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# INSECT CHITOSAN BIOINSPIRED APPROACH FOR HAEMODIALYSIS TREATMENT

by
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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Biomedical Engineering

by
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# Resumo

A doença renal crónica (CKD) é caracterizada por um declínio contínuo na função renal, muitas vezes persistindo por meses ou mesmo anos. A prevalência da CKD tem vindo a aumentar ao longo dos anos, representando um desafio crescente para a saúde pública, prevendo-se que se torne a quinta principal causa de morte até 2040. Para pacientes com doença renal em fase terminal (ESKD), a terapia de substituição renal (RRT), seja através de diálise (hemodiálise, HD e diálise peritoneal, PD) ou transplante renal, são tratamentos cruciais, mas dispendiosos.

No entanto, para alguns pacientes com ESKD pode não ser adequado o transplante devido a contraindicações médicas, tornando a diálise a abordagem apropriada nessas situações. De facto, ambas as modalidades de diálise, HD ou DP, podem oferecer resultados semelhantes a longo prazo.

Esta tese apresenta uma exploração inovadora na extração e aplicação de quitosana de inseto (*Tenebrio molitor*) para fins biomédicos, especificamente visando a HD. Inicialmente, é demonstrado o potencial da quitosana derivada de insetos como uma alternativa sustentável e eficaz às fontes tradicionais (i.e. crustáceos marinhos). Foram desenvolvidos métodos eficientes de extração (num total entre 6 a 12 h), produzindo quitosana com propriedades físicas e químicas comparáveis à quitosana comercial proveniente de crustáceos. Primeiramente, a quitina foi isolada de *T. molitor* através de desproteinização e desmineralização, com rendimentos de aproximadamente 5%. Posteriormente, obteve-se quitosana a partir da quitina extraída utilizando um processo acelerado, resultando em rendimentos entre 65,0 ± 0,8% e 79,3 ± 0,8%. A caracterização através da espectroscopia de infravermelho com transformada de Fourier (FTIR) confirmou semelhanças estruturais com a quitosana comercial e graus de desacetilação na faixa de 73 - 75%. As propriedades bioativas da quitosana obtido de *T. molitor*, incluindo atividades antimicrobiana e antioxidante, foram avaliadas. Todos os microrganismos testados (*Escherichia coli, Staphylococcus aureus, Candida albicans, Staphylococcus epidermidis* e *Pseudomonas aeruginosa*) foram inibidos, exibindo concentrações letais mínimas entre 2 e 8 mg/mL, confirmando a atividade antimicrobiana da quitosana extraída. Além disso, a quitosana mostrou atividade antioxidante na faixa de 60 a 65 µmol equivalente Trolox/g, sugerindo o seu potencial para diversas aplicações médicas.

Nesta tese foi também desenvolvida uma membrana à base de quitosana (CH – M) especificamente para fins de HD, culminando numa fase de prova de conceito, concentrando-se na avaliação dos parâmetros de permeabilidade de difusão e retenção. As características de permeação da CH – M para ureia e albumina foram estudadas, *in vitro*, para viabilidade como membranas de HD. A ureia foi permeável para valores superiores a 70% e a albumina retida na totalidade.

Esta abordagem abrangente garantiu uma exploração e desenvolvimento de uma solução inovadora e bioinspirada para a HD, com ênfase na sustentabilidade e inovação, embora mais estudos terão de ser efetuados relativamente à biocompatibilidade, tempo de duração de simulação de tratamento, testes de porosidade, permeabilidade e, também, de hemocompatibilidade.

**Palavras – chave:** Doença renal crónica, hemodiálise, inseto, quitosana, membrana bio-inspirada.

# Abstract

Chronic kidney disease (CKD) is characterised by a continuous decline in kidney function, often persisting for months or even years. The prevalence of CKD has been increasing over the years, representing a growing public health challenge and is expected to become the fifth leading cause of death by 2040. For patients with end-stage kidney disease (ESKD), renal replacement therapy (RRT), either through dialysis (haemodialysis, HD, and peritoneal dialysis, PD) or kidney transplantation, are crucial but expensive treatments.

However, for some ESKD patients' transplantation may not be appropriate due to medical contraindications, making dialysis the appropriate approach in these situations. In fact, both dialysis modalities, HD or PD, can offer similar long-term results.

This thesis presents an innovative exploration into the extraction and application of insect chitosan (*Tenebrio molitor*) for biomedical purposes, specifically targeting HD. Initially, the potential of insect-derived chitosan as a sustainable and effective alternative to traditional sources (i.e. marine crustaceans) is demonstrated. Efficient extraction methods were developed (totalling between 6 h and 12 h), producing chitosan with physical and chemical properties comparable to commercial chitosan from crustaceans. Firstly, chitin was efficiently isolated from *T. molitor* through deproteinisation and demineralisation, with yields of approximately 5%. Subsequently, chitosan was obtained from the extracted chitin using an accelerated process, resulting in yields of between 65.0 ± 0.8% and 79.3 ± 0.8%. Characterisation using Fourier transform infrared spectroscopy (FTIR) confirmed structural similarities with commercial chitosan and degrees of deacetylation in the 73 - 75% range. The bioactive properties of chitosan obtained from *T. molitor,* including antimicrobial and antioxidant activities, were evaluated. All the microorganisms tested (*Escherichia co*li, S*taphylococcus aureus*, *Candida albicans*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*) were inhibited, exhibiting minimum lethal concentrations between 2 and 8 mg/mL, confirming the antimicrobial activity of the extracted chitosan. In addition, chitosan showed antioxidant activity in the range of 60 to 65 µmol Trolox equivalent/g, suggesting its viability for various medical applications.

This dissertation also developed a chitosan-based membrane (CH - M) specifically for HD purposes, culminating in a proof-of-concept phase for haemodialysis, focusing on the evaluation of diffusion and retention permeability parameters. The permeation characteristics of CH-M for urea and albumin were studied *in vitro* to assess their suitability as HD membranes. Urea was permeable to values of over 70 per cent and albumin was fully retained.

This comprehensive approach ensured the exploration and development of an innovative, bio-inspired solution for HD, with an emphasis on sustainability and innovation, although further studies will have to be carried out on biocompatibility, treatment simulation duration, porosity, permeability and haemocompatibility tests.



**Keywords:** Chronic kidney disease, haemodialysis, insect, chitosan, bio-based membrane

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Aos meus pais, Carlos e Sandra, aos meus avós, Maria e Alcides e à minha madrinha, Teresa.
Sem dúvida que foram as pessoas que me moldaram e fizeram a pessoa que sou hoje e que conseguiu conquistar esta (difícil) etapa. Obrigada, do fundo do coração, por me darem todo o amor possível, todos os dias cresço com vocês. E isto é literalmente verdade, porque todos os dias o meu Alcides diz "Ó Maria! Como tu cresceste, como estás tão grande!".
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***Jorge Coutinho***

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# List of abbreviations

ABTS - 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid
ACR – Albumin – to – creatinine rate
AER – Albumin excretion rate
AT-R – Attenuated total reflectance
AVFs – Arteriovenous fistulas
AVGs – Arteriovenous grafts
CH-M – Chitosan – based HD membrane
CFU – Colony – forming units
CKD – Chronic kidney disease
DD – Degree of deacetylation
E4 – Insect powder treated for 4 hours
E4D2 – Insect powder treated for 4 hours and deacetylated for 2 hours
E4D4 – Insect powder treated for 4 hours and deacetylated for 4 hours
E8 – Insect powder treated for 8 hours
E8D2 – Insect powder treated for 8 hours and deacetylated for 2 hours
E8D4 – Insect powder treated for 8 hours and deacetylated for 4 hours
eGFR – Estimated glomerular filtration rate
ESKD – End – stage kidney disease
FTIR – Fourier – transform infrared spectroscopy
GFR – Glomerular filtration rate
HD – Haemodialysis
HF – High – flux dialyzer
LF – Low – flux dialyzer
MHB – Mueller Hinton broth
MLCs – Minimum Lethal Concentrations
MRSA – Methicillin-resistant *Staphylococcus aureus*
MSSA – Methicillin-sensitive *Staphylococcus aureus*
PA – Polyamide
PD – Peritoneal dialysis
PES – Polyethersulfone
PMMA – Polymethylmethacrylate
PSf – Polysulfone
PVC - Polyvinyl chloride
PVP – Polyvinylpyrrolidone
RRT – Renal replacement therapy
µmol TE/g - µmol/Trolox equivalent per gram
URR – Urea reduction rate
ZP – Zeta potential

# CHAPTER 1

# WORK OUTLINE

*“Every champion was once a contender who refused to give up.”*

*Rocky Balboa*

# CHAPTER 1: WORK OUTLINE

## 1.1. Aim of the Thesis

The aim of this work was to develop a new bio-inspired HD membrane, using insect chitosan from *T. molitor.* Within this framework, the project focuses on the value-adding of biopolymers, particularly in the development of dialysis membrane, to improve the technology's economic feasibility, opening up the potential of membrane reutilization and sustainability. In order to achieve this objective, several steps were established, namely:

- Development of a new-fast method for chitosan extraction from *T. molitor*;
- Determination of Physico-chemical properties of insect-chitosan;
- Determination of Bioactive properties of insect-chitosan;
- Design of a chitosan-based membrane for HD purpose;
- Proof-of-concept of HD, regarding diffusion and retention permeability parameters.

## 1.2. Thesis Organisation

The present thesis is organized in 5 chapters. **Chapter 1** is composed by a theoretical context what chronic kidney disease is, its prevalence, its different stages and how it is characterised. It also presents the different possible treatments, the pros, and cons of each, as well as the prevalence of patients on RRT. After presenting the three types of RRT, the two types of dialysis (HD and PD) were discussed and, after a thorough comparison, the HD process, the different types of membranes used in dialysers and their performance characteristics were discussed in depth.

After this, it was presented information about the chitosan extracted from insects and its potential for biomedical application including its potential for HD membranes. In **Chapter 2** the aims of the project and the organization of the thesis is detailed as a guideline of the work. **Chapter 3** describes the materials and methodology used, including the extraction and characterization of the insect chitosan and of the HD membrane. **Chapter 4** presents the results obtained and their interpretation. The chitin and chitosan were characterised by FTIR and zeta potential as well as were characterised by their antimicrobial activity against six different microorganisms and Minimum Lethal Concentrations (MLC) values were achieved; the antioxidant activity of the samples was also analysed. The chitosan-based HD membrane was also tested based on the diffusion of urea and albumin. Finally, the overall conclusions of the developed work as well as the emerged limitations and suggestions for future research are presented in **Chapter 5**.

## 1.3. Outputs

**Fellowships**

Research Fellowship at Center for Biotechnology and Fine Chemistry with a “Programa de Recuperação e Resiliência (PRR)” BSc SCHOLARSHIP, project reference: “Agenda VIIAFOOD – Plataforma de Valorização, Industrialização e Inovação comercial para o Agroalimentar; PPS5.1 “- Novas formulações de produtos “verdes/biológicos”. (august 2023 – present)

Research Fellowship at Center for Biotechnology and Fine Chemistry with an “ANI – Associação Nacional de Inovação” BSc SCHOLARSHIP, project reference: “BUGS@PETS – Desenvolvimento de novos ingredientes de insectos para incorporação em rações para petfood”. (april 2022-october 2022)

**Research Articles**

Martingo, M., Baptista-Silva, S., Borges, S., & Pintado, M. (2024). An uplifting avenue upon mealworm chitosan bioactivities. (submission process)

# CHAPTER 2

# INTRODUCTION

*“Do or do not. There is no try.”*

*Yoda*

# CHAPTER 2: INTRODUCTION

## 2.1. Chronic Kidney Disease

The kidneys work as the body's excretory system, responsible for the removal of metabolic waste products, toxins, and surplus fluids from the bloodstream. Impairment in kidney function, leading to the accumulation of excessive fluids and waste substances, can precipitate a spectrum of associated health complications, contributing to cardiovascular conditions, such as heart disease and stroke.[1]

The chronic kidney disease (CKD) is marked by an ongoing decline in renal function, often persisting for months or even years.[2]

The prevalence of CKD has been increasing over the years, presenting a growing public health challenge and it is expected to become the fifth leading cause of death by 2040.[3] These prevalence data exhibit regional disparities, with a higher incidence observed in low and middle-income countries. In specific regions like Southeast Asia and the Western Pacific, CKD prevalence exceeds 10% of the population.[4]

While CKD is more frequently identified in older age groups and its risk escalates with advancing age it is by no means limited to a specific demographic. It is important to note that this disease can also affect younger individuals, particularly those harboring risk factors such as diabetes, hypertension, or a family history of kidney disease.[5]

The aging demographic in many countries adds complexity to the CKD landscape, as older adults are particularly vulnerable to CKD-related complications. Age-related declines in kidney function increase susceptibility to CKD-related health issues.[6]

As the global population continues to expand and age, the prevalence of CKD is on the rise, further underscoring its significance as a public health concern.[7]

So, it is underscored the urgent need for comprehensive strategies aimed at preventing and managing CKD effectively.[8]

### 2.1.1. Exploring Chronic Kidney Disease: Definition and stages

The symptoms of CKD are often vague and nonspecific, making it challenging to detect the condition in its early stages.

Many individuals may not experience severe symptoms until their renal disease has progressed significantly.

Common nonspecific symptoms include increased fatigue and decreased energy levels, difficulty concentrating, a diminished appetite, disrupted sleep patterns, dry and itchy skin, and increased frequency of urination, particularly at night.[9]

As the renal function deteriorates, an array of supplementary symptoms may arise, contingent upon the extent and duration of CKD, such as hypertension (high blood pressure), accumulation of urea leading to uremia, elevated levels of potassium and/or phosphates in the blood (hyperkalemia and/or hyperphosphatemia), reduced production of erythropoietin (a hormone responsible for red blood cell

production), fluid volume overload resulting in edema, deficiency of vitamin D and iron-deficiency anemia.[10,11]

In fact, a concerning aspect of this condition is the elevated risk it presents several complications and mortality, particularly those ones related to cardiovascular health, as mentioned above.[12]

By definition, CKD is characterised by a sustained reduction in glomerular filtration rate (GFR) persisting for a duration of three months or longer, without regard to its underlying etiology. [13]. The differentiation between chronic and acute kidney disease is primarily established by the requisite three-month duration.[14]

CKD is typically defined and diagnosed following the criteria outlined in the Kidney Disease: Improving Global Outcomes (KDIGO) guidelines.[13]

These guidelines specify two key diagnostic criteria: 1) a persistent glomerular filtration rate (GFR) below 60 mL/min/1.73m² for less than three months, regardless of associated renal structural damage, and 2) kidney impairment persisting for three months or longer, marked by structural or functional renal anomalies, with or without concurrent GFR decline, confirmed through pathological, compositional, or imaging abnormalities indicative of renal injury.[15]

However, these measurement methods are complex to implement. Thus, in clinical practice, GFR is typically estimated (eGFR) from the serum concentration of creatinine, an endogenous filtration marker. Hence, the GFR is the most comprehensive indicator of renal function in both healthy stages and disease contexts, while albuminuria (which signifies heightened glomerular permeability) stands as the most extensively studied marker of kidney damage.[16]

In one hand, GFR is more commonly used to classify the renal filtration rate and it is defined as the flow rate of filtered fluid through the kidneys, requiring a blood test to measure the creatinine, which is usually produced at a constant rate by the body and is normally cleared from the blood by the kidneys. If the kidneys are damaged and the glomeruli are not filtering as much as normal, the level of creatinine in the blood increases.[17]

**Table 1 -** Glomerular filtration rate categories in chronic kidney disease. [18]

| Category | GFR (ml/min/1.73 $m^2$) | Description |
|---|---|---|
| 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 | Kidney failure |

Also, it is relevant to refer that neither GFR categories G1 nor G2 meet the requirements to be considered chronic kidney disease in the absence of damage to this organ.[19]

In other hand, albuminuria is a pathological condition characterized by the presence of the protein albumin in the urine. In typical renal function, large molecules such as albumin are not filtered into the urine, making albuminuria a valuable indicator of kidney impairment. As shown in table 2, the

categorization of albuminuria levels is determined through assessment methods like the albumin excretion rate (AER) over a 24 h period or the albumin-to-creatinine ratio (ACR).

**Table 2 -** Albuminuria categories in chronic kidney disease.[18]

| Category | AER (mg/24 h) | ACR (mg/g) | Description |
|---|---|---|---|
| A1 | < 30 | < 30 | Normal to mildly increased |
| A2 | 30 – 300 | 30 – 3000 | Moderately increased |
| A3 | > 300 | > 300 | Severely increased |

Conversely, values exceeding these thresholds are regarded as indicative of pathological conditions, often necessitating further monitoring and potential intervention.[18]

### 2.1.2. Renal Replacement Therapy

The prevalence of CKD patients has also been rising not only due to life expectancy, but also due to the risk factors including obesity and diabetes mellitus have increased.[20] Up to 30% of adults 70 years of age and beyond may have chronic kidney CKD, which has a strong age correlation[10], as mentioned before. It is crucial to account for these distributions when comparing CKD prevalence because of the substantial correlation between age and the variance in the overall population age distributions throughout locations.[21]

Despite the fact that end-stage kidney disease (ESKD) patients' mortality has decreased[22], the Global Burden of Disease studies have revealed that CKD has risen to become a major global cause of death.[23,24]

For patients with ESKD, renal replacement therapy (RRT), either dialysis (subdivided in haemodialysis, HD, and peritoneal dialysis, PD) or renal transplantation, are a lifesaving but expensive treatment.[25]

For almost 50 years, high-income nations have had access to it; throughout that time, the number of patients treated has rapidly increased. Due to variations in ESKD frequency, access to and availability of RRT, and population demographics, there are significant regional variations in the use of dialysis as a treatment for end-stage kidney disease.[26,27]

The demographic shift causing this increase is anticipated to happen more in poorer nations than in developed ones, which will put financial pressure on many of them to treat more patients with ESKD using RRT.[28–30]

Around the world, 2.6 million people were undergoing kidney replacement therapy in 2020, however it's possible that a similar number of people also passed away in the same year due to a lack of access to dialysis and transplantation.[31] In contrast to this situation, the mortality impacts of other major chronic illnesses including cardiovascular disease and respiratory disease are on the decline[32].

The last report presented by EDTA-ERTA was in 2021 and the regions that contributed with data to it were: Austria, Belgium (Dutch-speaking), Belgium (French-speaking), Bosnia and Herzegovina, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France (26 of 27 regions), Greece, Hungary, Iceland, Israel, Italy (7 of 20 regions), Kosovo, Latvia, Lithuania, Montenegro, North

Macedonia, Norway, Poland, Portugal, Romania, Serbia, Slovakia, Spain (Andalusia, Aragon, Asturias, Basque Country, Canary Islands, Cantabria, Castile and León, Castile-La Mancha, Catalonia, Community of Madrid, Extremadura, Galicia, La Rioja, Murcia, Navarre, Valencian Region), Sweden, Switzerland, the Netherlands, Tunisia (Sfax region), Turkey, Ukraine, United Kingdom (England/Northern Ireland/Wales, Scotland).

According to this report and among with the contributing regions, the 3 countries or regions with the highest prevalence of patients on RRT (**Figure 1**) were Portugal, with 2003 *per million population* (pmp), Spain – Canary Islands region with 1556 *pmp* and Spain – Catalonia with 1525 *pmp*.[33]

The data depicted in **Figure 1** underscores the regional disparities in RRT prevalence, emphasizing the importance of tailored healthcare strategies and resource allocation to meet the specific needs of each population. These insights are invaluable for policymakers, healthcare providers, and researchers in the field of nephrology, facilitating targeted interventions and improved patient care.[34]

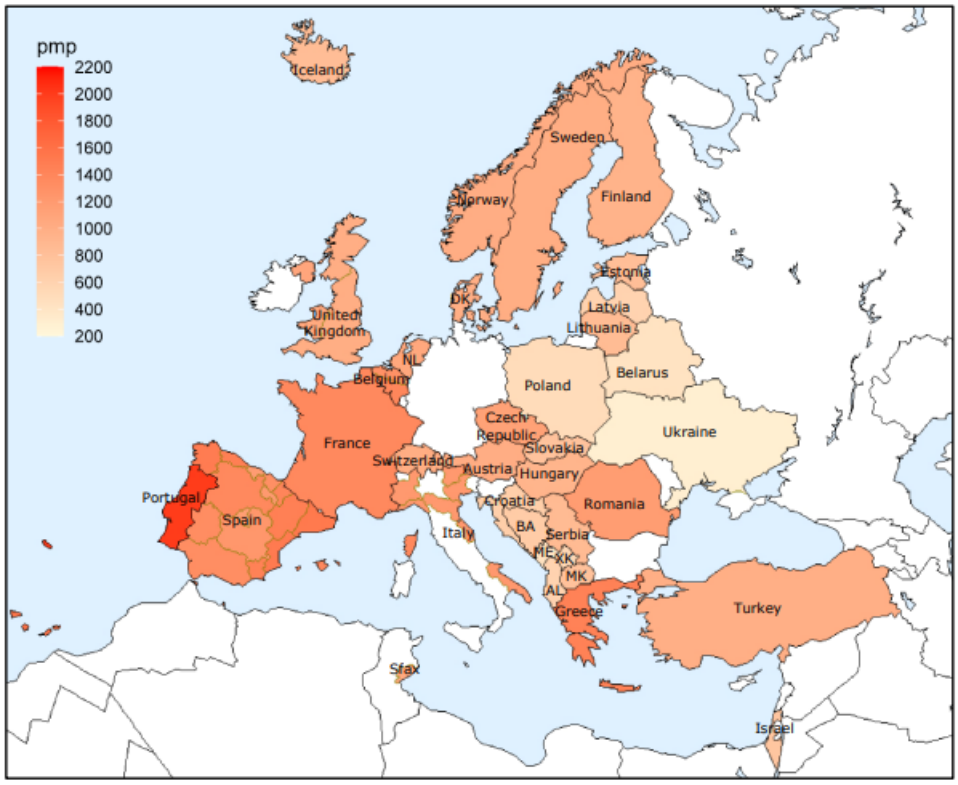


**Figure 1 -** Regional disparities in RRT Prevalence (2021).[33] This figure and its data highlighted portrays the regional disparities in the prevalence of patients on RRT during the year 2021.

The patient population receiving RRT in 2021 had a number of significant clinical and demographic characteristics. These important numbers are outlined in this scientific explanation, which emphasises their importance in the field of renal medicine and, according to EDTA-ERTA, in the same year, 62% of the prevalent patients receiving RRT in 2021 were of the male gender, indicating a noticeable gender disparity within this patient population.[33] Also, 47% of prevalent RRT patients were aged 65 years or older[33], signifying that a substantial proportion of individuals requiring RRT were in the geriatric age

group. This observation underscores the importance of tailoring renal care and treatment protocols to accommodate the unique needs of elderly patients.[35]

Within this cohort of RRT patients, the major identified cause of primary renal disease was glomerulonephritis/sclerosis, highlighting the critical role of these conditions in driving the need for RRT.[33]

The primary source of difficulties in kidney transplantation is the ongoing lack of eligible donor organs. The stringent requirements necessary for a transplant to be effective, including matching of tissue and blood types, result in a major mismatch between supply and demand.[36]

Consequently, transplant candidates frequently end up on extended waiting lists, which means they must endure the challenges of prolonged dialysis.[37,38]

Even with the amazing advancements in immunosuppressive drugs, organ rejection remains a constant concern[39], the transplanted kidney may be recognised by the patients' immune system as an alien object, starting the rejection process. A recurring difficulty for both transplant patient and healthcare professionals is striking a balance between the delicate process of preventing rejection and avoiding infections and other medication-related consequences.[40]

Other problem is the requirement for long-term immunosuppressive drugs, which presents several difficulties. Despite being necessary to prevent organ rejection, these medications have side effects that include an increased risk of infections, cardiovascular problems, and some types of cancer.[41] For the transplanted kidney to survive, patients need to carefully follow a rigorous medication schedule.[42]

Nowadays, in fact, kidney transplantation faces several challenges. The limited availability of suitable donor organs hinders transplantation efforts is more cost-effective, more survivable, and improves quality of life rather than dialysis.[43]The process of transplant surgery is complicated, and issues can occur before, during, or after the treatment. These include bleeding, infection, and problems unique to the place. Despite major advancements in surgical methods, inherent hazards still exist.

The distribution of donor organs presents difficult moral conundrums. Several variables, including waiting times, organ availability, and medical urgency, affect the decision-making process regarding transplant patients. Maintaining fair distribution of organs is still a moral concern that requires ongoing consideration and examination.[44]

Despite immunosuppression, there remains a risk of organ rejection, necessitating further transplant or a return to dialysis.[45]

Ence, a sizable portion of ESKD patients might not be a good fit for transplantation due to medical contraindications, making dialysis (i.e. HD and PD) the appropriate approach in those situations.

### 2.1.2.1. Haemodialysis vs. Peritoneal dialysis

HD and PD (**Figure 2**) are the two primary modalities employed for RRT, each offering distinct clinical, social, and economic pros and cons.

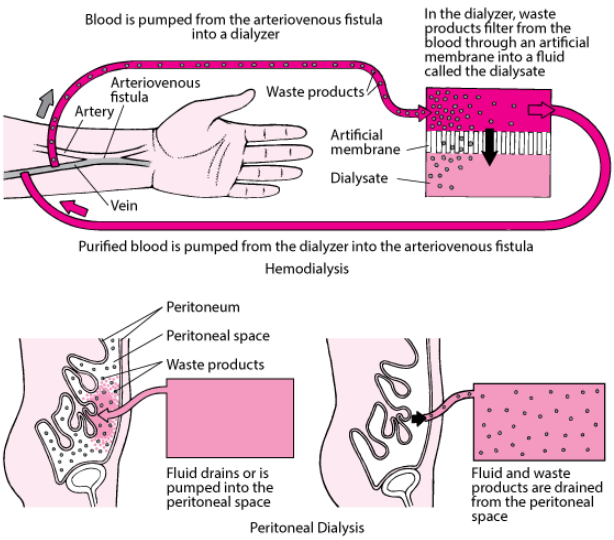


**Figure 2 -** Comparison between the mechanism of HD and PD.[46]

HD is a widely used RRT characterised by efficient toxin removal. It provides an effective clearance of waste products and excess fluids from the bloodstream.[47]

Patients receive HD under the professional oversight of healthcare providers in dialysis centers. However, this treatment demands a significant time commitment, necessitating multiple in-center weekly sessions. This schedule can disrupt patients' routines and restrict their mobility.[48] Additionally, the use of vascular access, may lead to access-related complications, being the infections and thrombosis the leading ones. Managing fluid balance can also be challenging, as rapid fluid removal may cause fluid overload or dehydration.[49]

Conversely, PD provides ongoing elimination of toxins, possibly maintaining residual kidney function.[50] It is a home-based therapy that gives patients more freedom and adaptability hence the treatment takes place at the patient's home.[51]

The fact that PD does not require invasive vascular access reduces related problems, which is a considerable benefit.[52] However, daily encounters may cause problems for patients in their daily lives. Adequate catheter self-care is essential to prevent issues such as peritonitis and other complications associated to catheter use.

Additionally, PD places a strong focus on psychological preparedness, asking people to show perseverance, adaptability, and determination in the face of potential at-home problems and daily roadblocks.[53]

Taking into the account social and economic considerations, human interactions are encouraged by in-center HD, which presents the opportunity for a positive dialysis community. But it restricts movement and can cause problems in day-to-day living.[54]

Patients' financial well-being may be impacted by losing employment opportunities and expenses associated with travelling to dialysis facilities.[55] In contrast, since PD is home-based, it improves the quality of life for its patients. It lessens the financial load and minimises interruptions to regular activities.[56] But compared to in-center HD, it could restrict social engagement, and patients would need room at home for equipment storage. Additionally, supplies and equipment such dialysate bags, tubing, and a cycler are needed (if automated PD is being utilised). These materials must be kept in storage at home[57], as shown in **Figure 3**. In contrast, patients receiving in-center HD do not have to handle the storage of their dialysis equipment because it is provided by the dialysis center.[58]


**Figure 3 -** Example of a room set-up for peritoneal dialysis.[59]

However, HD remains the dominant modality, making up around 89% of dialysis and over 69% of all RRT.[60]

It is immediately accessible and can be initiated without delay and any patients and physicians prefer the professional supervision offered by in-center HD.[61] Moreover, healthcare policies in many regions favor the expansion of in-center HD, influencing the treatment choices of patients and practitioners.[62]

In the evaluation of patient outcomes, it was revealed that a 2-year survival rate was comparable between patients' receiving PD and those on HD.

This observation suggests that both modalities may offer similar long-term outcomes, providing valuable insights for clinicians when making treatment decisions for RRT patients.[34]

In this thesis, it is placed particular emphasis on the detailed and scientific exploration of HD as a pivotal RRT modality.

### 2.1.3. The HD treatment

The blood-purifying treatment known as HD is usually administered three times a week for three to four hours each time.[63] Some patients require longer sessions to achieve adequate waste removal and fluid balance. Treatment frequency varies as well; most patients receive treatment three times a week, but some may require more frequent sessions.[64]

Blood is circulated through a device known as a dialyzer during HD, which removes extra fluid and solutes from the patient.[65] A blood circuit, a dialysate, a dialyzer, a dialysis machine, and a vascular access are the requests for this treatment[66] (**Figure 4**).

**Figure 4 -** Real-time image of a HD machine / process.

For the blood to enter the HD circuit, vascular access (**Figure 5**) is required. Currently, the options are Arteriovenous (AVFs), which are formed by joining a patient's artery to a vein.

Also, arteriovenous grafts (AVGs), which are created by joining a patient's artery to a vein using a hollow tube, and central nervous catheters, which are catheters inserted into the femoral, jugular, or subclavian vein.[67]

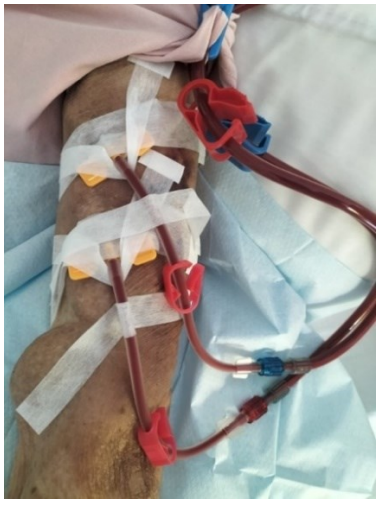

**Figure 5 -** Real-life image of a patient's AVF.

The process of cannulation, initiating the blood flow, involves the insertion of two needles by healthcare professionals. The "arterial" needle removes blood from the patient, while the "venous" needle returns cleaned blood. Proper needle placement and site rotation are crucial to ensure efficient blood flow and prevent complications.[68]

In fact, the HD machine is the heart of the process, including the blood pump to control blood flow through the dialyzer, being this one the epicenter of this therapeutic intervention, being colloquially denominated as the “artificial kidney”. This apparatus is the principal site where the sanguineous purification transpires, mirroring the filtration functionality characteristic of a healthy nephron.[69]

As the HD machinery propels blood through the dialyzer, its intricate configuration is operationalised. The dialyzer’s design incorporates a semipermeable membrane constituted of multifarious hollow fibres, which orchestrate the selective transmembrane movement of solutes. This membrane facilitates the diffusion of toxins and the osmotic flux of excess fluids from the blood, effectuating a purification process that is pivotal in the maintenance of the patient’s electrolytic and metabolic equilibrium.[70]

### 2.1.4. The role of the dialyzers in HD treatment and the interaction between its membrane, the dialysate, and the blood

In the dialyzer, as is an example of a real one in **Figure 6**, both blood and dialysate are pumped counter currently to enhance the concentration difference of the solutes and facilitate the removal of uremic toxins.[71]

**Figure 6 -** FX® CorDiax dialyzer by Fresenius Medical Care.

The dialysate fluid dialysate is a carefully formulated fluid that helps remove waste products from the blood and maintain the body's electrolyte balance (**Table 3**) and is prepared through a series of water detoxifications using activated carbon filter, reverse osmosis and distillation.[72]

**Table 3 -** Quantitative composition and therapeutic implications of dialysate constituents in HD regimen. It is relevant to note that the constituents' concentrations can vary based on individual patient needs and the specific prescriptions of their healthcare providers. This table gives a general idea of the dialysate composition used in standard HD treatments.[73]

| Component | Concentration (mmol/L) | Therapeutic implications |
|---|---|---|
| Sodium (Na+) | 135-145 | Regulates osmotic balance; Fluid removal. |
| Potassium (K+) | 0-4 | Management of the serum potassium; Crucial for cardiac function. |
| Calcium (Ca2+) | 1.25-1.75 | Essential for neuromuscular activity; Vascular activity. |
| Magnesium (Mg2+) | 0.5-1 | Required for various biochemical reactions; Blood pressure regulation. |
| Chloride (Cl-) | 100-108 | Balances cationic charges to prevent electrolyte disturbances |
| Bicarbonate (HCO3-) | 32-40 | Corrects metabolic acidosis; Maintains acid-base balance. |
| Glucose (C6H12O6) | 0-5.5 | Provides caloric substrate; Aids in osmotic fluid removal. |

During HD, the dialysate fluid's solute content is carefully controlled to maintain physiological homeostasis.[74] In adults, dialysate flow rate is normally set to 500 mL/min primarily to achieve adequate removal of small solutes (e.g. urea, creatinine, and phosphate).[75]

As mentioned, the purpose of the dialyzers is to balance the electrolyte content of the patient's blood and eliminate uremic poisons and extra water from it. **Table 4** illustrates how uremic toxins are categorised according to their molecular weight.[76] In HD, the molecular weight of solutes is integral to their selective transmembrane transport.[77]

Low molecular weight compounds such as urea (60 Da), creatinine (125 Da), and phosphate (134 Da) are efficiently cleared from the bloodstream, given their facile diffusivity through the semipermeable dialysis membrane. Conversely, macromolecules like albumin (68 kDa) and transferrin (90 kDa) are retained due to their substantial molecular dimensions, which exceed the membrane's pore size threshold. This selective permeability is pivotal, as it ensures the removal of metabolic waste while preserving critical plasma proteins essential for homeostatic functions.

**Table 4 -** Small and large molecules and corresponding molecular weights.[78]

| Molecule size | Molecule name | Molecular Weight |
|---|---|---|
| Small | Urea | 60 Da |
| | Creatinine | 125 Da |
| | Phosphate | 134 Da |
| Large | Albumin | 68 (kDa) |
| | Transferrin | 90 (kDa) |

In an HD system, the interaction between the dialysate and blood occurs through the semi-permeable membrane within the dialyzer, based on the principles of diffusion and osmosis. This process is crucial for effectively cleansing the blood of patients with kidney failure.[79]

The patient's blood, containing waste products (urea, creatinine, and phosphate) flows inside the hollow fibre membranes of the dialyzer. Surrounding these fibres is the dialysate, a specially formulated fluid that flows outside the fibres in a counter-current direction to the blood flow. This setup is essential for maximising the efficiency of waste removal.[80]

As the blood passes through the fibres, waste products and excess water in the blood diffuse through the pores of the membrane into the dialysate.

This diffusion is driven by the concentration gradient, where these waste products are in higher concentrations in the blood compared to the dialysate. Simultaneously, excess water moves from the blood into the dialysate by osmosis, facilitated by the concentration gradient of solutes.[81]

As mentioned before, the membrane's selective permeability plays a pivotal role in this process. It allows waste products and excess water to pass through while retaining larger blood components such as red blood cells and proteins. This selective barrier ensures that essential elements of the blood are preserved while unwanted substances are removed. Once the exchange is complete, the cleansed blood is returned to the patient's body, and the dialysate, now containing the removed wastes and excess fluid, is discarded. This interaction within the dialyzer is critical for the effective and safe treatment of patients undergoing HD.[81]

### 2.1.5. The type of membranes used for HD treatment.

Hollow fibre membranes (**Figure 7**), the predominant type used in contemporary HD, are designed to maximize the surface area for dialysis in a compact form. These dialyzers consist of thousands of tiny, hollow fibres, each acting as a semipermeable membrane.

Blood flows through these fibres, while dialysate flows around them in the outer compartment. This arrangement facilitates the efficient transfer of solutes and fluids across the membrane, adhering to the principles of diffusion and convection.[82]

The fibres themselves are typically made from synthetic materials like polysulfone (PSf) or polyethersulfone (PES). The diameter and wall thickness of these fibres are precisely engineered to optimize treatment efficacy and patient safety.

Such design innovations in hollow fibre dialyzers have significantly improved the quality of HD treatment, making it more efficient and safer for patients with renal failure.[83]

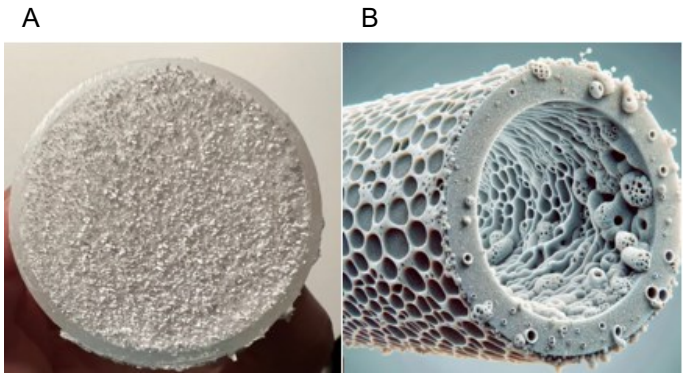


**Figure 7 -** (A) - Top view of FX® CorDiax haemodialysis dialyzer by Fresenius Medical Care displaying the arrangement of the hollow fibre membranes; (B) - Magnified illustration of the interior of a hollow fibre membrane used in a dialyzer for haemodialysis, the intricate structure and texture of the membrane wall. This image was created with the assistance of DALL·E 2.

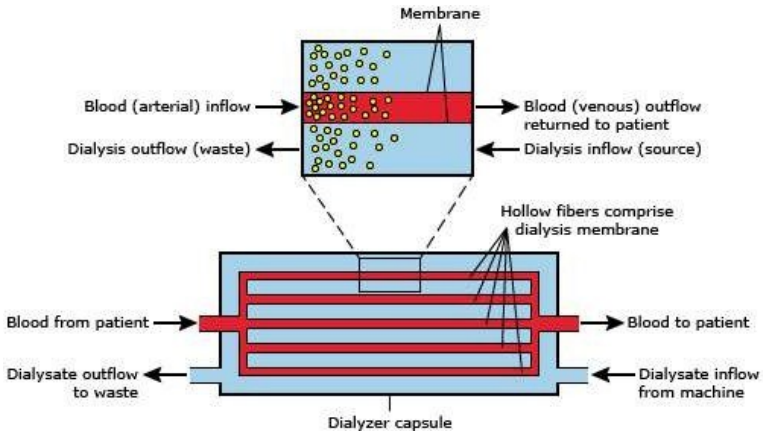


**Figure 8 -** Schematic of the solute movement in dialyzers.[82]

The dialysate circulates in countercurrent around the capillaries as blood flows inside of them (**Figure 8**). Via its pores, the dialyzer membrane functions as a sieve for the passage of fluids and solutes.
Dialyzers are categorised as either high flux (HF) or low flux (LF) based on the membranes' capacity to permeabilize water. Larger solutes cannot be effectively removed by LF dialyzers (ultrafiltration coefficient less than 15 mL/h/mmHg), however minor uremic toxins can be removed with sufficient effectiveness.
On the other hand, both tiny and big uremic toxins may be removed with comparatively satisfactory results using HF dialyzers (ultrafiltration coefficient more than 15 ml/h/mmHg). The ultrafiltration profile is volumetrically controlled by an ultrafiltration pump which creates a negative pressure gradient in the dialyzer.[80]

### 2.1.5.1. Dialyzers' membranes over the time

The composition of the membrane in a dialyzer is a critical determinant of its performance in facilitating the removal of waste products during the dialysis process.[84]

Historically, cellulose-based membranes were extensively utilized in dialyzers' making. Among these, cellulose acetate and cellulose triacetate were common choices. These materials offered a natural and porous structure, facilitating the effective removal of waste products from the blood. The inherent porosity of cellulose-based membranes played a crucial role in separating toxins while preserving essential blood components. However, as technology progressed, there was a notable shift towards synthetic alternatives.[85]

In modern dialyzer designs, there is a prevalent adoption of synthetic membranes made from materials such as PSf or PES. These synthetic alternatives bring several improvements to the area.[86] Firstly, they provide enhanced control over pore size[87], allowing for more precise regulation of the substances that can pass through the membrane. This attribute contributes to improved biocompatibility, as it minimises the risk of adverse reactions during the dialysis process.[87]

Furthermore, synthetic membranes reduce the risk of complications associated with cellulose-based materials, such as inflammation or clotting. The evolution towards synthetic alternatives reflects a commitment to enhancing the overall effectiveness and safety of the dialysis procedure.[88]

### 2.1.5.2. Current membranes used in HD.

Dialyzer performance may be maximised by carefully selecting the kind of membrane, considering aspects like cost-effectiveness, permeability, and biocompatibility.

These days, a few businesses dominate the worldwide HD market, and they are all competing for the same customers by producing high-quality membranes for dialysis machines.

To date, Fresenius's PSf-based dialyzer is known as one of the best options in the market since offers optimal biocompatibility and solute removal.[89] This dialyzer has always been used as the main reference to the development of new dialyzers over the recent years. Recently, Fresenius presented a new class of PSf-based dialyzers, which combine a new housing design and an advanced membrane (FX-class).[89]

Dialyzers can be made from various polymeric materials, including PES and polymethylmethacrylate (PMMA), in addition to PSf.

For instance, Baxter's Polyflux® and Revaclear® dialyzers employ membranes composed of polyamide (PA), polyvinylpyrrolidone (PVP), and PES, while Toray Medical Co. Ltd.'s Filtryzer® series uses PMMA membranes. The aim of this product, manufactured in Tokyo, Japan, is to offer high biocompatibility and effective removal of intermediate and small-molecular toxins.[90]

The natural and porous nature of these materials made it easier to efficiently remove waste from the blood while preserving vital blood components, thanks in large part to the intrinsic porosity of cellulose-based membranes. However, with the development of technology, there has been a noticeable shift towards synthetic substitutes.[91]

Recently, big names in the biomedical industry like WEGO in Shenzen, China, and Nipro in Osaka, Japan, have been making strides in dialyzer production. **Table 5** shows all the dialyzers the major companies are making.

**Table 5 -** Commercial dialyzers in the current market.[83]

| Country | Series Name | Brand | Polymeric Material(s)[a] |
|---|---|---|---|
| Germany | FX-class | Fresenius | PSf |
| | F-Series | | PSf |
| | Hemoflow™ | | PSf |
| | Purema | Membrana | PES |
| The United States of America | Polyflux | Baxter | PVP and PA |
| | Theranova | | PAES and PVP blend BPA-free |
| | Revaclear | | PAES and PVP blend BPA-free |
| | Xevonta | B-Braun | PSf |
| | Diacap Pro | | PSf |
| Japan | ELISIO S | Nipro | PES |
| | Sureflux | | CTA |
| | SolaceaTM | | CTA |
| | APS-U | Asahi | PSf |
| | ViE Series | | Vitamin E-coated PSf |
| | Rexeed Series | | PSf |
| | Toraysulfone TS | Toray | PSf |
| | Filtryzer | | PMMA |
| | Renak | Kawasumi | PSf |
| China | F15 | WEGO | PSf |
| | HF15 | | PSf |

[a] - BPA (bisphenol A); CTA (cellulose triacetate); EVAL (ethylene vinyl alcohol copolymer); PA (polyamide); PAES (polyarylethersulfone); PES (polyethersulfone); PMMA (polymethylmethacrylate); PSf (polysulfone); PVP (polyvinylpyrrolidone).

Synthetic membranes composed of PSf or PES are widely used in dialyzer designs nowadays. These artificial substitutes provide various enhancements.

In first place, they offer improved control over pore size, making it possible to regulate the molecules that can flow through the membrane with more precision. This characteristic reduces the possibility of negative responses during the dialysis procedure, which improves biocompatibility.[92]

In fact, the transition from cellulose to synthetic membranes in HD can be attributed to advancements in biocompatibility, performance, and patient outcomes, supported by technological innovations.[93]

Nevertheless, cellulose membranes were once widely used in HD, but were associated with biocompatibility issues. These primarily included complement activation, leading to inflammatory responses in patients. This activation was observed due to the direct contact of blood with the cellulose membrane, triggering the body's immune response and resulting in complications such as dialysis-related amyloidosis.[94]

In addition, compared to synthetic membranes, cellulose membranes demonstrated lower efficiency in removing middle molecules and uremic toxins. Synthetic membranes like PSf and PES showed superior clearance rates for these molecules, contributing to more effective dialysis treatment.[95]

The limited permeability of cellulose membranes restricted their ability to filter larger molecular weight solutes, a limitation addressed by the high-flux design of many synthetic membranes.[89]

The development of new synthetic materials revolutionised HD. These materials offered enhanced biocompatibility, higher flux rates, and better clearance profiles. The introduction of these advanced membranes allowed for more efficient and safer dialysis procedures, addressing many of the limitations inherent in cellulose membranes.[85]

Patient outcomes with synthetic membranes were significantly improved. The high-flux synthetic membranes were associated with lower rates of dialysis-related complications and better overall health outcomes, including improved control of uremic symptoms. Despite the shift to synthetic materials, modified cellulose membranes are still used in certain scenarios, especially where cost considerations are paramount.[96]

Modifications to cellulose membranes, such as acetate-free biofiltration (AFB) membranes, have improved their biocompatibility. However, they generally do not match the performance metrics of their synthetic counterparts.[97] The development of synthetic membranes like those made from PSf and PES has been a significant step forward in improving patient outcomes in dialysis. However, modified cellulose membranes still find niche applications, underscoring the complexity of membrane selection in HD based on individual patient needs and treatment objectives.

A development in the quest for improving dialyzer membranes involves the incorporation of chitosan. It is derived from chitin found in the exoskeletons of crustaceans, insects, and other sources and is a biocompatible and biodegradable material.[98]

Chitosan has antimicrobial properties, potentially reducing the risk of infections during dialysis. Moreover, it may contribute to the overall biocompatibility of the membrane, making it a promising avenue for further research and innovation in dialyzer technology.[99]

In conclusion, the composition of dialyzer membranes has evolved over time, transitioning from cellulose-based materials to synthetic alternatives with improved biocompatibility and control over pore

size. The exploration of materials like chitosan highlights the continuous efforts to enhance the performance and safety of dialyzer membranes, ultimately aiming to improve the quality of care for individuals undergoing RRT.[100]

## 2.2. Insect as a source of chitosan and its use in biomedical fields

As natural sources of chitin and chitosan, edible insects are boundless. As part of their life cycle, insects must frequently modify their structures for growth and development, which requires continual synthesis and decomposition of chitin. Due to their strong reproductive capabilities and small size, insects also have a short generation time and breeding period. Additionally, environmental contamination for breeding can be decreased, and it's critical to develop alternative sources of chitin and chitosan because sea pollution is reducing the harvest of snow crabs, a key source of chitin and chitosan.[101]

Insects are now increasingly used as a source of food due to their sustainability, nutritional value, and potential to address global food security challenges. Research has shown that insects are a highly efficient source of protein, requiring less feed, water, and land than traditional livestock, while emitting fewer greenhouse gases and contributing to a more sustainable food system overall.[102] They are a diverse and abundant food source, with over 1,900 species currently consumed worldwide. Popular species include crickets, mealworms, grasshoppers, and beetles, and they can be consumed as a whole or processed into powders and incorporated into a variety of food products such as energy bars, snacks, and even pasta.[102]

In Europe, insect consumption was approved by the European Food Safety Authority in 2015, and several companies have since begun selling insect-based products. In the United States, the FDA approved the use of crickets as a food ingredient in 2018, paving the way for wider acceptance of insects as food.[103]

In addition, insect farming can provide income and employment opportunities for communities in rural areas.[104]

Despite the potential benefits of insect consumption, there are still challenges to be addressed, including consumer aversion, regulatory hurdles, and the need to develop sustainable and scalable insect farming practices. However, with increasing demand for sustainable and nutritious food sources, it is likely that insects will play an increasingly important role in our diets in the future.[105]

A typical edible insect that is found all over the world is the mealworm (*T. molitor*), which is a member of the Coleoptera family.[106]

Mealworms have a brief life cycle, lasting just three months on average[107], making quick scale manufacturing practicable. Due to their fast rate of reproduction and the ease of breeding, mealworms are extremely simple to industrialise.[108]

Chitin, which serves as the exoskeleton's primary building block, is produced when mealworm larvae transform into pupae.[109] Chitin (poly (β-(1 → 4)-N-acetyl-D-glucosamine) can be extracted from the exoskeletons of crustaceans, such as shrimp and crab, by treating them with alkali and acid to remove proteins, lipids, and minerals[110], that, for commercial applications, are the most common ones, once they contain 15%-20% of chitin.[111]

Chitosan is a naturally occurring biopolymer composed of glucosamine and N-acetylglucosamine units[112] and is typically produced by the deacetylation of chitin, which involves the removal of the acetyl groups from the N-acetylglucosamine units in chitin (**Figure 9**).[113]

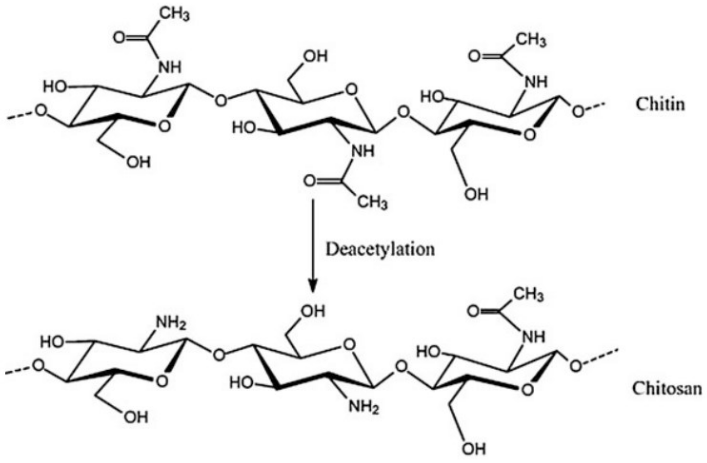


**Figure 9 -** Chemical structure of chitin and chitosan after deacetylation.[114]

Also, chitosan derived from insects is widely available by virtue of its high reproductive rate, ease of reproduction, and higher endurance to changes in their environment. Furthermore, it requires more moderate conditions of extraction, compared to the ones required for crustaceans. Also, insects have a larger production of chitosan material than crustaceans.[115]

Increasingly, chitosan has been serving a variety of functions in industries besides food, including sewage treatment, pesticides, disinfectants, and fisheries.[116]

Chitosan has been also widely used in medical fields (**Table 6**), such as anticoagulant, antihypertensive agent, to reduce the risk of vascular disease and to have anticancer effects that inhibit the growth of cancer cells, to promote the growth of beneficial bacteria in the intestine and activate cells by adsorbing and excreting too much harmful cholesterol from the body[117] and it revealed to have an immune-boosting action.[118]

**Table 6 -** Biomedical applications of the chitosan.

| Biomedical Application | Description |
|---|---|
| Wound Healing | Chitosan promotes haemostasis and accelerates tissue regeneration in wound sites.[119] |
| Drug Delivery Systems | Utilized for targeted and controlled drug delivery, especially in cancer treatment.[120] |
| Tissue Engineering | Serves as a scaffold in regenerative medicine, supporting cell growth and differentiation.[121] |
| Antimicrobial Agents | Exhibits antibacterial and antifungal properties, useful in preventing infections.[122] |
| Dental Applications | Used in dentistry for periodontal regeneration and as a component in dental implants.[123] |
| Vaccine Adjuvants | Enhances the efficacy of vaccines by improving immune response.[124] |
| Hemostatic Agents | Effective in blood clotting.[125] |
| Ophthalmic Applications | Employed in ocular drug delivery systems for treating eye diseases.[126] |
| Weight Loss Supplements | Used in weight management products due to its fat-binding properties.[127] |

Furthermore, synthetic membranes reduce the risk of complications associated with cellulose-based materials, such as inflammation or clotting. The evolution towards synthetic alternatives reflects a commitment to enhancing the overall effectiveness and safety of the dialysis procedure and, so, adding chitosan is an interesting advancement in the search for better dialyzer membranes.[128]

Due to its antibacterial properties, chitosan may lower the chance of infections when receiving dialysis, which makes it a viable opportunity for a future study and advancement in dialyzer technology.

In summary, the materials that make up HD membranes have changed over time, moving from cellulose to synthetic alternatives that have better biocompatibility and control over pore size. The investigation of materials such as chitosan underscores the continual endeavours to augment the efficacy and safety of dialyzer membranes, with the goal of elevating the standard of care for patients receiving RRT.

# CHAPTER 3

# MATERIALS AND METHODS

*“A dream is only a dream until you decide to make it real.”*
*Harry Styles*

# CHAPTER 3: MATERIALS AND METHODS

## 3.1. Standards

All standards and reagents, including commercial chitosan of medium molecular weight (degree of deacetylation in the range of 75%, viscosity-based molecular weight of 190,000-310,000 Da) were obtained from Sigma-Aldrich (Sintra, Portugal) unless otherwise noted.

## 3.2. Microbial strains used for antimicrobial activities

For the antimicrobial assay, *Staphylococcus aureus* (MRSA, DSM 11729), *Candida albicans* (DSM 3454) and *Staphylococcus epidermidis* (DSM 20044) were provided from DSM Pharmaceuticals Inc (Durham, North Carolina) and *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (MSSA, ATCC 29213) and *Pseudomonas aeruginosa* (ATCC 10145) were provided from American Type Culture Collection (Manassas, Virginia).

## 3.3. Microorganism inoculum preparation for the antimicrobial experiments

Loopful of culture from each previously inoculated MHA was transferred into Muller Hinton Broth (MHB) (Biokar, France).
The broth was then incubated at 37 °C overnight. Then the concentrations of these suspensions were adjusted with the turbidity of 0.5 McFarland equal to $1.5\times10^8$ colony-forming units (CFU)/mL). The turbidity of the microorganism suspension was prepared in sterile saline and measured at 600 nm using a Mini 1240 UV-Vis Spectrophotometer (Shimadzu, Japan).

## 3.4. Chitin and chitosan Extraction

The samples of *T. molitor* larvae (Gotanbug, Portugal) were grinded to obtain a powder to be used in the experiments. To extract the chitin and chitosan from *T. molitor*, the method from Chae-Shim Shin[129] et al. was used with modifications.

### 3.4.1. Chitin extraction

Two processes were tested varying the extraction time. In each, the dried insect powder (20 g) was alkaline treated with 10% (w/v) NaOH solution (Merck, Darmstad, Germany) at 90 °C for 2 h (E4) or 4 h (E8) with the objective to remove protein, fat, and color. The samples that were obtained were washed in the distilled water until the pH value became neutral. Subsequently, samples were acid-treated with 7% (v/v) HCl solution (Merck, Darmstad, Germany) at 55 °C for more 2 h (E4) or 4 h (E8), respectively, to remove minerals and then they were washed with distilled water until the pH value became neutral. So, the total time of the E4 process was 4h and for the E8 was 8 h.

After drying in the air oven at 60 °C for 24 h, the chitin was obtained, and its color corresponded to a light brown in both samples (E4 and E8) (**Figure 10**).

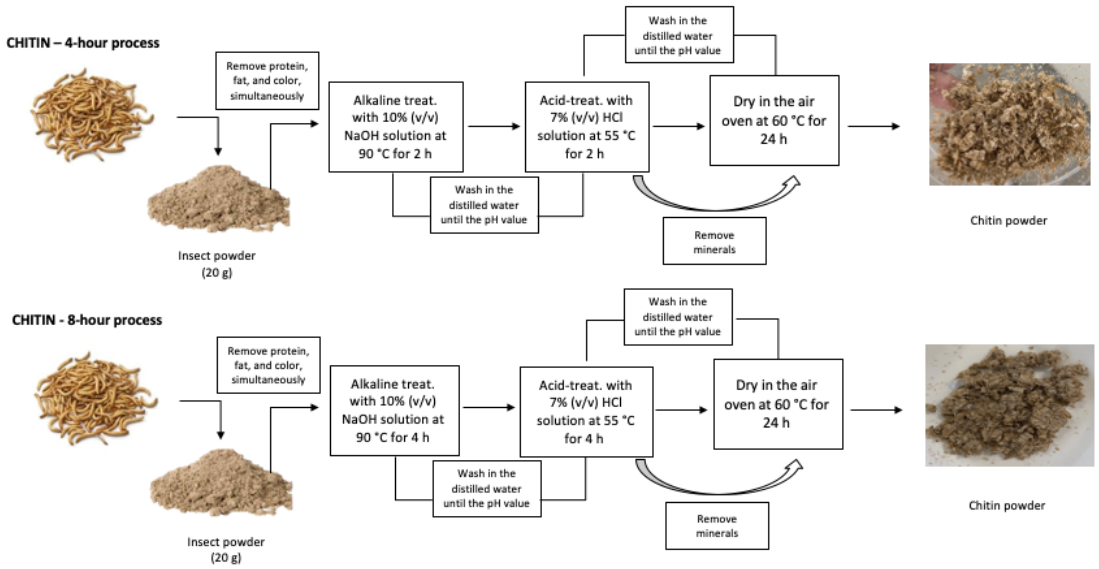


**Figure 10 -** Chitin extraction processes (i.e. extraction during 4 h and 8 h)**.**

After measuring the weight of the dried chitin, the yields were calculated according to the following equation:

$$yield\ of\ chitin\ (\%) = \frac{weight\ of\ chitin}{weight\ of\ insect} \times\ 100$$

### 3.4.2. Chitin deacetylation into chitosan

Two processes were performed, varying the deacetylation time.

In order to remove the acetyl groups, the powder of each sample of the chitin extracted (E4 and E8) from *T. molitor* was treated with a 55% (w/v) NaOH solution at 90 °C for 2 h (E4D2 and E8D2) or 4 h (E4D4 and E8D4), and it was washed in distilled water until the pH value became neutral (**Figure 11**).

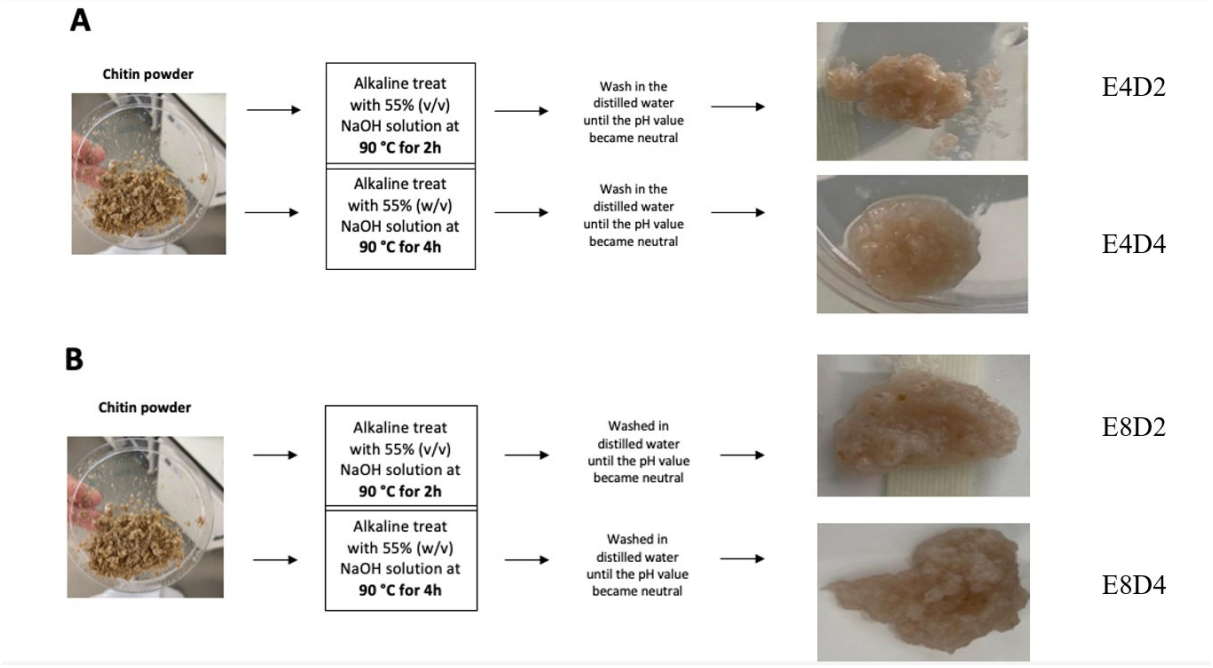


**Figure 11 -** Chitin deacetylation for chitosan extraction - (A) chitin powder, E4; (B) chitin powder, E8.

After drying in the air oven at 60 °C for 24 h, the weight of the dried chitosan was measured, and the yields of each chitosan sample were calculated according to the following equation:

$$\text{Yield of chitosan } (\%) = \frac{\text{weight of chitosan}}{\text{weight of chitin}} \times 100$$

## 3.5. Chitosan physico-chemical characterization

### 3.5.1. Fourier-transform infrared spectroscopy (FTIR)

The chemical composition of the extracted chitin and chitosan specimens underwent analysis through Fourier-transform infrared spectroscopy (FTIR), utilizing a Perkin Elmer spectrometer (Waltham, MA, USA) equipped with a diamond/ZnSe crystal and an attenuated total reflectance (ATR) sampling accessory from PIKE Technologies (Beaconsfield, UK). Each sample underwent 32 scans, covering the wavenumber range of 600-4000 cm, with a spectral resolution of 4 $cm^{-1}$. Additionally, baseline point correction and spectra normalization procedures were implemented.

The degree of deacetylation (DD) in the chitosan samples was determined via the absorbance ratio (A1655/A3450), which exhibits a linear correlation with the DD of *T. molitor*-derived chitosan. The following equation was employed for DD calculation:

$$\text{Degree of deacetylation (\%): } 97.67 - [26.486 \times (A1655/A3450)]^{130}$$

This method provided a quantitative assessment of the degree of deacetylation in the chitosan samples, aiding in the characterisation of the chitosan's structural properties.

### 3.5.2. Zeta potential

In a 1% (v/v) acetic acid solution, 10 mg of extracted and commercial chitosan were dissolved.
A NanoZSP (Worcestershire, UK) was used to evaluate the prepared samples' zeta potential (ZP).
All tests were performed using a disposable foldable capillary cell with a 90° laser angle (Malvern, Worcestershire, UK) at room temperature (25 °C).

## 3.6. *In vitro* bioactivities of the extracted chitosan

### 3.6.1. Antimicrobial activity

On Muller Hinton agar (Biokar, France), each microbe culture was grown aerobically for 24 h at a temperature of 37 ºC. The bacteria were then moved to a sterile saline solution, and their turbidity was adjusted to 0.5 MacFarland scale, which is equivalent to an optical density of 0.08-0.1 at a wavelength of 600 nm. Within a concentration range of 1–10 mg/mL, commercial and extracted chitosan solutions were made concurrently. In Muller-Hinton broth, these respective serial solutions were created. The chitosan solutions were then mixed with 2% (v/v) of each microbial inoculum, and the resultant mixtures were incubated at 37 ºC for 24 h. The microbial inoculum-containing chitosan solutions were then placed onto Muller Hinton agar plates and kept incubating for an additional 24 h at 37 ºC.
The lowest chitosan concentrations at which microbial growth was totally suppressed and the initial viability of the microbial population was reduced by at least 99.9% were identified as the Minimum Lethal Concentrations (MLCs).

### 3.6.2. Antioxidant activity

The antioxidant activity was assessed using the 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical as a photometric technique. The fundamental principle underlying ABTS involves the attenuation of a well-established metastable radical (ABTS+) by antioxidant compounds. The experimental procedure, as detailed in Coscueta et al. 2020[131] was faithfully executed within a 96-well microplate format. The assay was conducted using a multidetector plate reader (Synergy H1, Vermont, USA), operating under the guidance of Gen5 Biotek software version 3.04. To generate the ABTS radical cation (ABTS•+), a reaction mixture of 2.45 mM potassium persulfate and 7 mM 2,20-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt was initiated. An absorbance of 0.70 ± 0.02 at 734 nm was maintained, achieved by combining 180 µL of the ABTS•+ working solution with 20 µL of either the sample or Trolox as the standard calibration curve (ranging from 25 to 175 M).
The scavenging activity for the control was expressed as a percentage reduction in absorbance. Trolox concentration was determined through regression equations, and the results were presented in units of µmol/Trolox equivalent per gram (µmol TE/g).

## 3.7. Design and development of a bio-based HD membrane

The insect-chitosan method extraction E4D2 was the chosen, since it was the fastest and with the best antimicrobial activity.

For membrane production of polyvinyl chloride (PVC), 2% (w/v) in tetrahydrofuran, mixed until the PVC was dissolved in its fullest and then 1% (w/v) of the E4D2 chitosan powder was added. It was mixed until the solution became homogeneous.

After that, the PVC and chitosan solution was transferred to a petri dish, and it was left to dry in the air. After the membrane was dried, it was cut in approximately 6x1 cm batch.

### 3.7.1. Diffusion and retention parameters for permeation studies to simulate HD

The semi-permeability of the chitosan-based membrane was evaluated through a permeation study of the urea and albumin. This study was performed to understand the ability of the membrane to selectively allow the passage of certain molecules (albumin) while restricting others (urea), which is fundamental to its application HD. The permeation characteristics of the insect-chitosan membrane were studied *in vitro* at room temperature (approx. 25 ºC). Solutions were propelled by a Gilson Minipuls 3 peristaltic pump with PhthalateFREE® PVC pump tubes (**Figure 12**).

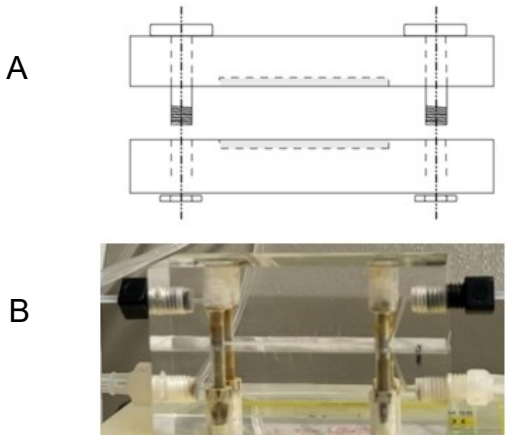


**Figure 12 -** (A) Lateral view of the configuration of the chambers[132]; (B) Lateral view of the chambers without the membrane.

The membrane was placed between the two chambers of equal volume (**Figure 13**).

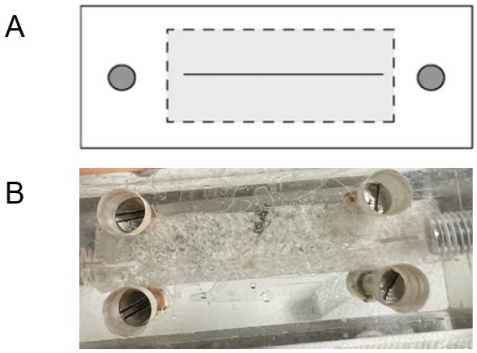


**Figure 13 -** (A) Top view of the configuration with a straight channel, length = 7.5 cm[132]; (B) Top view of the chamber with the membrane already placed.

One of the chambers, the donor chamber, was filled with solutions of known concentration of the solute of interest (urea 37 mg/dL and albumin 8 g/dL), one solution at a time, while the other chamber was filled with a deionised water[133] (**Figure 14**).

At appropriate time intervals (2, 4 and 6 minutes), 1.5 mL of the sample was collected, and the quantification of the solute diffused through the membrane was determined by spectrophotometry for urea UV spectrophotometry, at 200 nm.[134]

The linearity of the method was verified by a calibration with a serial dilution of a standard urea solution at 37 mg/dl and the Pierce BCA Protein Assay Kit method was used for albumin quantification.

**Figure 14 -** Schematic of the full process of the HD simulation.

## 3.8. Statistical Analysis

Statistical analysis was conducted using the IBM® SPSS® Statistics 26 software. For comparing the means across multiple groups, the analysis involved a one-way ANOVA for datasets with a normal distribution, complemented by Tukey's HSD for post hoc analysis. When comparing just two groups, the data underwent analysis with a Student's t-test if normally distributed. Threshold for statistical significance was established at a $p < 0.05$.

# CHAPTER 4

# RESULTS AND DISCUSSION

*“The only thing standing between you and your goal is the story you keep telling yourself as to why you can’t achieve it.”*

*Jordan Belfort*

# CHAPTER 4: RESULTS AND DISCUSSION

The chitosan extracted from insects using four different processes (varying extraction times, between 6 h and 12 h) was characterised in terms of physical properties, including analysis by FTIR (Zeta potential and degree of deacetylation).

In addition, for each sample some bioactivities were also characterised, namely antioxidant capacity and antimicrobial activity in a range of selected concentrations (1, 2, 4, 6, 8, 10 mg/mL) against MRSA, MSSA, *S. epidermis*, *E. coli*, *P. aeruginosa* and *C. albicans*.

Within that, the chitosan extracted by the E4D2 process was chosen for the development of the chitosan-based HD membrane (CH-M), since it was the one that showed the best performance in antimicrobial activity.

In order to simulate the HD process and to test the semi-permeability of the membrane, albumin retention and urea permeation studies were carried out. All these results are described in this chapter.

## 4.1. Extraction of chitin and chitosan

The procedure was broken down into two approaches, one lasting 4 h (E4) and the other 8 h (E8).

**Table 7 -** Comparison of chitin yield from *T. molitor* at different extraction times.

| Sample name | Extraction time (h) | Chitin Yield (%) |
|---|---|---|
| E4 | 4 | 5.2 ± 0.8%[a] |
| E8 | 8 | 5.0 ± 0.1%[a] |

[a] Same letters mean no statistically significant differences ($p > 0.05$)

The data presented in **Table 7** compares the chitin yield from *T. molitor* at two different extraction times and reveals E4 and E8 yields of 5.2% ± 0.8% and 5.0% ± 0.1% respectively. The difference in yield is not statistically significant ($p > 0.05$), suggesting that the additional time spent on extraction in E8 does not yield a proportionally higher amount of chitin.

Considering this it is suggested that the extraction efficiency of chitin from *T. molitor* does not vary significantly with time. The almost identical yields, coupled with the standard deviations, imply that any variations in the yields are within the error margin of the experiments and do not constitute a meaningful difference in the context of the research.

From an operational standpoint, favouring the E4 timeframe for chitin extraction might be more advantageous, once it implies a reduction in the time and potentially the resources, such as energy and reagents, required for the process. This efficiency is particularly relevant when considering the scale-up of production for commercial purposes, where cost and environmental impact are crucial factors.

In essence, the findings from **Table 7** reinforce the hypothesis that not only is it possible to derive chitosan from insects like *T. molitor* efficiently, but also that it can be done in a manner that is cognizant of economic and environmental sustainability.

Also, these yields results are in line with results reported in previous studies using *Beetle holotrichia* and *Melolontha melolontha*. These have indicated a range of values for chitin content from: 5.3% to 16%[135–139]. It is important to keep in mind that the chitin extracted from different insects might vary, presumably depending on the insect's species and stage of development and growth conditions[140].
Once the chitin is obtained and, in order to obtain chitosan from E4 and E8, a deacetylation procedure was used, varying also the deacetylation time.
The first deacetylation was 2 h (E4D2, E8D2), giving a total of a 6 h and a 10 h process, respectively and a 4 h (, E4D4, E8D4), giving a 8 h and 12 h process, respectively.

**Table 8 -** Chitin to chitosan conversion efficiency at different extraction times.

| Sample name | Chitin Extraction time (h) | Chitosan Extraction time (h) | Chitosan Yield (%) |
|---|---|---|---|
| E4D2 | 4 | 2 | 66.4 ± 0.2[a] |
| E4D4 | 4 | 4 | 73.2 ± 0.1[b] |
| E8D2 | 8 | 2 | 65.0 ± 0.8[a] |
| E8D4 | 8 | 4 | 79.3 ± 0.8[c] |

[a,b,c] Same letters mean no statistically significant differences ($p > 0.05$)

In the study outlined by **Table 8**, the chitosan yield from the E4D2 and E8D2 samples did not show a statistically significant difference ($p > 0.05$). This result suggested that doubling the chitin extraction time from 4 h to 8 h did not enhance the chitosan yield when the deacetylation time was held constant at 2 h. In contrast, extending the deacetylation time to 4 h in the E4D4 and E8D4 samples did lead to a significant increase in yield ($p > 0.05$), indicating that the duration of the deacetylation is a more critical factor than the duration of the chitin extraction.
The most notable increase in yield was observed when comparing the E4D4 and E8D4 samples to their E4D2 and E8D2 counterparts, highlighting the importance of the deacetylation phase. A longer period for this phase allows for a more complete conversion of chitin to chitosan, perhaps due to a more thorough deacetylation process.
However, despite the highest yield being obtained with the E8D4 sample, it's crucial to assess whether the increase justifies the additional resources and time spent on the process.
In conclusion, the findings suggest that optimising the deacetylation time, rather than the chitin extraction time, may be a more viable strategy for improving yield in a sustainable manner.
Also, in other studies, but differing time conditions, the insects *Beetle holotrichia* and *Melolontha melolontha* chitosan yields, ranged from 4% to 74%[136,141], which highlights the value of the extraction processes presented.
Additionally, the four chitosan samples obtained from the two chitin extraction procedures showed, empirically, very similar macroscopic features and texture close to commercial chitosan (**Figure 15**).

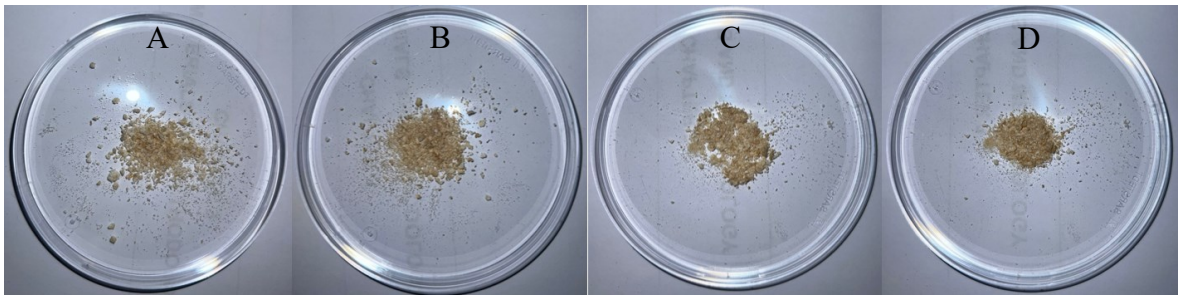


**Figure 15 –** Macroscopic features of the four chitosan samples. (A) - E4D2; (B) – E4D4; (C) – E8D2; (D) – E8D4.

The innovation compared to reported studies is the significantly reduced processing time from 1 ½ day to a timeframe of 6-12 h. Also, by employing a hot alkali treatment for decolorization, the method demonstrated remarkable efficiency in achieving similar macroscopic features to the commercial chitosan, offering a substantial advantage in terms of both time savings and resource optimisation.

## 4.2. Physico-chemical characteristics of chitosan

### 4.2.1. Fourier transform infrared spectroscopy (FTIR)

An infrared spectrum can be utilised to identify any material by recording the correlation between absorption intensity and wavenumber (measured in $cm^{-1}$), thereby providing a unique fingerprint that reflects the structural information of the substance.

The functional groups of chitosan and chitin produced from *T. molitor* and commercial chitosan are compared in **Figure 16** along with the FTIR spectrum profiles. The four samples of chitosan from this insect species' FTIR spectra were extremely comparable to those of commercial chitosan from crustaceans.

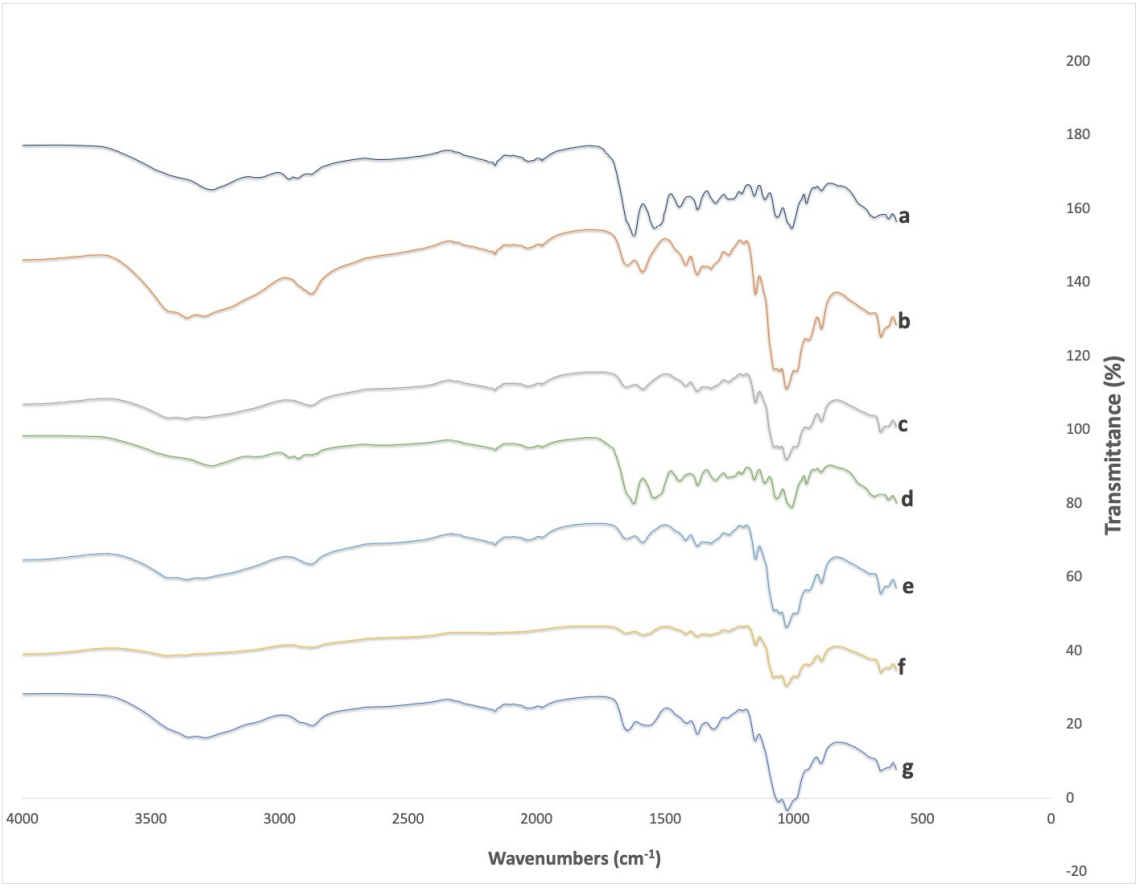


**Figure 16 -** FTIR spectra of the chitosan and chitin obtained from *T. molitor:* a) chitin (E8); b) chitosan (E8D4); c) chitosan (E8D2); d) chitin (E4); e) chitosan (E4D2); f) chitosan (E4D4); g) commercial chitosan.

A broad absorption peak centered around 3300 $cm^{-1}$ is observed in the IR spectrum of chitin (E4 and E8), indicating the presence of hydrogen bonding associated with O-H or N-H groups, which are typical functional groups found in chitin. Additionally, a sharp peak at approximately 1650 $cm^{-1}$ is discerned, signifying the presence of carbonyl (C=O) or potentially conjugated double bond (C=C) groups. Two additional peaks at 1450 $cm^{-1}$ and 1250 $cm^{-1}$ correspond to C-H bending in alkanes and C-O stretching in esters or ethers, respectively, suggesting the existence of these functional groups within the chitin structure. Chitosan E8D4 exhibited absorption peaks in the range of 3000-2850 $cm^{-1}$, indicative of C-H stretching vibrations in aliphatic (alkane) groups. Furthermore, a distinct peak near 1650 $cm^{-1}$ was observed, implying the presence of carbonyl (C=O) groups or potential double bonds (C=C) within the chitosan molecular framework. Chitosan E8D2 shares similarities with chitin in displaying a broad absorption peak around 3300 $cm^{-1}$, indicating the presence of O-H or N-H groups and the associated hydrogen bonding. Peaks near 1650 $cm^{-1}$, similar to chitin, signify the existence of carbonyl (C=O) groups. The appearance of peaks at 1450 $cm^{-1}$ and 1250 $cm^{-1}$ suggests the presence of C-H bending in alkanes and C-O stretching in esters or ethers, corroborating the molecular composition.

Chitosan E4D2 showcases a broad absorption peak around 3300 $cm^{-1}$, akin to other chitosan samples, suggesting the presence of O-H or N-H groups and associated hydrogen bonding. Chitosan E4D4 manifests sharp peaks within the 3000-2850 $cm^{-1}$ range, signifying C-H stretching vibrations in aliphatic (alkane) groups.

The sharpness of these peaks may suggest a more ordered or crystalline structural arrangement in comparison to other samples presented in literature, possibly due to differences in processing.[142–144]

### 4.2.2. Degree of deacetylation

In general, the amount of alkali solution, reaction temperature, reaction time, and solid chitin structure[145] affects the degree of deacetylation. There are two different ways to modify chitin to obtain chitosan: chemically (using intense alkali solutions, alkali catalysis, alkali fusion, and hydrazine hydrate techniques)[138] and biologically (using enzymes). Since the concentrated alkali solution technique is the most well-known and regularly used due to the reduced cost and efficiency, it was selected in this study to extract chitosan. To produce chitosan, raw materials were heated in a NaOH solution for 2 h or 4 h. As a result, the degrees of deacetylation of the raw materials were determined.

The DD of the four samples of chitosan obtained from *T. molitor* was determined using FTIR (**Table 9**).

**Table 9 -** Degree of deacetylation of chitosan samples.

| Sample name | Degree of Deacetylation (%) |
|---|---|
| E4D2 | 75.06 |
| E4D4 | 74.78 |
| E8D2 | 73.14 |
| E8D4 | 73.36 |
| Commercial | 70.22 |

The E4D2 sample exhibits the highest degree of deacetylation at 75.06%. E4D4, with a degree of deacetylation at 74.78%, demonstrates a lower deacetylation compared to E4D2.

This slight decrease may imply that increasing the chitosan extraction time from 2 h to 4 h might have negligible effect on deacetylation, given the extraction conditions remain the same.

The E8D2 sample shows a further reduced degree of deacetylation at 73.14%, indicating that prolonging the chitin extraction time to 8 h may not be optimal for maximizing deacetylation. Also, the E8D4 sample reflects a degree of deacetylation of 73.36%, which is slightly higher than that of E8D2 but still lower than the E4 series.

This could suggest that while extending chitosan extraction time does increase deacetylation, the initial 8 h chitin extraction may limit the potential for higher deacetylation levels.

The commercial sample presents the lowest degree of deacetylation at 70.22%, which could be due to various factors including the source of chitin, industrial processing techniques.

The results obtained are consistent with data previously reported in the literature, which indicates that the DD of medium-molecular-weight commercial chitosan ranges from 70 to 85% and from other insects, such as *Blaps lethifera, Pimelia fernandezlopezi, Musca domestica* ranges from 76.9 to 88%.[146]

### 4.2.3. Zeta Potential

Zeta potential is a crucial factor that controls the physicochemical characteristics of polymers.[147]
It stands for the electric surface charge that, as a result of the electrostatic attraction between the particles, has a substantial impact on the stability of particles in suspension. A high mutual repulsion between all the particles in the medium will prevent particle aggregation when they all have either a noticeably negative or positive zeta potential. Typically, compounds with zeta potentials greater than +30 mV or lower than -30 mV are regarded as being in a stable condition.[148]
The zeta potential value for commercial chitosan is found to be 65.5 mV and the values of the chitosan from the 4 processes presented an average value of 58.1 mV, which may guarantee the stability of the developed chitosan formulations and opening the range of its possible future applications.
This positive zeta potential is in accordance with previous studies on chitosan from crustaceans and other insects, which typically exhibits positive surface charges in aqueous solutions and, also, it is attributed to the presence of amino groups (NH2) on the chitosan molecule.

## 4.3. Bioactive properties of chitosan from *Tenebrio molitor*

In order to explore the potential application of chitosan extracted from *T. molitor* in various areas (especially biomedical applications), it was necessary to evaluate the antimicrobial and antioxidant activity of chitosan.

### 4.3.1. Antimicrobial activity

The extracted chitosan was evaluated for antimicrobial activity against a wide range of microorganisms associated with infections.
It was proved that all of the four extracted chitosan exhibited antimicrobial activity against *E. coli*, *S. aureus* (MSSA and MRSA), *C. albicans, S. epidermidis*, and *P. aeruginosa*. **Table 10** summarizes the minimum lethal concentration (MLC) values found for each extracted chitosan as well as for the commercial one.

**Table 10 -** Minimum lethal concentration (MLC) results, in mg/mL, of the antimicrobial activity of the extracted chitosan from *T. molitor* in comparison to commercial chitosan.

| | MLC (mg/mL) | | | | |
|---|---|---|---|---|---|
| **Microorganisms** | **E8D2** | **E8D4** | **E4D2** | **E4D4** | **Commercial** |
| **MRSA** | 6 | 4 | 4 | 6 | 4 |
| **MSSA** | 4 | 4 | 2 | 6 | 4 |
| ***Staphylococcus epidermidis*** | 6 | 2 | 2 | 6 | 4 |
| ***Escherichia coli*** | 4 | 4 | 6 | 6 | 4 |
| ***Pseudomonas aeruginosa*** | 6 | 6 | 6 | 6 | 6 |
| ***Candida albicans*** | 2 | 4 | 2 | 4 | 4 |

The MLC is the lowest broth dilution of antimicrobial compound that prevents the growth on an agar plate. The organism's failure to develop on the plate means that only nonviable microorganisms are available.[149]

The antimicrobial agent exhibiting the highest potency is characterized by possessing the lowest MLC value. All extracted chitosans exhibited antimicrobial properties similar to the commercial sample, the chitosan E4D2 demonstrated the most extensive inhibitory effects overall, as evidenced by the lowest MLC against the largest set of tested bacterium types.

In contrast, chitosan E4D4 showed the highest MLC for the greatest number of bacteria. Until now, the antimicrobial activity of insects was explored by limited number of studies, however, there is already some evidence for insects such as *T. molitor.*[129]

Hence, the potential applications of chitosan derived from *T. molitor*, commonly known as mealworms, extend across a diverse range of industries. Beyond its significance in the context of insects, *T. molitor* chitosan exhibits promising versatility with implications for various sectors, including but not limited to medicine, textiles, food and beverages, water treatment, cosmetics, biomedical engineering.

## 4.3.2. Antioxidant activity

Using the ABTS methods, the antioxidant activity was assessed, as shown in the **Table 11**.

**Table 11 -** Values of the ABTS antioxidant activity of the extracted and commercial chitosan, in µmol TE/g.

| µmol TE/g | | | | |
|---|---|---|---|---|
| **E8D2** | **E8D4** | **E4D2** | **E4D4** | **Commercial** |
| 62.97 ± 1.35$^{b}$ | 65.53 ± 1.42$^{a}$ | 65.62 ± 3.22$^{a}$ | 60.91 ± 1.45$^{b}$ | 64.17 ± 2.61$^{a}$ |

$^{a,b}$Same letters mean no statistically significant differences ($p > 0.05$)

E8D4, E4D2 and the commercial chitosan showed that their antioxidant activities are not significantly different ($p > 0.05$).

The results suggest that the extraction and deacetylation times have no impact on the antioxidant activity of the chitosan.

In conclusion, variations in processing times did not affect the antioxidant properties of chitosan. The commercial chitosan's antioxidant activity falls within the range of the extracted samples, suggesting that the extraction method used for the *T. molitor* samples is effective.

Also, this led to the conclusion that the extracted chitosans' antioxidant activity levels were within the range described in the literature, which ranged from 48.5 to 80.9 µmol Trolox equivalent/g for crustacean chitosan.[150]

## 4.4. Bio-based HD membrane development and HD simulation

For a HD purpose it is important to analyse whether the bio-based HD membrane (CH-M) effectively retains albumin[151], a critical protein in blood plasma, while allowing smaller molecular constituents (urea) to pass through.[152]

It is crucial to note, also, that this setup did not entirely replicate the typical HD process. The membrane used wasn't of the hollow fibre type, and the experiment did not involve blood contact, nor were hemocompatibility tests conducted. These are important distinctions from the clinical setting and should be taken into consideration when interpreting the results.

### 4.4.1. Analysis of the urea permeation

For the urea permeation, over a time range of 2 to 6 min, the urea removal analysis demonstrated a consistent permeation of urea across the membrane, with concentrations ranging from 0.27 to 0.31 mg/mL (**Table 12**), once the initial solution presented a concentration of 0.37 mg/mL.

Initially, after 2 min, the urea permeated was measured at 0.29 ± 0.019 mg/mL, which equates to 77.6 ± 5.24%. The high percentage in such a little time underscores the membrane's rapid response to urea diffusion, a critical aspect of efficient HD.

Subsequently, at 4 min, there was a slight increase in both the concentration and percentage of urea permeated, reaching 0.31 ± 0.013 mg/mL and 82.6 ± 3.72%, respectively. This can suggest that the membrane maintains its permeability over time, allowing for continued diffusion of urea without saturation or loss of function.

After this, at the 6-min interval, there was a slight decrease in urea permeation to 0.27 ± 0.003 mg/mL, which corresponds to 72.8 ± 0.87% permeation. This reduction could be attributed to the approach towards an equilibrium state, where the concentration gradient between the two sides of the membrane diminishes over time.[153]

**Table 12 -** Urea permeation results of the CH – M.

| Time (min) | Urea Permeated (mg/mL) | Urea Permeated (%) |
|---|---|---|
| 2 | 0.29 ± 0.019 | 77.6 ± 5.24 |
| 4 | 0.31 ± 0.013 | 82.6 ± 3.72 |
| 6 | 0.27 ± 0.003 | 72.8 ± 0.87 |

During the HD process, approximately 60 to 70% of urea is typically removed from the blood. While it's challenging to specify a definitive percentage for adequate dialysis, it is observed that patients tend to have a longer lifespan and fewer hospital admissions when the urea reduction ratio (URR) - a critical measure of the dialysis treatment's ability to remove urea from the blood - is 60% or higher.[153]

The choice of these specific time intervals was strategic, aiming to capture the initial efficiency and progression towards equilibrium of the membrane's filtration capacity. Nonetheless, the permeability rate is directly proportional to the filtration surface (i.e. filter and/or membrane). In this case, the surface with size (6x1cm) allows a test permeability of low volumes and caudal (i.e. 1.5 mL/min) and consequently a few minutes (i.e. less than 10 min), until establishing its equilibrium. Therefore, this was a pilot permeability test that is still far from real dialysis conditions.

The membrane's constant performance after continued exposure to the urea solution is reflected in the comparatively low standard deviation at this stage, which suggests an equilibrium in the permeation process.

### 4.4.2. Analysis of the albumin retention

In this study, when analyzing the samples collected at 2, 4, and 6-minute intervals, it was observed a complete absence of albumin in the filtrate. This was conclusively verified through BCA assay readings at 562 nm, which displayed no deviation from the baseline, clearly indicating the CH - M effectiveness in retaining albumin molecules.[154]

The ability of the membrane to selectively filter solutes while preventing the passage of larger molecules like albumin is of paramount importance in its application in HD, where it is intended to simulate the kidney's filtration function.

In typical HD processes, not all albumin is retained and, according to the literature, dialysis-related albumin loss is up to 26.4 g/4 h.[155]

The ability of a dialyzer to sieve or reject solutes during dialysis has been known as a marker for the clinician to determine the suitable dialyzer for patient. The sieving coefficient (that indicates the potential of different solutes to pass across a particular dialyzer membrane) is < 0.001 for albumin for the most commons commercial PSf dialyzers.[156]

The average amount of albumin retained in HD is a crucial consideration, as it indicates the efficiency of the CH - M in mimicking the selective permeability of the kidneys.[157]

However, the study revealed total retention of albumin, suggesting a very high level of selectivity.

Despite these promising results, several limitations must be recognized. Firstly, the time frame of the analysis was relatively short, restricted to intervals of 2, 4, and 6 minutes. This duration may not fully capture the membrane's performance over the extended periods typical of HD treatments, as mentioned previously.

Secondly, while the focus on albumin retention was critical, it does not cover the entire spectrum of molecules that are involved in HD, thereby necessitating a more comprehensive range of testing for a thorough evaluation.

Furthermore, the study was conducted under laboratory conditions, which may not completely reflect the complexities encountered in a clinical HD setup.

Another significant limitation was the absence of actual blood contact in the performance tests, a factor that is essential in real-world HD scenarios and can significantly impact the performance of the membrane. Lastly, the study did not include hemocompatibility tests, which are crucial for assessing how the membrane interacts with blood components in clinical applications[158].

Understanding these limitations is vital for interpreting the results within the appropriate context and for guiding future research aimed at optimising the membrane's design and functionality for practical HD applications. While the study lays a solid foundation for future advancements, it also highlights the need for further research to fully understand and enhance the membrane's capabilities in a real-world clinical setting.

# CHAPTER 5
# CONCLUSIONS AND FUTURE PROSPECTS

*“Dedicação, trabalho árduo e incessante devoção. Se achas que és perfeito, então nunca o vais ser.”*

*Cristiano Ronaldo*

# CHAPTER 5: CONCLUSIONS AND FUTURE PROSPECTS

## 5.1. Conclusions

As this thesis draws to a close, it is necessary to consider the broader implications of CKD, a pressing global health issue impacting millions. The increasing prevalence of CKD underscores an urgent need for advancements in RRT, HD playing a pivotal role. This study ventured into a novel territory, developing the CH – M, marking a significant innovation in this field. The choice to use chitosan extracted from insects breaks away from traditional sources, aligning with sustainable practices while showing potential for medical applications. Throughout the research process, from extraction to application, insect-derived chitosan has demonstrated its efficacy as a suitable material for dialysis membranes.

The exploration into the extraction times for both chitin and chitosan has demonstrated the nuanced relationship between process duration and yield. Notably, the study found that increasing the chitin extraction time does not necessarily result in a higher yield of chitosan. This was evidenced by the similar yields obtained from the samples treated for different durations, indicating a potential plateau in efficiency beyond a certain time threshold. This finding is particularly relevant for the industrial scalability of chitosan production, where time efficiency directly correlates with economic and environmental viability.

Moreover, the investigation into the deacetylation phase has underscored its critical role in the chitosan yield. It was observed that extending the deacetylation time significantly improves the yield, as seen in the difference between yields from E4D2 to E4D4 samples, as well as from the E8D2 to E8D4 samples. This enhancement in yield with prolonged deacetylation time could lead to a more effective use of resources, but it clearly implies the environmental footprint of the production process.

The physicochemical characterisation of chitosan, employing FTIR, along with the bioactive assessments through antimicrobial and antioxidant activities, has provided a robust understanding of the material's properties. The degree of deacetylation, a key parameter in determining the application potential of chitosan, was quantified, offering insights into the structural integrity and functionality of the biopolymer.

The antimicrobial efficacy, determined by the MLC against a spectrum of microorganisms. This notable antimicrobial activity leaves open not only the potential of bio-active membranes but also the possibility of their reuse in HD processes for each patient, being a system that is not only biological but also sustainable. Alongside the antioxidant capacity evaluated through ABTS radical scavenging, has reaffirmed chitosan's potential in biomedical applications, particularly in the realm of sustainable HD membranes.

Within this, the insect-chitosan method extraction E4D2 was the chosen one for the creation of the bio-based membrane, since it was the fastest and with the best antimicrobial activity.

The CH-M, through testing and evaluations, has displayed promising results. It possesses key characteristics such as semi-permeability and efficient filtration capabilities, crucial for HD efficacy. Additionally, it showed significant promise in urea and albumin permeability tests, reinforcing its potential in HD membranes.

This research paves the way for new directions in HD membrane technology, suggesting that the potential of chitosan-based membranes may extend well beyond the current findings. By addressing a critical need in CKD treatment, this thesis contributes significantly to the future of sustainable medical technology, bringing insect-sourced chitosan to the forefront and opening the opportunity for the first time on bio-inspired membranes with potential for reuse, given their outstanding antimicrobial activity.
However, it's important to recognise certain limitations in this new approach. The testing duration for the membrane was brief—limited to intervals of 2, 4, and 6 min—which doesn't reflect the extended period of actual HD treatments that often last for hours. This gap highlights the need for further research to evaluate the membrane's long-term performance, with high membrane surface and volume permeation tests. The CH-M was also not tested in a hollow fibre configuration, no blood contact was involved, and crucial hemocompatibility tests were omitted. These limitations are significant as they bear implications on the translatability of the results to a clinical context and to the potential reuse of membranes.
Acknowledging these limitations is crucial for a nuanced understanding of the study's outcomes and for guiding future research aimed at optimizing the membrane for practical HD applications. This thesis lays a solid foundation for such future advancements, marking a promising step in the evolution of HD treatment.

## 5.2. Future prospects

Looking towards future prospects, the goals englobe a different set of initiatives.
One of the greatest aims is to delve into porosity testing, which is a crucial step to fine-tune the membrane's filtration capacity, in order to get closer to HD membrane. Also, the intention is to broaden the scope of the urea and albumin permeability tests, extending them over more extended periods to rigorously assess long-term performance, simulating, in this way, a real HD process (that takes about 3-4 h).
So, a key focus will be on developing chitosan-based membranes through electrospinning, aiming to replicate the structure of commercial hollow fibre HD membranes. This step is pivotal in matching industry and scale-up standards. Additionally, thorough hemocompatibility testing is on the agenda to ensure patient safety.
Finally, comparing the antimicrobial efficacy of the CH – M against commercial counterparts will be vital, potentially leading to significant advancements in membrane reuse for multiple treatments, marking a stride towards sustainability in medical applications.
Moving forward from the findings of this thesis, the future of bio-based HD membrane technology looks toward addressing the limitations of current membranes and leveraging the benefits of biopolymers.
Currently, the prevalent HD membranes are designed as hollow fibres, providing a high surface area for filtration in a compact module. Future research should endeavor to engineer the CH - M to match this hollow fibre configuration, thus allowing for direct comparison with existing technologies and a seamless integration into the current HD infrastructure.
The key will be to fine-tune the chitosan membranes to achieve the selective permeability that is characteristic of modern HD membranes while maintaining the impressive albumin retention demonstrated in this study. Achieving a balance between permeability to compounds like urea and the

retention of crucial blood proteins will be vital. Furthermore, ensuring that these membranes can withstand prolonged contact with blood and exhibit hemocompatibility will be essential criteria for their success in clinical settings.

Additionally, the development of these membranes must consider the real-world conditions of HD treatment. This includes ensuring that the membranes can operate effectively for the typical duration of an HD session, which is substantially longer than the time frames examined in this research. Prolonged testing will be crucial to assess the durability and functional consistency of the membranes over time.

The study also highlights the need for a broader examination of the solute spectrum involved in HD. Beyond urea and albumin, a range of other molecules are typically filtered or retained during HD, and any new membrane technology must be evaluated against this full spectrum.

In conclusion, the progression from the current state of research to the practical application of the CH – M will require a multi-faceted approach. This approach should encompass structural design improvements, extended performance testing, and a more comprehensive understanding of the interaction between these bio-based membranes and the complex mixture of solutes in blood.

With these efforts, it is anticipated that the next generation of HD membranes will not only enhance patient outcomes but also introduce a new standard of sustainability and efficiency in RRT.

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