSA-84 varied more between the assays and a higher number of experiments (n = 4, for SIV n = 3) was needed to obtain statistically significant results. This could be caused by the fact that, as LUSA-84 is more non-polar, its solution presumably does not mix well with aqueous solutions. The 95% confidence interval (CI) of IC50 of SIV was also much narrower (CI = 24,43 to 39,16 nM) in comparison to LUSA-84 (CI = 76,8 to 99,9 nM). Although SIV proved to be a better inhibitor in terms of potency, the inhibitory activity in solution could altogether differ from their inhibitory activity when immobilized in membranes, which depends mainly on the adsorption capacity of HNEIs.

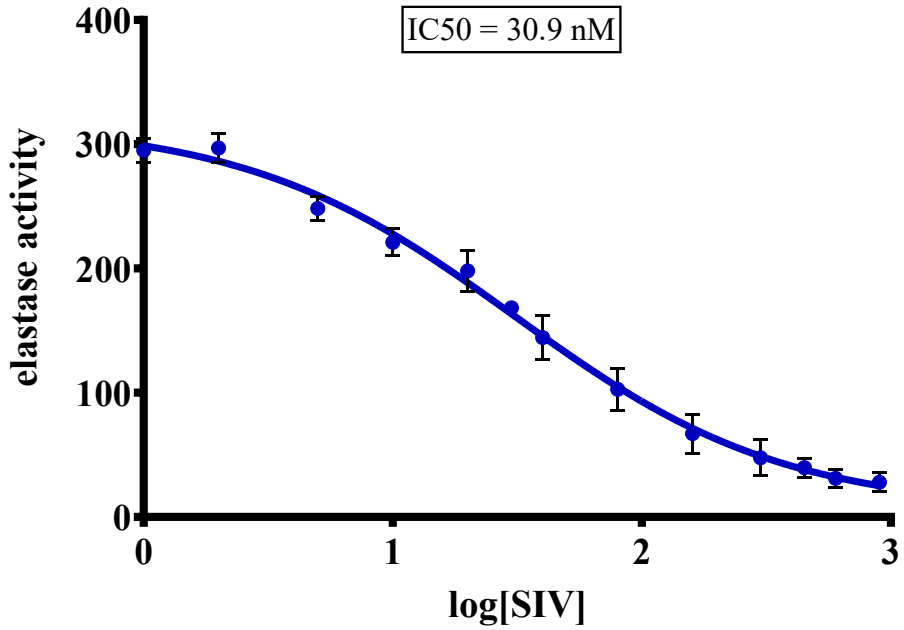


***Figure 10**: Inhibition curve of SIV in solution. The data are expressed as mean ± SEM (n=3).*

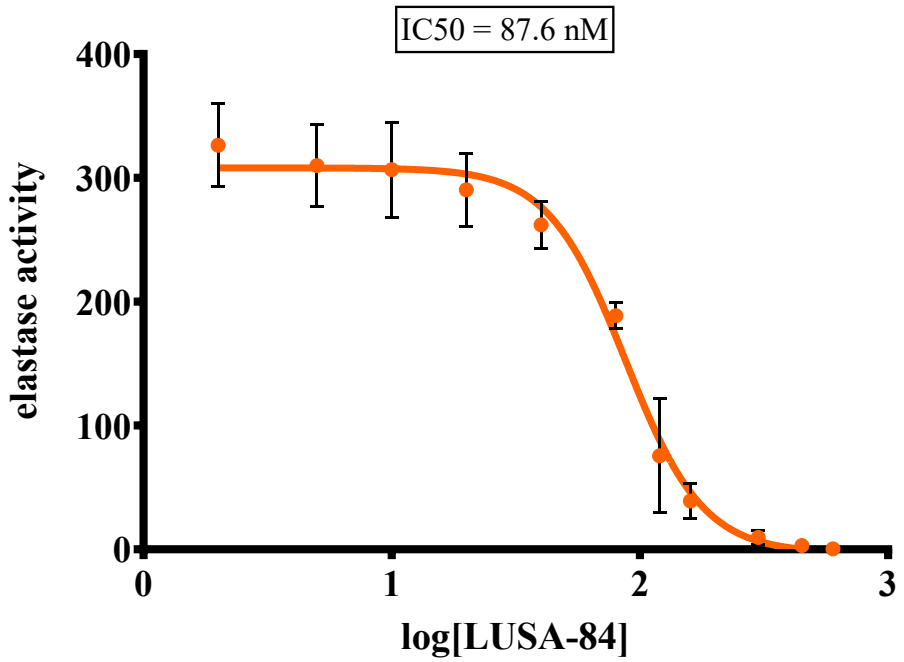


***Figure 11**: Inhibition curve of LUSA-84 in solution. The data are expressed as mean ± SEM (n=4).*

5.2 Inhibitory potential of membranes modified by coating

To modify PSf membranes with HNEIs, the process of immobilization by adsorption (coating) was selected as preferable due to low stability of HNEIs in aqueous solution. As described in 4.2.1, the final step in PSf membrane production is 24 hours washing process in ultrapure water, which might cause degradation of HNEIs, especially LUSA-84 (4-oxo-β-lactam ring opening). The HNE inhibitory activities of the modified membranes are shown in Figure 12.

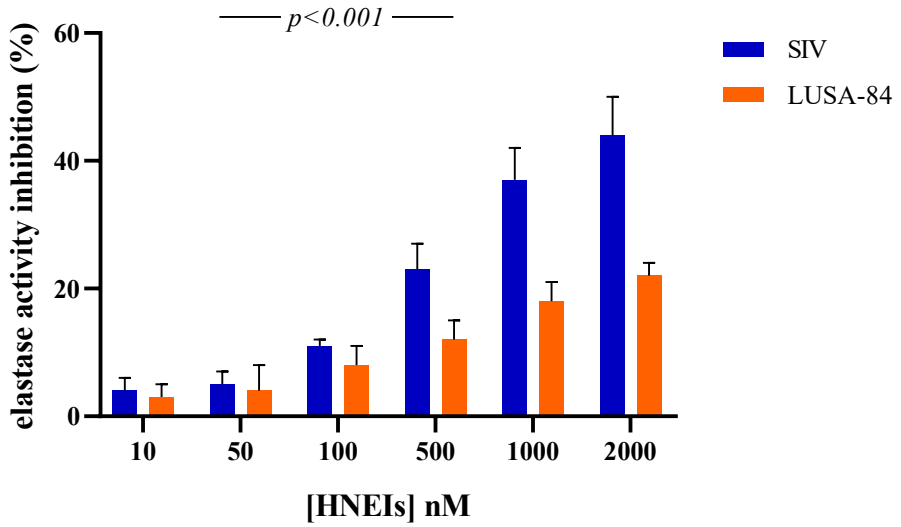


***Figure 12**: Inhibitory activity of modified membranes with HNEIs by coating. Data from 5 independent experiments are presented as mean ± SEM.*

From the results of elastase activity inhibition of modified PSf membranes with both HNEIs, we observed a positive association between concentration of the solution in which the PSf membranes were incubated (10, 50, 100, 500, 1000 and 2000 nM) and the inhibitory activity of these membranes. The maximal achieved inhibition was 44 % and 22 % for SIV and LUSA-84 membranes, respectively. These results confirmed the observations from the previous assay with SIV being the more potent inhibitor, causing also higher inhibition when adsorbed onto the membrane surface. This may be explained not only by the fact that SIV has a lower IC50 value but, also, as it is more hydrophilic inhibitor it can bind better to the membrane surface and pores with hydrophilic characteristics (hydrophilic additive). On the other hand, as it was already mentioned, LUSA-84 is not stable in aqueous solution and because of its hydrophobic nature it may not bind well onto the PSf membrane surface.

5.3 Modified membranes stability test

When we assessed the inhibitory capacity of the membranes in different time-points after immobilization of HNEIs, the difference in elastase activity inhibition for the same membrane along the storage time did not show to be statistically significant. However, it can be observed in Figures 13 and 14, that the bioactivity of LUSA-84-modified membranes loses the pattern of increasing inhibition with increasing concentration, while the bioactivity of SIV-modified membranes appears to be more stable. Nevertheless, the data presented are the results from only one experiment (performed in triplicate) and considering the variability of HNEIs' bioactivity in the EAA, especially for LUSA-84, the experiment should be repeated in order to support the given results.

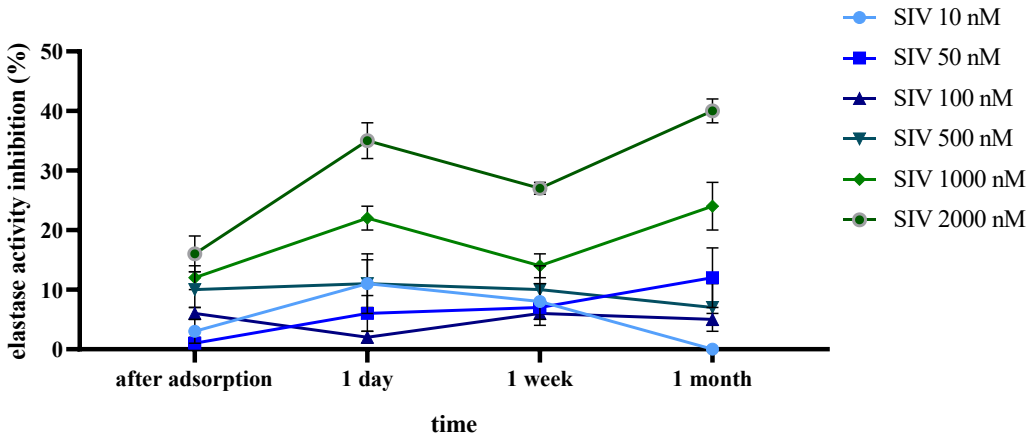


***Figure 13**: Bioactivity of SIV-modified membranes by coating along storage time (1 day, 1 week and 1 month).*

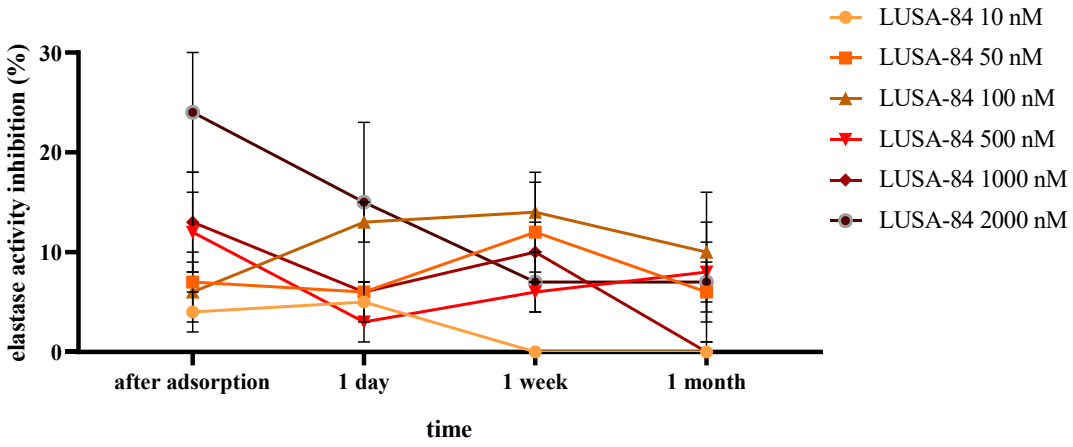


***Figure 14**: Bioactivity of LUSA-84-modified membranes by coating along storage time (1 day, 1 week and 1 month).*

5.4 Biocompatibility

5.4.1 Hemolysis

Due to possible hemolysis present in the blood alone, that cloud be caused by venipuncture, sample handling, or the incubation by itself, the results presented in Figure 15 were normalized by subtracting the concentration of Hb in blood incubated without membranes from all other samples (hemolysis = 0 mg/dL).

Concerning membrane-induced hemolysis, the concentration of plasma Hb present in uH_2O (blank) and DMSO 2.5% (reference) membranes was 33.5 ± 3.1 mg/dL and 32.2 ± 4.0 mg/dL (mean ± SEM; n = 6), respectively, being the highest values measured among all membranes. Both SIV and LUSA-84 modified membranes presented lower levels of plasma Hb. On average (compared to reference membranes), the decrease in plasma Hb concentration was 31 % and 51 % in SIV and LUSA-84 modified membranes, respectively. Even though it seemed like there was a connection between increase in hemolysis with increasing concentration of NEI in SIV-modified membranes, this was not observed in LUSA-84 modified membranes. Although none of the membranes presented plasma Hb values below cut-off level 10 mg/dL [53], the membranes modified with HNEIs appear to be biocompatible to the similar extent or even more than the unmodified PSf membrane itself. As it was already mentioned, even levels over this cut-off value could be acceptable depending on risk/benefit analysis.

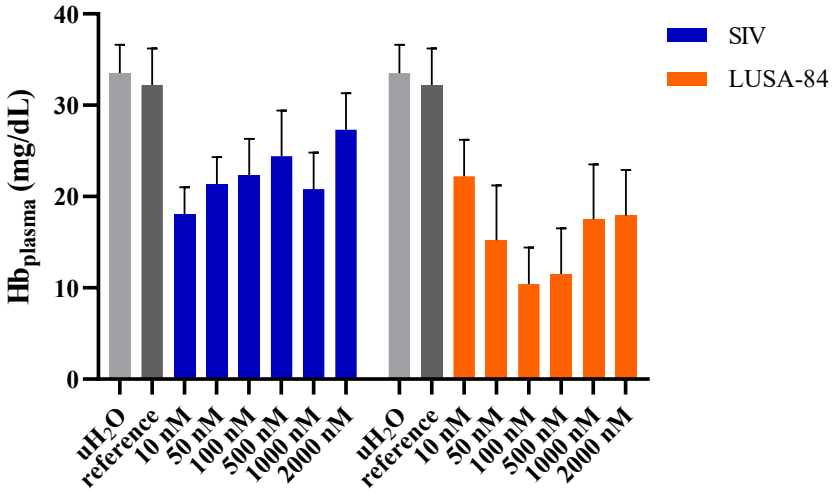


***Figure 15**: Plasma concentration of free Hb after 3h incubation at 37 ºC with modified membranes with HNEIs. Data are presented as mean ± SEM from 3 independent experiments.*

5.4.2 PLT activation

Results of PLT activation in samples (Figure 16) are presented as the percentage of activated PLTs from total number of platelets analyzed, subtracted by the percentage of PLT activation in blood incubated without membrane (negative control).

Regarding the PLT activation assay, uH_2O (blank) and reference (vehicle) membranes presented 2.1 ± 0.9 % and 1.7 ± 1.7 % (mean ± SEM) of activated PLTs respectively, without significant differences, meaning that incubation of DMSO 2.5% with membranes does not cause additional PLT activation. Modified membranes with SIV showed similar to higher levels of PLT activation (58 % increase in comparison with reference, on average), while LUSA-84 modified membranes showed similar to lower levels of PLT activation (25 % decrease in comparison with reference, on average). As above (section 5.4.1), there was no connection found between increasing concentration of HNEIs and percentage of PLT activation. Comparing modified membranes with unmodified PSf membrane (already considered biocompatible material), we can conclude that both types of new membranes, but especially the ones modified with LUSA-84 are biocompatible, with SIV-PSf membranes showing some potential for triggering pro-thrombotic events.

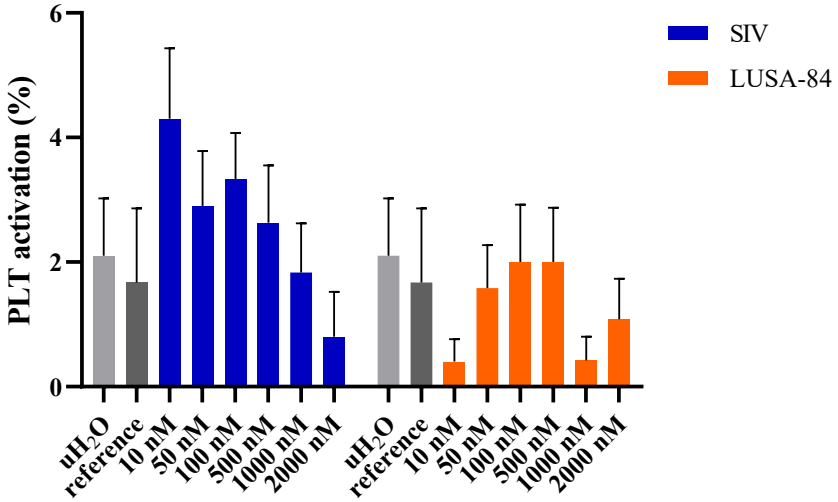


***Figure 16**: Percentage of activated PLTs after 3h incubation at 37 ºC with modified membranes with HNEIs. Data are presented as mean ± SEM from 3 independent experiments.*

However, a problem faced during this assay was the decreased count of cells after incubation. This was originally attributed to the possible binding of PLT to the membrane surface, nevertheless, decreased PLT count was also observed in the samples with blood incubated without membrane. Whether the PLTs bind to the membrane surface or not, should be further evaluated by SEM analysis.

5.4.3 RBC morphology

Observation of blood smears under optical microscope showed no changes to the RBC morphology (Figure 17), after the 3h incubation at 37 ºC. This means that contact between blood and modified membranes does not cause mechanical damage or alterations to cell shape.

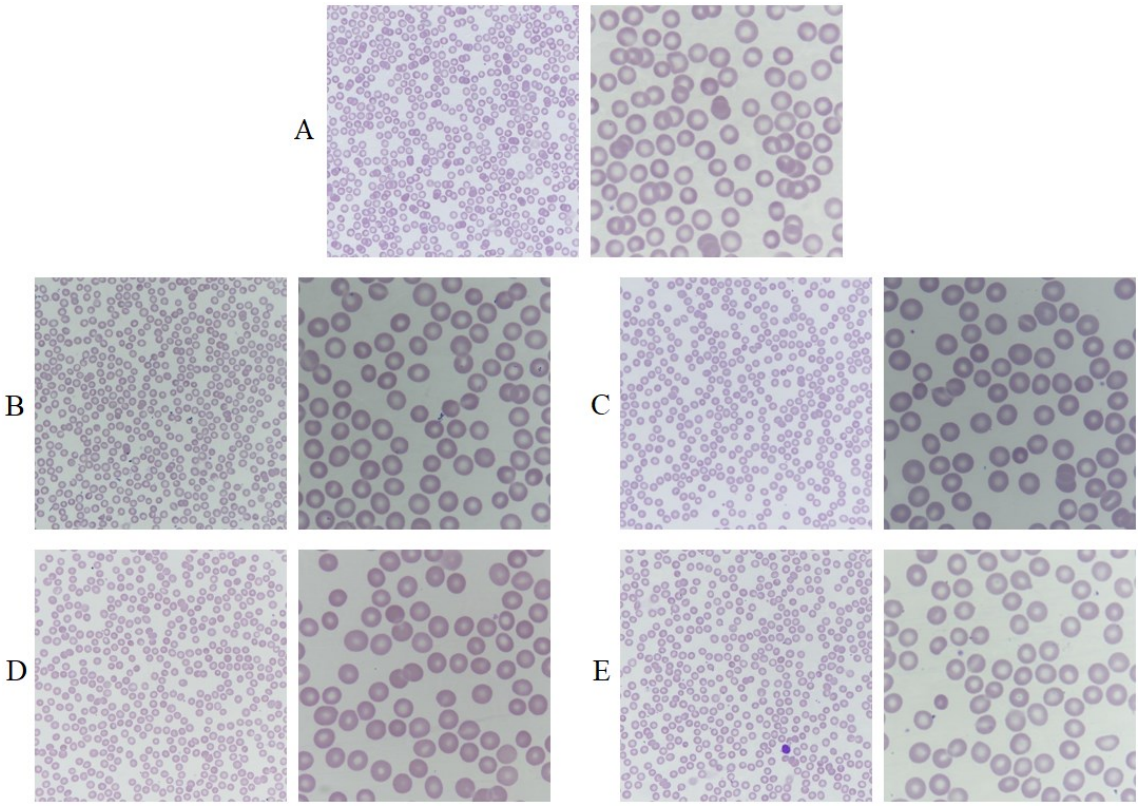


***Figure 17**: Optical microscopy images of Wright-stained blood smears after 3h incubation at 37 °C. A) blood t_0, B) unmodified PSf membrane, C) reference membrane (DMSO 2.5%), D) SIV 2000 nM modified membrane, E) LUSA-84 2000 nM modified membrane (left side – 40× magnification, right side – 100× magnification).*

5.5 Test of efficacy in plasma and leaching of HNEIs from modified membranes

Considering the test of efficacy (Figures 18 and 19) of the modified PSf membranes in plasma, it could be observed that both inhibitors presented the same pattern. Incubating modified membranes in HEPES buffer did not change their bioactivity since their inhibitory capacity remained the same as it was before incubation. However, as we expected, the membranes incubated with plasma completely lost their elastase inhibitory activity, which means that during incubation they formed an enzyme-inhibitor complex on the membrane surface, making HNEIs no longer available for the later on added elastase. Additionally, HNEIs from the membranes leached into plasma establishing the enzyme-inhibitor complex and inactivating free plasma elastase (Figure 20). In either case, we believe that the decrease of inhibitory capacity of modified membranes is caused by binding of free elastase to HNEIs, which proves the concept of our work that it is possible to reduce the amount of free circulating elastase in blood through contact with HNEI-modified membranes during HD procedure.

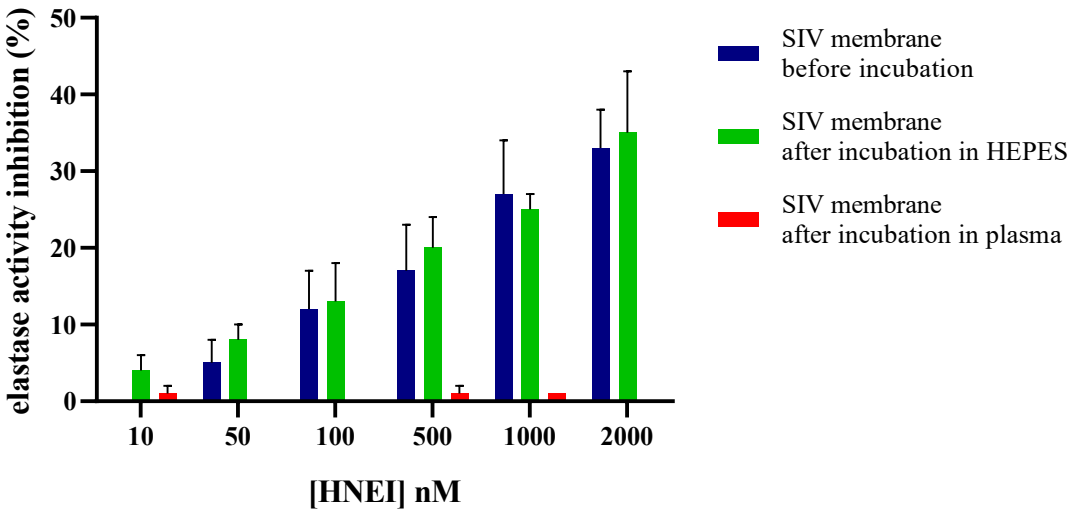


***Figure 18**: Efficacy of SIV-modified membranes after 4h incubation in plasma/HEPES buffer at 37 °C. Data from 3 independent experiments are presented as mean ± SEM.*

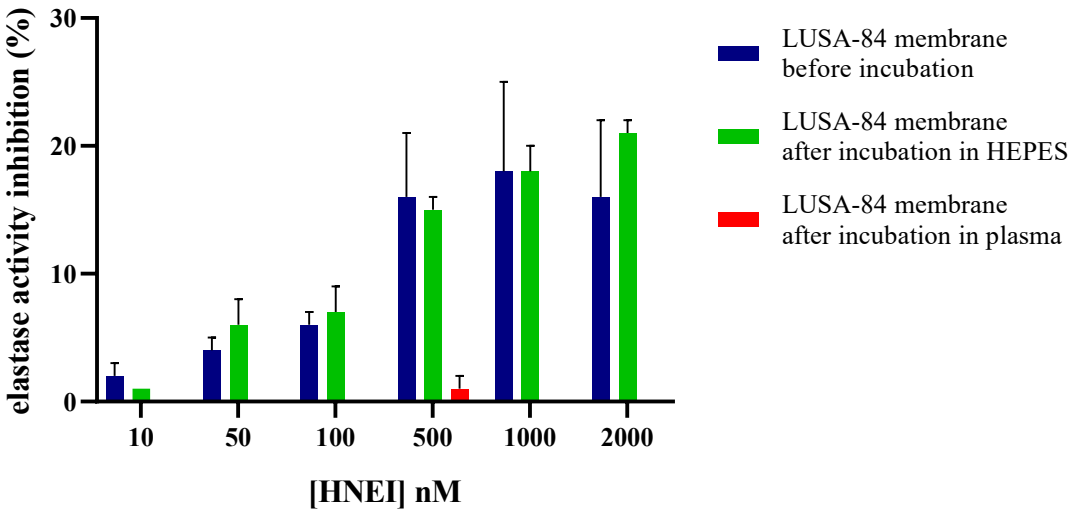


***Figure 19**: Efficacy of LUSA-84-modified membranes after 4h incubation in plasma/HEPES buffer at 37 °C. Data from 3 independent experiments are presented as mean ± SEM.*

Assessing elastase activity inhibition after 4h incubation with modified membranes (Figure 20) it can be observed, that in both plasma and HEPES buffer, from the wells in which the membranes were incubated, was expressed inhibitory activity when compared to the reference plasma/HEPES. There are no statistically significant differences between the results of SIV and LUSA-84 leaching into plasma. Although LUSA-84 presented similar and SIV higher levels of leaching into HEPES buffer, this did not result in decreased inhibitory capacity by the membranes as it can be observed above. Therefore, we presume, that the inhibition of free plasma elastase is a result of the combination of both the binding of elastase by HNEIs onto modified membranes and by leaching of inhibitors into plasma. Considering this results, further experiments on the biocompatibility of these compounds in solution are necessary to be performed.

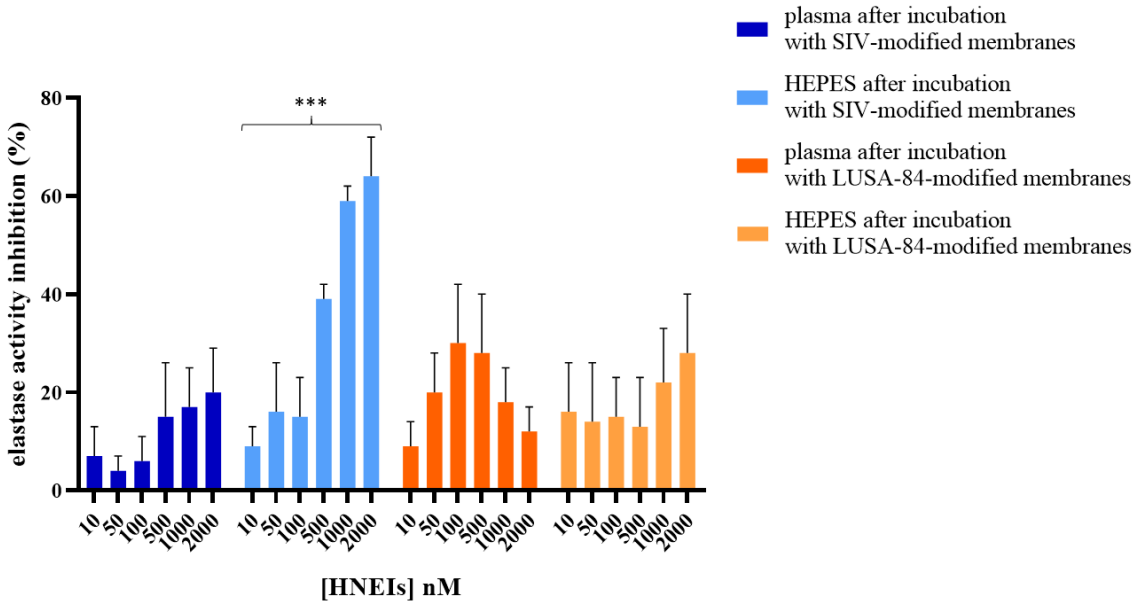


***Figure 20**: Elastase activity inhibition of plasma/HEPES buffer after 4h incubation at 37 ºC with modified membranes. Data are expressed as mean ± SEM (n=3). *** $p < 0.001$.*

6. Conclusions

This work was focused on the development and characterization of HNEI-enriched HD membranes using a novel potent and selective inhibitor with the 4-oxo-β-lactam scaffold designed by the group of Medicinal Chemistry, Faculty of Pharmacy, University of Lisbon and a well-known 2nd generation inhibitor, sivelestat. Their properties such as inhibitory activity and biocompatibility were compared using simultaneously prepared modified membranes.

We successfully managed to manufacture PSf membranes with immobilized HNEIs that proved to be effective in reducing active free HNE in plasma. Coating modification method confirmed to be an optimal method for modification of membranes with these HNEIs as we successfully produced bioactive materials. Considering the inhibitory activity, SIV-modified membranes showed to be more potent than LUSA-84-modified membranes, which followed the trend of potency of HNEIs in solution (IC50). Additionally, bioactivity of SIV-modified membranes also appeared to be more stable throughout storage (up to 1 month). On the other hand, LUSA-84-modified membranes presented a slightly better safety profile, especially regarding PLT activation. However, when evaluating overall biocompatibility, both types of modified membranes were similar to unmodified PSf HD membranes.

Considering our findings of HNEIs leaching from modified membranes into plasma, the biocompatibility of these inhibitors in solution should be tested. Moreover, additional extensive studies should be carried out using SEM analysis in order to understand better the binding of HNEIs on the membrane surface, change of the membrane structure during storage time, and possible PLT adhesion.

This work leaves an open door for further studies of inhibitors from the library of 4-oxo-β-lactam molecules and their applicability for production of HNEI-doped membranes with the best equilibrium between bioactivity and biocompatibility profiles. As the goal for these membranes is the use as medical devices, HNEI-modified membranes with the best results will be selected for further experiments testing their efficiency and selectivity in flow conditions and in *in vivo* bioactivity and biocompatibility.

The significance of development of modified membranes targeted on lowering inflammatory response in CKD patients during HD procedure is supported by the fact, that membrane modification with bioactive molecules was successfully accomplished

[55] and is now used in practice. Example of such membranes are vitamin E-coated membranes that proved its effect on reduction of oxidative stress biomarkers such as malondialdehyde and thiobarbituric acid reactive substances as well as inflammation biomarker IL-6. Additionally, these membranes presented reduction in EPO resistance index which is a common problem for anemia control in these patients. While having positive effect on oxidative stress and inflammation, these membranes do not affect dialysis adequacy. [56, 57, 58]

7. References

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