UNIVERSIDADE FEDERAL DE SANTA CATARINA
CENTRO TECNOLÓGICO
PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA QUÍMICA

ENZYMATIC RING OPENING POLYMERIZATION OF POLY(GLOBALIDE-CO-ε-CAPROLACTONE) BY MEANS OF SUPERCRITICAL TECHNOLOGY AND POST FUNCTIONALIZATION BY THIOL-ENE REACTIONS

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FLORIANÓPOLIS - SC

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Enzymatic Ring Opening Polymerization of Poly(globalide-co-ε-caprolactone) by Means of Supercritical Technology and Post Functionalization by Thiol-ene Reactions

por

Camila Guindani

Tese julgada para obtenção do título de **Doutor em Engenharia Química**, na área de Concentração de **Desenvolvimento de Processos Químicos e Biotecnológicos** e aprovada em sua forma final pelo Programa de Pós-graduação em Engenharia Química da Universidade Federal de Santa Catarina.

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An expert is a person who has made all the mistakes that can be made in a very narrow field.

Neils Bohr

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RESUMO

Durante as últimas décadas, muitos esforços têm sido realizados no desenvolvimento de novos dispositivos biocompatíveis e biorreabsorvíveis para aplicação biomédica, visando melhorar a qualidade de vida de pacientes. Poliésteres são uma das classes de polímeros mais estudadas para estas aplicações, devido a sua capacidade de serem bioreabsorvidos e/ou biodegradados, além de serem biocompatíveis. A polimerização de poliésteres por abertura de anel pode ser realizada por enzimas, consideradas catalisadores *green*, visto que o seu uso não gera resíduos tóxicos e a reação pode ser conduzida em condições brandas de forma eficiente. O uso de fluidos supercríticos como substituintes de solventes orgânicos tóxicos também vem provando ser uma alternativa limpa para a produção de polímeros para aplicação em dispositivos biomédicos. Neste contexto este trabalho relata a síntese de poli(globalide-co-ε-caprolactona) (PGlCL) por polimerização por abertura de anel via enzimática (e-ROP) utilizando tecnologia supercrítica e sua posterior funcionalização via reações tiol-eno. PGlCL foi sintetizada por e-ROP utilizando como solventes o dióxido de carbono supercrítico ($scCO_2$) e a mistura $scCO_2$+diclorometano (DCM). Foram utilizadas diferentes razões de comonômeros Gl/CL. Com relação aos valores de massa molar, maiores teores de Gl (relativo a quantidade total de monômeros) levaram a maiores os valores de massa molar. O uso de $scCO_2$ + DCM causou redução nos valores de massa molar, em comparação com os valores obtidos para $scCO_2$ puro. O uso de DCM como cossolvente também causou maior produção de oligômeros cíclicos, causando um comportamento de fusão duplo. Amostras de PGlCL com diferentes razões de Gl/CL sintetizadas utilizando $scCO_2$ puro foram então funcionalizadas com N-acetilcisteína através de reações tiol-eno. A insaturação presente nas unidades de Gl permitiu esta conjugação, e o PGlCL funcionalizado apresentou menor cristalinidade (sendo totalmente amorfo para teores de Gl maiores que 50%), e maior hidrofilicidade, além de potencial antioxidante. Estas características devem melhorar a degradação do material e são interessantes para aplicações biomédicas, especialmente quando a biorreabsorção é desejada. Visando futuras aplicações na nanomedicina, nanopartículas (NPs) de PGlCL foram também produzidas. A superfície das NPs foi funcionalizada via reações tiol-eno com a proteína BSA de forma bem-sucedida, reduzindo a internalização das NPs por células imunológicas. O PGlCL mostrou ser um copolímero muito funcional com forte potencial para futuras aplicações como um biomaterial.

Palavras-chave: ε-caprolactona, globalide, dióxido de carbono supercrítico, funcionalização, reações tiol-eno.

ABSTRACT

During the last decades, many efforts have been made in the development of new biocompatible and bioresorbable polymeric devices for biomedical application, aiming to improve the quality of life of the patients. Polyesters are one of the most studied polymers to these applications, due to its capacity of being bioresorbed/biodegraded, besides being biocompatible. The ring opening polymerization reaction of polyesters may be catalyzed by enzymes, considered as *green* catalysts, since it does not generate toxic residues and the reaction can be carried on under mild conditions in an efficient way. The use of supercritical fluids, in substitution to toxic organic solvents is also proving to be a clean alternative to the production of polymers for application in biomedical devices. In this context, this work reported the synthesis of poly(globalide-ε-caprolactone) (PGlCL) by enzymatic ring opening polymerization (e-ROP) using supercritical technology and its subsequent functionalization via thiol-ene reactions. PGlCL was synthesized by e-ROP using supercritical carbon dioxide ($scCO_2$) and the mixture $scCO_2$ + dichloromethane (DCM) as solvents. Different Gl/CL comonomer ratios were used. Regarding the molecular weight values, higher Gl contents (relative to the total monomers amount), led to higher PGlCL molecular weight values. The use of $scCO_2$ + DCM caused a decrease on molecular weight values, in comparison to the use of only $scCO_2$. The use of DCM as cosolvent also increased the production of cyclic oligomers, causing a double melting point behavior. Samples of PGlCL with different Gl/CL ratio synthesized using only $scCO_2$ were then successfully functionalized with N-acetylcysteine by thiol-ene reaction. The unsaturation present in Gl units enabled this conjugation, and the functionalized copolymer PGlCL-NAC presented lower crystallinity (being totally amorphous for Gl contents higher than 50%) and higher hydrophilicity, besides presenting antioxidant potential. These characteristics should improve the material degradation and are interesting for biomedical applications, especially when bioresorption is desired. Aiming future applications in nanomedicine, nanoparticles (NPs) made of PGlCL were also produced. The surface of the NPs was successfully functionalized by thiol-ene reactions with the protein BSA, reducing the internalization of the NPs by immune cells. PGlCL showed to be a very functional copolymer with strong potential for future applications as a biomaterial.

Keywords: ε-caprolactone, globalide, supercritical carbon dioxide, functionalization, thiol-ene reactions.

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LIST OF ABBREVIATIONS AND SYMBOLS

ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AIBN –Azobisisobutyronitrile
BSA – Bovine serum albumin
BSA-FITC – Bovine serum albumin labeled with fluorescein isothiocyanate
BSA-NP – Bovine serum albumin covalently conjugated with nanoparticle
CALB – *Candida antartica* Lipase B
$CDCl_3$ – Deuterated chloroform
CL – Caprolactone
CO_2 – Carbon dioxide
DCM – Dichloromethane
DHB – 2,5 - Dihydroxybenzoic Acid
DLS – Dynamic light scattering
D_p – Particle diameter
DPPH – 1,1-diphenyl-2-picrylhydrazil
DSC – Differential scanning calorimetry
EAM – Enzyme-activated monomer
EAPC – Enzyme-activated polymer chain
e-ROP – Enzymatic ring opening polymerization
EtOH - Ethanol
Gl – Globalide
FACS – Fluorescence-activated cell sorting
FBS – Fetal bovine serum
FCS – Fluorescence correlation spectroscopy
FDA – Food and drug administration
FITC – Fluorescein isothiocyanate
GPC – Gel permeation chromatography
KPS – Potassium persulfate
MALDI-TOF – Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MIX – Mixture of dichloromethane and monomers
M_n – Number average molecular weight
M_w – Weight average molecular weight
NAC – N-acetylcysteine
NMR – Nuclear magnetic resonance
NP – Nanoparticle
PCL – Poly(ε-caprolactone)
PDI – Polydispersity index

PEG – Polyethilene glycol
PGl – Polyglobalide
PGlCL – Poly(globalide-co-ε-caprolactone)
PGlCL-NAC – Poly(globalide-co-ε-caprolactone) functionalized with N-acetylcysteine
R – Degree of randomness
ROP – Ring opening polymerization
$scCO_2$ – Supercritical carbon dioxide
SDS – Sodium dodecyl sulfate
SEM – Scanning electron microscopy
T_c – Crystallization temperature
TEM – Transmission electron microscopy
T_g – Glass transition temperature
THF – Tetrahydrofuran
T_m – Melting temperature
TMS – Tetramethylsilane
UV – Ultraviolet
X_c – Degree of crystallinity
Đ – Dispersity
ΔH_m – Fusion Heat

SUMMARY

CONCEPTUAL DIAGRAM

"ENZYMATIC RING OPENING POLYMERIZATION OF POLY(GLOBALIDE-CO-ε-CAPROLACTONE) BY MEANS OF SUPERCRITICAL TECHNOLOGY AND POST FUNCTIONALIZATION BY THIOL-ENE REACTIONS"

What?
Enzymatic synthesis of poly(globalide-co-ε-caprolactone) (PGlCL) using supercritical carbon dioxide ($scCO_2$) as solvent, and production of PGlCL nanoparticles (NPs). Subsequent thiol-ene functionalization of PGlCL and PGlCL NPs with N-acetylcysteine (NAC) and bovine serum albumin (BSA), respectively.

Why?
• There is a growing interest in the use of biocompatible and bioresorbable polymers in biomedical devices, aiming to improve medical treatments efficiency and give more comfort and practicality to the life of patients; • Both globalide (Gl) and ε-caprolactone (CL) can be used to produce polymers with very interesting properties for biomedical and pharmaceutical applications; • Through functionalization reactions it is possible to produce tailored biomaterials for different purposes; • The use of enzymes and $scCO_2$ is considered *green*, and ideal for biomedical and pharmaceutical applications, since it does not leave toxic residues in the final product.

State of the art
• The use of $scCO_2$ as solvent in reaction media has been employed for the enzymatic ring opening polymerization (e-ROP) of several monomers, including ε-caprolactone, providing good results. • The literature has few reports on the polymerization and functionalization of globalide, but none using N-

acetylcysteine (NAC) or the protein bovine serum albumin (BSA);
- The conjugation of proteins to NPs is usually done through simple non-covalent methods. However, protein-NPs conjugates produced by covalent methods are more uniform and stable in a biological environment.
- This is the first study to propose a platform of biomaterials based on PGlCL synthesized using *green* technologies.

Hypotheses

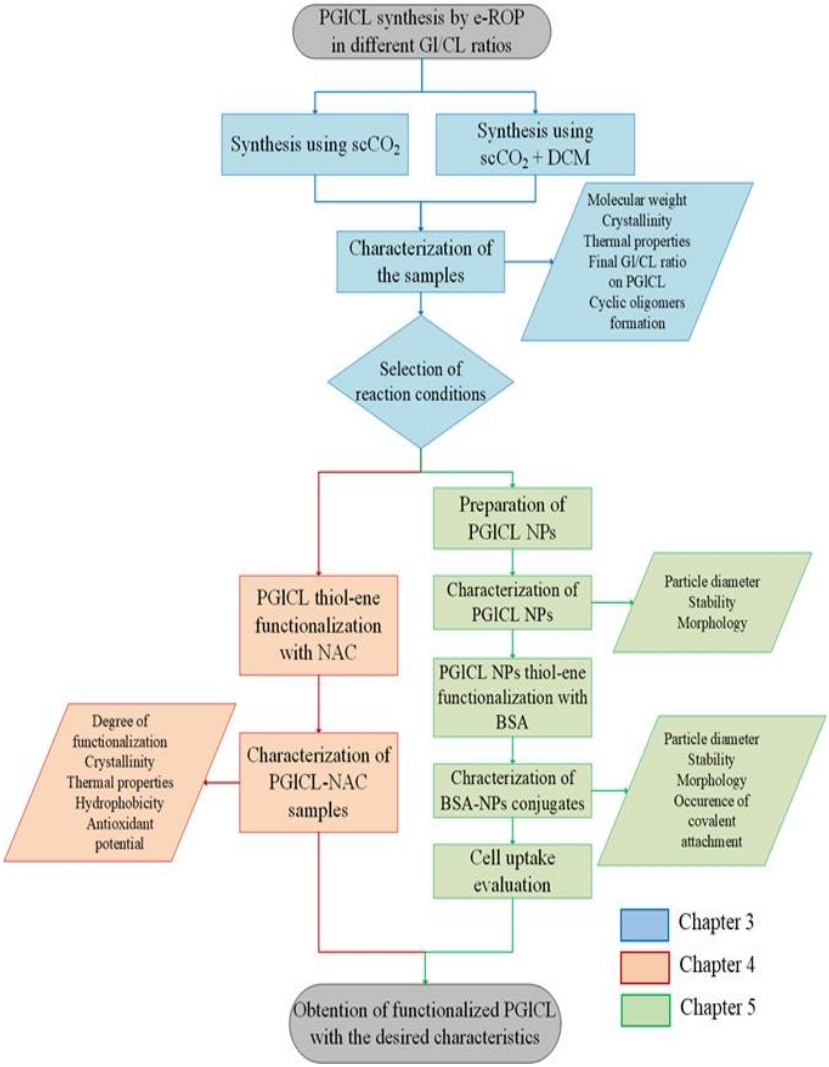
- Different Gl/CL feed ratios lead to different PGlCL molecular weights and thermal properties;
- The use of dichloromethane (DCM) as cosolvent affects the phase equilibria and the mass transfer in the system, causing changes in PGlCL molecular weights;
- The formation of oligomeric cycles is affected by the use of DCM;
- Thiol-ene functionalization of PGlCL with N-acetylcysteine (NAC) reduces the crystallinity and hydrophobicity of the material, besides conferring it an antioxidant character;
- It is possible to produce BSA-NPs covalent conjugates functionalizing the surface of PGlCL NPs with BSA by thiol-ene reaction;
- After covalent conjugation with BSA, NPs present reduced uptake by immune cells (stealth properties).

Which steps?

- e-ROP of PGlCL with different Gl/CL feed ratios using pure $scCO_2$ and $scCO_2$ + DCM;
- Characterization of the obtained PGlCL;
- Functionalization of PGlCL with NAC by thiol-ene reaction;
- Characterization of the functionalized copolymer PGlCL-NAC;
- Preparation of PGlCL NPs by the solvent evaporation method;
- Functionalization of PGlCL NPs with BSA by thiol-ene reaction;
- Characterization of BSA-NPs covalent conjugates and conduction of cell uptake assays.

Expected results
• To comprehend the effect of Gl/CL ratio and kind of solvent on the phenomenology involved on PGlCL e-ROP and its relation with the product final properties; • To produce amorphous and hydrophilic functionalized PGlCL-NAC copolymers, with antioxidant characteristic; • To produce stable and irreversible BSA-NPs covalent conjugates with stealth properties; • To produce PGlCL copolymers with tunable properties, for different biomedical applications.

THESIS METHODOLOGICAL SEQUENCE FLOWCHART



CHAPTER 1

1. INTRODUCTION

The use of biomaterials dates back to the ancient civilization, where artificial eyes, teeth, and noses were found on Egyptian mummies, and waxes, glue and tissues were used to reconstruct missing or defective body parts by Indians and Chinese (BUCHANAN, 2008). The advances in medicine, engineering and materials science have allowed the progress on the development of new biomaterials. During the last decades, many efforts were concentrated on the development of biocompatible and bioresorbable/biodegradable polymers, used on medical applications, in substitution to traditional materials, as metal and ceramics, as well as on food and pharmaceutics area, on packaging and controlled drug delivery devices.

The application of polymers in the human body requires a high purity degree of the material, being free of any toxic residue. In this context, the use of non-toxic biocatalysts as immobilized enzymes is gaining more and more space in academia and industry. The synthesis of polymers by enzymatic ring opening polymerization (e-ROP) is a promising technique, since enzymes are considered *green* catalysts, being obtained from animal, plant or microbiological sources (KOBAYASHI, 2010; KOBAYASHI et al., 2001). In addition, enzymes have high chemo- regio- and enantioselectivity, which makes them highly specific for different reaction media (ZHANG et al., 2014).

In spite of the frequent use of enzymes, known as *green* catalysts, most e-ROP studies use organic solvents such as toluene (ATES et al., 2014; CLAUDINO et al., 2012; GEUS et al., 2005; KUNDU et al., 2011; VAN DER MEULEN et al., 2008, 2011), which is a solvent of high toxicity, that may leave residues in the final product (NIH, 2009; SMYTH et al., 1969). $scCO_2$ has been shown to be relatively inexpensive, non-toxic, non-flammable (KUMAR; MADRAS; MODAK, 2004) and exhibit transport properties that can accelerate mass transfer in enzymatic reactions (OLIVEIRA; OLIVEIRA, 2000). In addition, $scCO_2$ can be easily separated from the final product by simply depressurizing the system, generating free-solvent products, and being able to be reused in the process. Supercritical carbon dioxide ($scCO_2$) has been recently used in several studies (COMIM ROSSO et al., 2013; LOEKER et al., 2004; POLLONI et al., 2017; THURECHT et al., 2006; VENERAL, 2014) as solvent in e-ROP of lactones and macrolactones, showing to be a promising alternative to the use of organic solvents.

Poly(ε-caprolactone) (PCL) is a well-studied polymer due to its ease of molding and manufacturing, and capacity of application in a wide variety of biomedical devices. In addition, PCL exhibits mechanical and kinetic degradation properties suitable for driving the growth of living tissues and for controlled release of drugs contained within their matrix (WOODRUFF; HUTMACHER, 2010). PCL has a high crystallinity, which makes its bioresorption by the organism slower (2-3 years). Another important fact is that PCL-manufactured drug release devices have Food and Drug Administration (FDA) approval and mark registration (CE Mark), which allows a faster entry route to the market (WOODRUFF; HUTMACHER, 2010).

Polyglobalide (PGl) is a biocompatible and non-toxic polyester (VAN DER MEULEN et al., 2008), obtained by polymerization of the monomer globalide (Gl). Globalide is typically used in the fragrances and cosmetics industries, since it possesses musky aroma and a quality of slowly losing its perfume (VAN DER MEULEN et al., 2008). Globalide, also known as 15-pentadecenolide or oxacyclohexadecen-2-one, is derivative from hydroxy fatty acids, being a 16-membered macrolactone, with a double bond in its structure. In comparison to the smaller lactones, such as ε-caprolactone (CL) for example, polymerization via chemical catalysis of macrolactones proceeds slowly and gives low molecular weight polymers (FOCARETE et al., 2001; NOMURA; UENO; ENDO, 1994). However, enzymatic ring-opening polymerization has been shown to be very efficient (FOCARETE et al., 2001; GEUS et al., 2010; POLLONI et al., 2017). The interest in polyesters derivative from macrolactones comes from the excellent mechanical and chemical properties of their homopolymers, which are interesting features for application in biomedical devices. However, in general, the homopolymers of macrolactones are poorly biodegradable/bioresorbable due to their semi-crystalline structure and high hydrophobicity (VAN DER MEULEN et al., 2011). This fact makes it difficult to apply PGl in cases where bioresorption is desired. However, biocompatible and non-bioresorbable materials can also be applied in permanent applications such as bone implants (VAN DER MEULEN et al., 2008). In addition, the double bond present in globalide structure enables the attachment of various interest molecules to the polymer chain by thiol-ene reaction. The functionalization of the polymer has the ability of giving the material completely new features, depending on the type of desired application (ATES et al., 2014; ATES; THORNTON; HEISE, 2011).

The development of a copolymer derived from a lactone (ε-caprolactone) and an unsaturated macrolactone (globalide) is a very

functional alternative for biomedical applications. By the synthesis of this copolymer, it becomes possible to add new properties to the well-known PCL through the introduction of globalide units, which are able to be functionalized. Variables of the copolymer synthesis, such as the ratio between the monomers (unsaturation density) and the type of molecule to be attached to the main chain can be modulated. It means that, by these simple changes, it can be produced a wide range of engineered materials with different characteristics: tailored bioresorption behavior, enhanced mechanical properties, compatibility with specific types of tissue and optimized cell uptake (in the case of nanomaterials) are some examples.

This work proposed the synthesis and functionalization of the copolymer poly(globalide-co-ε-caprolactone) (PGlCL), produced using clean technologies. PGlCL and PGlCL nanoparticles (NPs) were functionalized respectively with N-acetylcysteine (NAC) and bovine serum albumin (BSA), aiming to obtain biomaterials with enhanced properties for application in a biological environment. Besides, this work also approached the study of the process phenomenology involved in the e-ROP of PGlCL in $scCO_2$. This is an original research that started the design of a PGlCL based biomaterial platform. This study should contribute to the development of novel engineered biomedical devices that improve healthcare and promote more effective medical treatments.

1.1. OBJECTIVES

1.1.1. General objective

To develop, using clean technologies, a copolymer composed of ε-caprolactone and globalide units and functionalize it with different molecules, obtaining materials with enhanced properties for biomedical applications.

1.1.2. Specific objectives

a) To obtain PGlCL copolymers by the use of $scCO_2$ and $scCO_2$ + DCM as solvents in different Gl/CL feed ratios, and evaluate the effect of these process variables in PGlCL molecular weight (M_n), dispersity (Đ), melting temperature (T_m), degree of crystallinity (X_c), final Gl/CL copolymer composition and formation of cyclic oligomers;
b) To select process conditions that yield PGlCL samples with the most interesting characteristics, in terms of biomedical applications, for subsequent functionalization by thiol-ene reactions;
c) To functionalize PGlCL copolymers with N-acetylcysteine (NAC) by thiol-ene reaction and characterize the functionalized copolymers regarding the number of functionalizations, T_m, X_c, contact angle with water and antioxidant potential;
d) To produce PGlCL nanoparticles (NPs), functionalize its surface with bovine serum albumin (BSA) by thiol-ene reaction, and characterize the uncoated NPs and BSA-NPs covalent conjugates regarding the particle diameter, stability, occurrence of the covalent attachment, morphology and evaluate cell uptake behavior.

CHAPTER 2

2. LITERATURE REVIEW

2.1. BIOCOMPATIBLE, BIODEGRADABLE AND BIORESORBABLE POLYMERS

The term biocompatibility, according to Williams (2008) refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting
any undesirable local or systemic effects in the recipient or beneficiary of that therapy but generating the most appropriate beneficial cellular or tissue response in that specific situation and optimizing the clinically relevant performance of that therapy.

The American Standard for Testing and Materials states that biodegradable polymers are those in which degradation results primarily from the action of microorganisms such as naturally occurring bacteria, fungi and algae (ASTM STANDARD D883-11, 2011).

On the other hand, bioresorbable polymers are those that show degradation through size reduction and are resorbed *in vivo*, for example: Materials that are eliminated by the metabolic routes of the organism. Bioresorption is a concept that reflects the total elimination of the material, and its by-products degradation (low molecular weight compounds), without residual side effects. The word "bioresorption" is used when the elimination is complete (VERT et al., 1992).

Biodegradable and bioresorbable polymers currently have two main applications. The production of biomedical polymers that contribute to patient care; and their use as *green* polymers that keep the environment clean. Most of the biodegradable and bioresorbable polymers available are used for one or other of the two purposes, but some of them are applicable to both (IKADA; TSUJI, 2000).

2.2. POLYMERS FOR BIOMEDICAL APPLICATIONS

The key to the success of polymer-based biomaterials is in the fact that they can be synthesized with a wide range of properties and functionality in a relative easy and inexpensive way. Because most of the structural living tissues (like bone, ligament, connective tissue, etc.) are macromolecular composites (combinations of different macromolecules and possible other components), synthetic polymeric composites are an

attractive group of materials for development of new, tailor-made biomaterials for replacement, support, augmentation or fixation of living tissues (TÖRMÄLÄ; POHJONEN; ROKKANEN, 1998). In many cases the tissues need only the temporary presence of a biomaterial to support, augment or replace tissues or to guide their regrowth. In such cases bioresorbable polymeric materials are very interesting alternatives, capable of being bioresorbed by the human body when, after healing, the biomaterial presence is no longer necessary (TÖRMÄLÄ; POHJONEN; ROKKANEN, 1998).

For such applications, the use of devices such as scaffolds are very interesting. Scaffolds are porous or semiporous materials that have suitable properties for the growth and interaction with cells of living organisms. Scaffolds have the function of mimetizing the extracellular matrix of the native tissue and providing a suitable environment to promote cellular migration and adhesion through the pores, repairing the native tissue (COSTA-PINTO; REIS; NEVES, 2011).

Nanoparticles are another important type of biomedical device. From the biomedical point of view, the main interest in nanoparticles comes from the fact that they are small enough to interact with cellular machinery and reach difficult access targets in the human body (MAHMOUDI et al., 2011). There are many applications of nanoparticles in medical treatments, such as nanocarriers for drug delivery systems, magnetic hyperthermia for tumor treatment and nanoparticle uses with diagnostic purposes.

For drug delivery applications, the main idea is to avoid systemic drug effects and guide the medicament to the desired location in the body. The nanocarrier also have the function of protect the drug from degradation before it reaches the target location, so the circulation time in the blood stream can be prolonged (WINZEN, 2015). On the other hand, magnetic hyperthermia treatment treats tumors by heating them to above 42 °C to destroy the cancerous cells. The benefit of this technique over chemotherapy is that it specifically targets the tumor and does not damage the surrounding healthy tissue (MCNAMARA; TOFAIL, 2017). The application of nanoparticles in for diagnosis aims to visualize pathologies and to improve the understanding of important (patho-) physiological principles of various diseases and disease treatments (BAETKE; LAMMERS; KIESSLING, 2015). For this application, diagnostic nanoparticles should possess a good and efficient delivery to the target site and should exhibit highly specific binding and internalization capabilities (BAETKE; LAMMERS; KIESSLING, 2015). Nanoparticles can be coupled with radiolabels and detected by positron emission

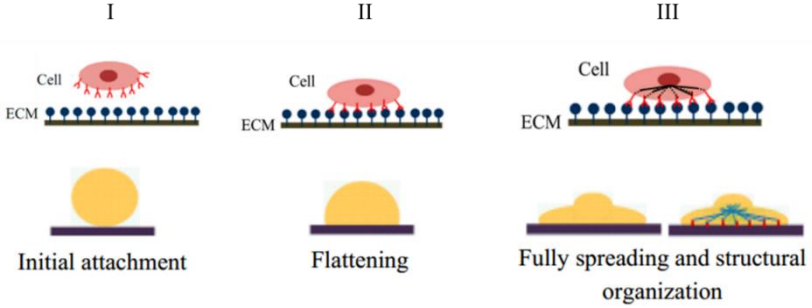
tomography (PET) or single photon emission computed tomography (SPECT) and contrast agents can be encapsulated and used to increase the sensitivity of imaging methods, such as magnetic resonance imaging (MRI) (WINZEN, 2015).

2.2.1 Interaction between polymers and biomolecules in a biological environment

2.2.1.1. Interaction between cells and polymer surfaces for tissue repairing

In a biological environment, when a cell first contacts a polymer surface, cells receptors (specially integrins) primarily interact with the polymeric devices via chemical groups (ligands) on the material surface. In case of materials that mimic extracellular matrix (such as scaffolds or guided tissue regeneration membranes for tissue repairing), integrin-ligand binding is followed by the extension of pseudopodia from the cell body, starting cell adhesion process. The adhesive bond is defined as the sum of non-covalent interactions, e.g., hydrogen bonds, electrostatic interactions, van der Waals forces, dipole-dipole interactions between two macro molecules (MCEVER; ZHU, 2010). As the cell begins to flatten against the substrate, it forms additional bonds with the extracellular matrix, rearranging its cytoskeleton to form focal adhesions (BURRIDGE, 1988). To spread, the cell must exert force against the surface to allow pseudopodial extension and the stabilization of new bonds as its contact area expands (REINHART-KING; DEMBO; HAMMER, 2005). Figure 2.1 illustrates the cell adhesion stages in a surface.

Figure 2.1 - Stages of cell adhesion in a surface.



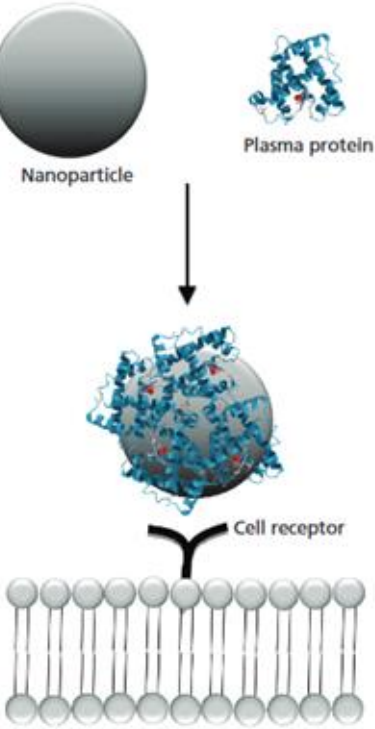
Source: Adapted from Khalili and Ahmad (2015).

2.2.1.2. Interaction between cells and polymeric nanoparticles

Nanomaterials are usually administered intravenously to be distributed over the body though the bloodstream (DUNCAN, 2006; WINZEN, 2015). Blood plasma is the non-cellular component of blood and consists of 3,700 identified proteins that are capable of interacting with the nanomaterial surface. The adsorption of this proteins to the nanoparticle surface gives rise to the so called “protein corona” (LYNCH; SALVATI; DAWSON, 2009; MONOPOLI et al., 2011). The formed corona creates a new interface and gives a “new identity” to the nanoparticle, which influences the recognition of the nanomaterials by the cells and determine its biological fate (LYNCH; SALVATI; DAWSON, 2009; WINZEN, 2015).

Typically, attractive forces (some generic and some highly specific, such as receptor–ligand interactions) will lead to the binding of the complex particle-protein to the cell surface. The proteins present in the protein corona that remain adsorbed on the particle surface for longer times will affect, indeed mediate, the nanoparticle–cell interactions. The scheme in Figure 2.2 illustrate this interactions.

Figure 2.2 - Interactions between cells and protein-nanoparticle complexes.



Source: Adapted from Caracciolo (2013).

The composition of the protein corona strongly influences the cellular uptake and biodistribution of the nanoparticles (EHRENBERG et al., 2009; SCHÖTTLER et al., 2016). For example, a protein corona rich in opsonins like fibrinogen and Immunoglobulin G (IgG), etc. is believed to promote phagocytosis and removal of the NPs from the bloodstream (ISHIDA; HARASHIMA; KIWADA, 2001; OWENS; PEPPAS, 2006), while binding of dysopsonins like apolipoproteins, promotes prolonged circulation time in blood (CARACCIOLO et al., 2015; MONOPOLI et al., 2011). Proteins that are not recognized by any receptors on the cell will make the particle less "attractive" to the cell. After all, cells associate and take up only the proteins they need (LYNCH; SALVATI; DAWSON, 2009).

2.3. POLYESTERS

In recent years, environmental concerns have led to a renewed interest in biodegradable polyesters as an alternative to commodity plastics. Since ester linkages are frequently encountered in nature it is reasonable to assume that at least a subset of the polyester family will be environmentally degradable (MILETIĆ; LOOS; GROSS, 2010).

Polyesters are versatile materials, being the lead candidate in the biomedical and pharmaceutical applications. Its good mechanical properties, hydrolysability, biodegradability and biocompatibility make polyesters suitable for a variety of medical applications (e.g. as prosthetics, artificial skin, etc.) and in pharmaceutical industries for drug delivery applications.

The biodegradation and the bioresorption of polyesters occur initially through the hydrolysis of the ester bonds, resulting in the decrease of the polymer molecular weight, but without the loss of mass. This initial degradation occurs until molecular weight less than 5,000 Da, at which point cellular degradation takes over. The final degradation and resorption of the material proceed through cells of the organism such as macrophages, lymphocytes and neutrophils (PACHENCE; BOHRER; KOHN, 2007). This second step of degradation is characterized by mass loss, molecular weight reduction, structural changing and loss of mechanical properties as tensile strength, compressive strength and hardness (ATHANASIOU et al., 1998; BARBANTI; ZAVAGLIA; DUEK, 2005).

Different factors affect the degradation kinetics of polyesters, such as: chemical composition and configurational structure; processing history; molecular weights (M_n and M_w); dispersity (Đ); environmental conditions; mechanical stress; crystallinity; chain orientation; distribution of chemically reactive compounds within the matrix; additives; overall hydrophilicity and morphology (porosity, for example); and size of the polymeric device (DUNN; CAMPBELL; MARRA, 2001; HEIDEMANN et al., 2001).

The physical and biodegradable properties of aliphatic polyesters can be adjusted by changing the structure and composition of the main polymer chain, chain flexibility, presence of polar groups, molecular weight, crystallinity and orientation, in order to match the material to a given application (ALBERTSSON; VARMA, 2003; COULEMBIER et al., 2006).

2.3.1. Poly(ε-caprolactone)

Poly(ε-caprolactone) (PCL) is a well-studied polymer, derivative from the monomer ε-caprolactone (also known as 2-oxepanone), being one of the first polymers synthesized by the Carothers group in 1930 (VAN NATTA; HILL; CAROTHERS, 1934).

During the resorbable-polymer-boom of the 1970s and 1980s, PCL and its copolymers were used in a number of drug-delivery devices. Attention was drawn to these polymers owing to their numerous advantages over other polymers in use at that time (WOODRUFF; HUTMACHER, 2010). PCL has mechanical properties, permeability and degradation kinetics suitable to enable controlled release of drugs contained within its matrix, besides having the ability of being fully excreted from the body once bioresorbed. (WOODRUFF; HUTMACHER, 2010). PCL biodegrades within several months to several years, depending on the molecular weight, the degree of crystallinity of the polymer, and the conditions of degradation (LABET; THIELEMANS, 2009).

In the 1990s and the 2000s, there was the birth of a new area, called "tissue engineering". The arising of this area generated many studies on PCL, due to its superior rheological and viscoelastic properties over many other resorbable polymers. These properties make PCL easy to manufacture and manipulate into scaffolds (three-dimensional structures designed to support cell growth, allowing the regeneration of tissues) with controlled pore size, enabling appropriate tissue growth (WOODRUFF; HUTMACHER, 2010). PCL can be used in a wide range of scaffold fabrication technologies, and its relatively inexpensive production routes (compared with other aliphatic polyesters) is hugely advantageous (WOODRUFF; HUTMACHER, 2010).

PCL and its copolymers have demonstrated excellent properties such as high flexibility, biodegradability and biocompatibility, as well as non-toxicity. These favorable attributes give PCL the characteristic of being the most attractive and useful class of biodegradable polyesters (PITT; MARKS; SCHINDLER, 1980). PCL is a very interesting polymer to be applied on drug delivery devices/long-term vaccines and suture materials, since it degrades at a slower rate (2-3 years) than polyglycolides and polylactides for example, besides having high permeability to many drugs (NAIR; LAURENCIN, 2006; WOODRUFF; HUTMACHER, 2010). Some drug delivery devices made from PCL have FDA (Food and Drug Administration) approval and trademark registration (CE Mark), which

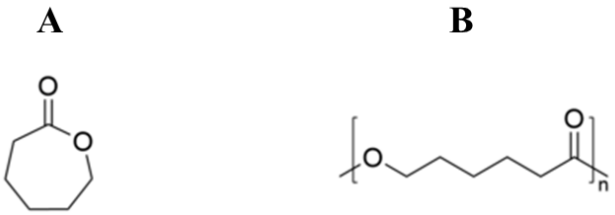
allows a faster way out to the market (WOODRUFF; HUTMACHER, 2010).

There are several examples of commercial products based on PCL: the long-term contraceptive device, Capronor™, that could yield a zero-order release of levonorgestrel for over one year (NAIR; LAURENCIN, 2006); the ε-caprolactone/glycolide copolymer suture fibers called Monocryl™; a multiblock copolymer composed of bioresorbable ε-caprolactone, glycolide, lactide and polyethylene glycol, used as a vehicle for drug delivery, called SynBiosys™ (NAIR; LAURENCIN, 2006); a bioresorbable implant that is used for covering trephination burr holes in neurosurgery, Osteoplug™ (LOW et al., 2009); and a thermoplastic synthetic polymer based root canal filling material made of 25-40% of poly(ε-caprolactone), called Resilon™ (HIRAISHI et al., 2007; JIA, 2005; JIA; ALPERT, 2003; JIA; TROPE; ALPERT, 2005).

PCL is a semi-crystalline polymer, which has glass transition temperature of -60 °C and melting point ranging between 59 and 64 °C, depending upon its crystalline nature of PCL (SINHA et al., 2004). At room temperature, PCL is highly soluble in chloroform, dichloromethane, carbon tetrachloride, benzene, toluene, cyclohexanone and 2-nitropropane; slightly soluble in acetone, 2-butanone, ethyl acetate, dimethylformamide and acetonitrile; and insoluble in alcohols, petroleum ether, diethyl ether and water (SINHA et al., 2004). Bordes et al. (2010) determined the solubility of PCL of different molecular weights in 99 analytical grade solvents, and determined in which solvents PCL is soluble, partially soluble or insoluble, at room temperature.

Industrially, high molecular weight PCLs are synthesized by ring opening polymerization of ε-caprolactone (CL) monomer, which can be obtained by the traditional Baeyer-Villiger reaction using cyclohexanone. In the last decades, another route has been developed to produce CL monomer. This route is a ‘‘green synthesis’’, which requires hydrogen peroxide as oxidizer and zeolite/tin catalysis (WILSON, 2001 apud COULEMBIER et al., 2006). This process looks very promising since the waste product is water and the tin-impregnated zeolite is an ecologically friendly catalyst. Figure 2.3A presents the structure of the monomer ε-caprolactone, while Figure 2.3B presents the repeating unit structure of PCL.

Figure 2.3 - (A) Structure of the monomer e-caprolactone; (B) Structure of PCL repeating unit.

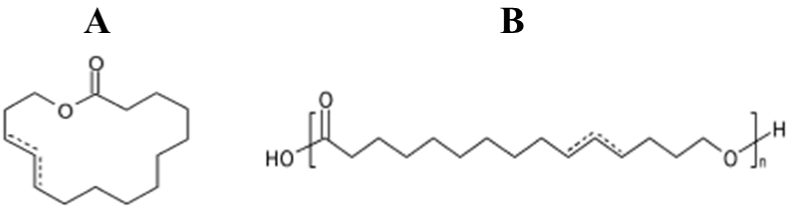


Source: Adapted from Labet and Thielemans (2009).

2.3.2. Polyglobalide

Polyglobalide (PGl) has been recently studied as aliphatic polyester on biomedical application (ATES et al., 2014; ATES; HEISE, 2014; CLAUDINO et al., 2012; VAN DER MEULEN et al., 2008, 2011). Until then, its monomer, globalide (Gl) (Figure 2.4), also known as 15-pentadecenolide or oxacyclohexadecen-2-one (IUPAC name), was most often used as a fragrance in the perfumery industry because of its musky odor and its slow loss of aroma. This way, very few studies on synthesis of polyglobalide are found in literature.

Figure 2.4 - (A) Structure of the monomer globalide; (B) Structure of the repeating unit of PGl.



Source: Van der Meulen et al. (2008).

In recent works on synthesis and functionalization of PGl, it was possible to verify that it is a biocompatible and non-toxic polymer (ATES et al., 2014; ATES; HEISE, 2014; ATES; THORNTON; HEISE, 2011; VAN DER MEULEN et al., 2008,VAN DER MEULEN et al., 2011). As well as other polymers derived from macrolactones, PGl has the

characteristic of being a very hydrophobic and semi-crystalline material. These characteristics are very similar to the characteristics of polyethylene, one of the most versatile polymers used in the industry. Despite of being very interesting for a large number of applications, these characteristics generate limitations for biomedical applications, where polymer bioresorption is desired (ATES; HEISE, 2014). In their work, van der Meulen et al. (2008) observed that there was no hydrolytic or enzymatic degradation of PGl over 100 days, and concluded that the high crystallinity and hydrophobicity are responsible for this behavior.

These limitations can, however, be overcome by the functionalization and/or crosslinking of the double bonds present in the PGl by thiol-ene "click" reaction. Functionalization enables the introduction of a wide range of chemical groups, changing the characteristics of the polymer as desired (ATES et al., 2014; ATES; HEISE, 2014; ATES; THORNTON; HEISE, 2011; CLAUDINO et al., 2012; VAN DER MEULEN et al., 2011).

PGl has a glass transition temperature lower than -60 °C and a melting temperature ranging from 46 to 50 °C (VAN DER MEULEN et al., 2008). According to the literature, PGl showed to be soluble in dichloromethane, toluene, tetrahydrofuran (THF) and chloroform (ATES; THORNTON; HEISE, 2011; VAN DER MEULEN et al., 2008). PGl, as well as other lactones and macrolactones, can be synthesized by ring opening polymerization of its monomer, globalide. Studies of the ROP of cyclic esters have primarily focused on the small-rings (glycolide, lactide, butyrolactone, valerolactone, etc.) and medium-rings (ε-caprolactone (CL), 1,5-dioxepan-2-one (DXO), etc.) rings, which have angular or transannular strains. Large-rings, on the other hand, are defined as a ring containing 12 or more atoms. There is no upper limit to this number, although very few reports are available on lactones with more than 16 atoms. This way, globalide can be considered a large-ring lactone. Unlike the small and medium-rings, which are considerably strained, these large rings are relatively strain-free, which ease ROP reactions (ALBERTSSON; VARMA; SRIVASTAVA, 2009).

Most studies involving PGl synthesis used enzymes as catalyst on ROP reactions, since the use of conventional chemical catalysts on macrolactones ROP generates only oligomeric, low molecular weight and low yield materials (ALBERTSSON; VARMA; SRIVASTAVA, 2009; BISHT et al., 1997). Besides, the reaction proceeds much faster, under mild conditions, and yields materials free of any metal contamination, which can be considered a great advantage for biomedical applications in

view of the toxicity of many metal containing catalysts (ALBERTSSON; SRIVASTAVA, 2008).

2.3.3. Copolymers of lactones and unsaturated macrolactones

Many different copolymers based on all kinds of lactones were made during the last decade. However, the study of copolyesters based on lactones and unsaturated macrolactones is little reported until now. The interest in polyesters derived from macrolactones stems from the excellent mechanical properties of the polymers and the fact that degradation products should be harmless, as they resemble fatty acid derivatives (ATES; HEISE, 2014; VAN DER MEULEN, 2010; VAN DER MEULEN et al., 2008). However, homopolymers of macrolactones are non-bioresorbable under physiological conditions due to their semi-crystalline morphology and high hydrophobicity (VAN DER MEULEN et al., 2008, 2011).

These problems can be overcome by the introduction hydrophilic or a bulky comonomer to reduce the crystallinity. At the same time, the transition from a semi-crystalline to an amorphous or low-melting temperature material may also requires a post-synthesis crosslinking step, in order to produce copolymers able to be processed into stable shapes for biomedical applications (VAN DER MEULEN, 2010; VAN DER MEULEN et al., 2011). The main chain unsaturation provides a straightforward functionality for crosslinking, since simple free radical reaction can be employed.

Van der Meulen et al. (2011) investigated the synthesis of copolymers from unsaturated macrolactones, aiming to increase the biodegradability of the materials. It was investigated the copolymerization with smaller biocompatible comonomers like 1,5-dioxepan-2-one (DXO) and 4-methyl caprolactone (4MeCL), and their influence on the copolymer properties. DXO and 4MeCL typically generates amorphous homopolymers. Thermal investigation showed that the melting point was lowered upon incorporating the comonomers, and the decrease was dependent on the comonomer ratio. The authors also showed that crosslinking of the polymer can be performed using dicumyl peroxide, generating completely degradable crosslinked polyesters. Preliminary degradation tests confirmed that the crosslinked copolymers are enzymatically degradable and that the incorporation of hydrophilic comonomers like DXO enhances degradation through crystallinity and hydrophobicity reduction.

In their work, Claudino et al. (2012) synthesized by e-ROP with different monomer ratios of poly(globalide-co-ε-caprolactone) copolyesters. The aim of the work was to investigate if thiol-ene crosslinking by UV curing could be used with unsaturated copolymers derived from macrolactones to provide films with efficient curing and high conversions. The authors were interested in determining the relationship between functional density (related to the amount of globalide) and the resulting thiol-ene conversion. It were obtained copolymers of molecular weight up to 23,000 Da (M_n). All compositions tested (globalide content ranging from 10% to 100%) were successfully crosslinked by equimolar reaction of "ene" groups and thiol groups from trimethylolpropane tris(3-mercaptopropionate), affording fully transparent amorphous elastomeric materials with different thermal and viscoelastic properties. The authors concluded that the addition of ε-caprolactone to globalide is a good way to increase the mobility of the chains while providing high thiol-ene conversions (>80%, based in the residual unsaturation after curing) and maintaining the cure behavior irrespective of the functional density.

2.4. ENZYMES ON POLYESTER SYNTHESIS

In nature, enzymes can be divided into six classes: oxide-reductases, transferases, hydrolases, lyases, isomerases and ligases. Hydrolases are the most-investigated enzymes for *in vitro* synthesis. This class includes lipase, which is used for the hydrolysis of fatty esters in nature, being particularly interesting for polymer synthesis. Lipases are known to catalyze reactions in organic media, since they are active on the water-fat interface in cells. Moreover, lipases do not require any co-catalyst, and proved to be the most efficient enzyme for *in vitro* polyester synthesis. Lipase can be used for polycondensation and poly-transesterification reactions, ring-opening polymerizations, and polymer modifications reactions (GEUS, 2007; MILETIĆ; LOOS; GROSS, 2010).

The most common lipase-catalyst used for polyester synthesis is *Candida antarctica* lipase B (CALB). The immobilized CALB catalyst that has been primarily used is Novozym® 435, manufactured by Novozymes (Bagsvaerd, Denmark). Novozym 435 consists of CALB physically adsorbed within the macroporous resin Lewatit VPOC 1600 (poly[methyl methacrylate-co-butyl methacrylate], supplied by Bayer) (ZHANG et al., 2014). CALB is composed of 317 amino acids and has a molecular weight of 33,000 Da. This lipase belongs to the alpha/beta hydrolases family, and was initially obtained from the yeast *Candida*

antarctica (UPPENBERG et al., 1994). Novozym 435 is a highly versatile catalyst with thermosetting immobilization and activity on a wide variety of substrates in various organic solvents (GEUS, 2007).

The high cost of the enzymes for a long time was considered as a barrier to their use in commercial processes. However, technological advances that include the study of the use of solvent-tolerant lipases and immobilized lipases, allowing reuse of the catalyst, have made possible the development of low cost systems (FUKUDA; KONDO; NODA, 2001)

Enzymatic polymerizations are a promising strategy under study by many groups throughout the world to develop environmental friendly processes for polyester synthesis (MILETIĆ; LOOS; GROSS, 2010).

2.5. ENZYMATIC RING OPENING POLYMERIZATION (e-ROP)

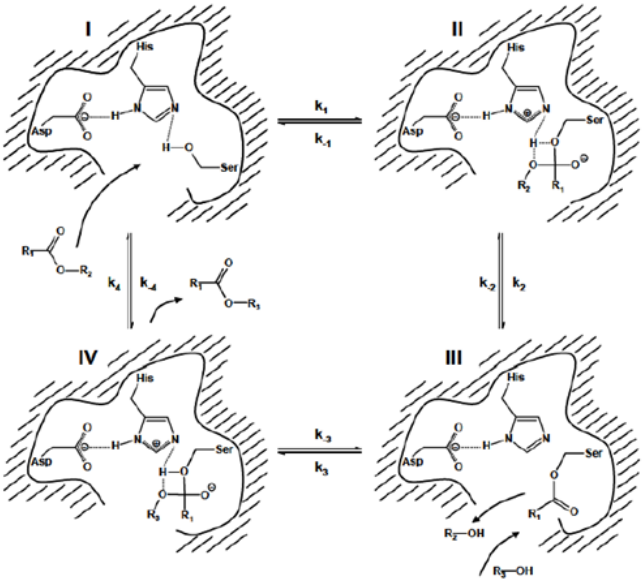
The most commonly used route for the production of PCL, PGl, and its copolymers, is the ring-opening polymerization (ROP), since it is able to give polymers with high molecular weight and low dispersity (CLAUDINO et al., 2012; GEUS, 2007; LABET; THIELEMANS, 2009; PENCZEK et al., 2007; THURECHT et al., 2006; VAN DER MEULEN et al., 2008, 2011). This reaction can be performed by a wide variety of catalysts, usually based on tin, zinc and aluminum. However, residues of organometallic catalysts are not tolerated in biomedical applications due to their toxicity (LI et al., 2011). In this context, the use of biological catalysts, such as enzymes, has been gaining more space, being an efficient alternative to avoid the toxicity problem (VENERAL, 2014).

The first e-ROP of lactones was presented in 1993 when Knani, Gutman and Kohn (1993) and Uyama and Kobayashi (1993) independently published works on e-ROP of ε-caprolactone using a lipase enzyme. Since then, many different lipases have been studied in e-ROP, transesterification and polycondensation reactions, such as lipases from *Candida antarctica, Candida cylindracea, Candida rugosa, Pseudomonas fluorescens*, *Pseudomonas cepacia* and from porcine pancreas (ALBERTSSON; SRIVASTAVA, 2008). Among these, lipase from *Candida antarctica* B is the most applied enzyme on e-ROP of lactones and macrolactones (ZHANG et al., 2014).

Lipase catalyzes ester bond hydrolysis by means of a catalytic triad, composed of a nucleophilic serine residue activated by a hydrogen bond in relay with histidine and aspartate or glutamate. This catalytic triad is responsible for the ROP of lactones (ALBERTSSON; SRIVASTAVA, 2008).

Figure 2.5 presents e-ROP mechanism proposed in details by Geus (2007), based on the works of Henderson et al. (1996) and Uyama et al. (1995). The catalytic triad in the active site of a lipase is electronically stabilized. In the e-ROP mechanism, a cyclic ester function as substrate molecule and undergoes a nucleophilic attack from the primary alcohol group of serine in the active site of the enzyme (I), leading to the formation of an intermediate specie (II). In the transesterification, the original alkoxy group (R2-OH) is then released (III), forming the enzyme-activated monomer species (EAM) (III). However, in the case of an e-ROP the alkoxy group will not be released, since the lactones are cyclic. Subsequently, a nucleophile (a primary alcohol, water, amine or thiol group) (R3-OH) can attack this EAM-species (III) forming a new intermediate specie (IV). Then the final product, a short chain polymer is released and the enzyme is regenerated (GEUS, 2007; VENERAL, 2014).

Figure 2.5 - Enzymatic transesterification mechanism proposed by Geus (2007).



Source: Geus (2007).

The nucleophile R3-OH can be considered as the initiator of the polymerization, and it is necessary to regenerate the enzyme and create the (ring-opened) product. This initiator can be water or any other nucleophile (such as alcohols amines and thiols). The ring-opened

product (oligomer) formed after a catalytic cycle has in its structure a hydroxyl molecule on one side and the functional initiator on the other. In this way it is possible to identify the initiator that gave origin to the molecule (GEUS et al., 2005).

Characteristics such as molecular weight control and functionalization of the terminal chain can be obtained by the addition of different nucleophiles in the polymerization, replacing the water in the e-ROP (KNANI; GUTMAN; KOHN, 1993). However, since the enzyme needs water to maintain its structure, the use of other nucleophiles results in products where fractions of the chains produced were initiated by water, resulting in a mixture of chains with different terminal groups (HENDERSON et al., 1996).

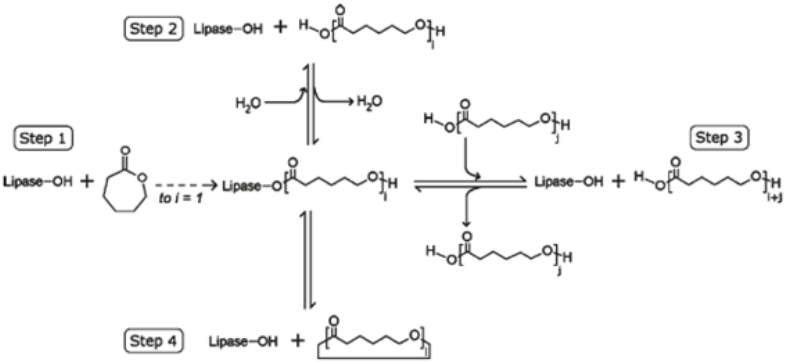
The reaction propagation stage occurs by a nucleophilic attack of the hydroxyl molecule present in the oligomer chain (formed in the previous cycle), to the EAM complexes (ALBERTSSON; SRIVASTAVA, 2008). New complexes, called enzyme-activated polymer chain (EAPC) are generated, resulting in the addition of one more monomeric unit in the polymer chain (as final product of one more cycle). In this way, the process occurs successively.

During the propagation step via e-ROP, different products can be obtained. Johnson, Kundu and Beers (2011) described the kinetic pathway of the polymerization of lactone ε-caprolactone using the commercial lipase Novozym 435 as a biocatalyst. The authors concluded that the proposed model is applicable for lactones, macrolactones, lactams and cyclic carbonates. The following scheme (Figure 2.6) was made by Johnson, Kundu and Beers (2011), based on the reaction pathways proposed by Mei, Kumar and Gross, (2002) and shows the kinetic modeled reactions in PCL synthesis by e-ROP in four steps.

All reaction steps require enzyme to be present, and include the reaction for ε-caprolactone ring-opening, enzyme and polymer chain interactions with water, chain propagation or degradation, and cyclic formation.

The first chain conformation possible to be obtained is the polymer chain activated by the enzyme, which is a PCL chain bounded to the active site of the enzyme (EAPC). The second consists of a PCL linear chain in solution. The third possibility is the formation of cyclic chains, which are polymer chains in solution that form a cyclic ring from an enzyme-activated polymer chain.

Figure 2.6 - Reaction pathways for PCL synthesis by e-ROP.



Source: Adapted from Johnson et al. (2011).

The first reaction (step 1) involved in the mechanism comprises the formation of the enzyme-activated polymer chain (EAPC) complex. This step is considered irreversible, as ring strain should prevent the ring from reforming. The linear chain formed is more thermodynamically stable when compared to the cyclic ring. The product generated is an EAPC with an ester bond at the active site (JOHNSON; KUNDU; BEERS, 2011).

In the second reaction (step 2), a water molecule is consumed in the EAPC complex, breaking the ester bond between the polymer chain and the active site of the enzyme, which leads to the formation of a polymer chain in solution and the regeneration of the active site. In the reverse pathway, occurs the formation of the active site-polymer chain ester bond (EAPC), generating a water molecule (JOHNSON; KUNDU; BEERS, 2011).

Enzymatic polycondensation reactions (step 3) may occur when an EAPC reacts with the hydroxyl group from a linear chain in solution. In this case, occurs the regeneration of the active site and formation of chains with high molecular weights. In the reverse reaction, the enzymatic degradation occurs when the active enzyme site cleaves an ester bond at a random position of a PCL chain, reforming the ester bond with the enzyme active site. This results in a lower molecular weight linear chain and an EAPC (JOHNSON; KUNDU; BEERS, 2011).

In the last reaction (step 4), the formation of cyclic compounds occurs when the polymer chain backbites on itself at the active site, forming an ester bond with itself and generating a cyclic chain and regenerating the enzyme active site. The reverse reaction consists in the opening of the cyclic ring, similar to step 1, but with a larger ring. This

reaction results in the formation of an EAPC (JOHNSON; KUNDU; BEERS, 2011).

2.5.1. The role of water on e-ROP

In e-ROP reaction, the control over the amount of water plays a crucial role, having very strong influence in the size of the polymer chains obtained (MEI; KUMAR; GROSS, 2002). When in excess, the water content can affect the polymerization, causing the degradation of polymer chains and reducing its molecular weight (GEUS, 2007; SIVALINGAM; CHATTOPADHYAY; MADRAS, 2003).

In their work, Thurecht et al. (2006) and Mei et al. (2003) studied the effect of different amounts of water as an initiator of poly(ε-caprolactone) e-ROP using the enzyme *Candida antarctica* B (CALB). The authors observed an inverse relationship between the initiator content and the molecular weight of the polymers. At higher water concentrations, it were obtained polymers of lower molecular weight, due to the hydrolysis of the aliphatic ester bonds of the poly(ε-caprolactone) chains (VENERAL, 2014).

Geus (2007) performed poly(ε-caprolactone) e-ROP reactions, initiated by HEBI, using dried and non-dried Novozym 435 as catalyst. The author noted that after 120 minutes, monomer conversion was almost identical using both dried and non-dried enzymes. However, the evolution of monomer consumption during the reaction was different. When more water was present, the initial rate of monomer consumption was significantly higher. At the same time, the observed M_n was lower, which can be expected, since water will also act as initiator, together with HEBI. Besides, the excess of water probably was favoring the degradation of the polymeric chains, reducing its molecular weight.

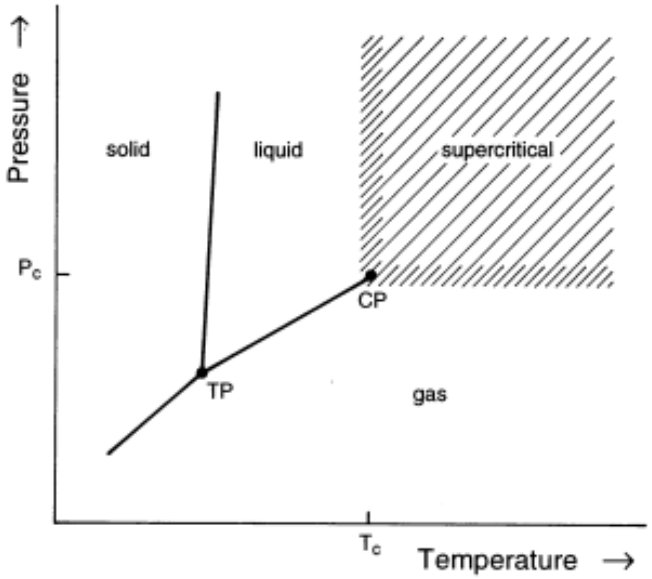
Besides acting as a nucleophile, creating carboxylic acid end-functionalized polymer chains, water has great influence in the conformation and flexibility of the enzymes, ensuring its stability (ADLERCREUTZ, 1991; SIVALINGAM; CHATTOPADHYAY; MADRAS, 2003), which means it cannot be completely removed from the enzyme.

Enzymes require a small amount of water to retain their active three-dimensional conformation even when they are covalently attached to a support. Water also contributes to the structural integrity, active site polarity and protein stability, and may also limit the solubility of hydrophobic substrates around the enzyme (DALLA-VECCHIA; NASCIMENTO; SOLDI, 2004).

2.6. E-ROP REACTIONS USING SUPERCRITICAL CARBON DIOXIDE AS SOLVENT

A supercritical fluid (Figure 2.7) can be defined as a substance that is under conditions of temperature and pressure above its critical values (above the critical point) and has intermediate properties between a gas and a liquid (BRUNNER, 2004).

Figure 2.7 - Carbon dioxide phase diagram in the pressure-temperature plane.



Source: Brunner (1994).

The most important supercritical fluid properties related to its use as a solvent are density, viscosity and diffusion coefficient. The density values are close to the values of liquids, which strengthens its solvent power. The viscosity and the diffusion coefficient, on the other hand, are close to the values of gases, which makes the transport properties of the fluid very favorable to the process. In the vicinity of the critical point, small changes in pressure and temperature lead to large variations in the density of the supercritical fluid and consequently of its solvent power (BRUNNER, 1994). All these unique properties make supercritical fluids a very interesting medium for chemical reactions. These transport

properties, similar to gases, facilitate the mass transfer rates between reactants and catalysts. In reactions limited by diffusion, kinetics will generally be more favored in supercritical fluids than in conventional liquid solvents (MESIANO; BECKMAN; RUSSELL, 1999).

The most widely used supercritical fluid is carbon dioxide (CO_2), due to its low toxicity, low cost, non-flammability and critical parameters of easy access (critical properties: 31.4 ° C and 78.8 bar). In addition, CO_2 is widely available in high purity from commercial and industrial sources and can also be reused. At ambient temperature and pressure, CO_2 is gaseous, which makes the recovery of the reaction products very simple and free of solvents (BRUNNER, 1994; JÉRÔME; LECOMTE, 2008).

Because there is no residual CO_2 remaining in the material after processing, CO_2 is not considered exactly a solvent, so it does not require reassessment by the United States FDA. Only water also enjoys this special situation. Most commercial operations using CO_2 as solvent have been initiated to take advantage of such particular advantages of CO_2 in products designed for intimate contact with the human organism, like food, biomedical and pharmaceutical devices (BECKMAN, 2004).

Some studies report the use of $scCO_2$ in lactones e-ROP. Loeker et al. (2004) reported for the first time the synthesis of poly (ε-caprolactone) via e-ROP in $scCO_2$, using the enzyme Novozym 435 as catalyst. The authors obtained poly(ε-caprolactone) with molecular weights (M_n) of 12,000 to 37,000 Da, dispersity of 1.4 to 1.6 and yields of up to 98% in 24 hours of polymerization at 65 °C. Thurecht et al. (2006) performed kinetic studies of ε-caprolactone e-ROP in a high-pressure reactor using $scCO_2$ as solvent. The authors obtained materials with high molecular weight (up to 50,000 Da, M_n), yields around 80% and could conclude that the amount of water in the reaction medium has a direct influence on molecular weight. Additionally, the authors concluded that e-ROP kinetics of ε-caprolactone in $scCO_2$ exhibited a similar behavior to e-ROP under analogous conditions, but using toluene as solvent.

More recently, poly(ε-caprolactone) was synthesized using $scCO_2$ as solvent in different types of reactors: fixed volume reactor (SANTOS et al., 2012); variable-volume reactor (COMIM ROSSO et al., 2013); and in a continuous packed bed reactor (PBR) (COMIM ROSSO et al., 2015). Comim Rosso et al. (2013) obtained poly (ε-caprolactone) with a M_n of up to 13,700 Da and dispersity of 1.2 to 1.7, while Santos et al. (2012) obtained poly (ε-caprolactone) with 7,400 Da (M_n) and dispersity of 1.96. Comim Rosso et al. (2015), using a PBR, obtained poly (ε-caprolactone) with molecular weights up to 35,800 Da and dispersity ranging from 1.5 to 2.1.

In spite of the good results of yield, molecular weight and dispersity, shown by the works that performed e-ROP of ε-caprolactone in $scCO_2$ (COMIM ROSSO et al., 2013, 2015; LOEKER et al., 2004; SANTOS et al., 2012; THURECHT et al., 2006), up to the present moment it was not possible to find in the open literature works covering e-ROP of globalide, using $scCO_2$ or any other supercritical fluid as solvent.

2.7. POST-POLYMERIZATION FUNCTIONALIZATION OF POLYESTERS

Material modification allows specific interactions within biological systems, which significantly enhances biomaterial performance, offering the material a dynamic role in its application. However, as highlighted by Pounder and Dove (2010), the introduction of functional groups throughout the polymer chain via ROP remains highly labored. Despite this hindrance, there are numerous examples of side chain functionalized linear polyesters produced from ROP which underlines the demand for these materials. Most examples reported are produced via rigorous, often multi-step, synthetic procedures to derivatize lactone or lactide monomers (ATES; THORNTON; HEISE, 2011).

Chain modification by post-polymerization allows the production of functional polymers that cannot be prepared by direct polymerization of the respective monomers. After the functionalization process, the physical and chemical properties of the polymers can be modified, such as viscosity, solubility, adhesion, reduction of crystallinity, increase of hydrophilicity, and the possibility of synthesis of more complex structures to obtain new materials.

In polymers from unsaturated macrolactones, such as globalide, the unsaturation provides a chemical handle for functionalization. A chemically straightforward modification of aliphatic polyesters is possible, by using highly efficient and robust thiol-ene “click” reactions (section 2.7.1) directly on unsaturated polyesters, obtained from ROP of the unsaturated macrolactone. This reaction may be employed for the post-polymerization modification of unsaturated polyesters to allow the introduction of numerous different chemical groups to polymeric backbones, thereby potentially enabling the formation of biofunctional materials (ATES; THORNTON; HEISE, 2011; DONDONI, 2008; LOWE, 2009).

Some authors reported the synthesis of polyglobalide (PGl) via e-ROP using CALB lipase, where the double bond in the formed polyester chain was functionalized via thiol-ene reaction, using different thiol

groups. These works aimed to change characteristics of polyglobalide and its copolymers, such as thermal and mechanical properties, crystallinity, hydrophilicity and ability for bioconjugation (ATES et al., 2014; ATES; HEISE, 2014; ATES; THORNTON; HEISE, 2011; CLAUDINO et al., 2012).

2.7.1. Thiol-ene "click" reactions

Kolb, Finn and Sharpless (2001) described a new concept for the conduction of organic reactions, which follows examples of nature, generating substances by joining small units together with heteroatom links. This concept is called "click" chemistry. Click reactions must have the following characteristics: being modular (capable of connecting two molecules), being wide in scope, obtaining high reaction yields, forming harmless byproducts (which can be removed by non-chromatographic methods), and being stereospecific. The required process characteristics include simple reaction conditions, readily available starting materials, solvent exclusion of the reaction medium or use of non-toxic solvents and ease of purification of the product.

Thiol-ene reactions consists of a simple and adaptable methodology to prepare functionalized polymers and polymer networks using combinations of multifunctional alkenes and thiols (HOYLE; LEE; ROPER, 2004). Thiol-ene addition reactions are considered click reactions, since they meet several attributes of this reactions class. Thiol-ene reactions have high yields and fast reaction rates, need small concentrations of catalysts, can be conducted without the use of solvent or using non-toxic solvents, can be carried out under mild conditions, are insensitive to air/oxygen, and generate pure products (HOYLE; BOWMAN, 2010; HOYLE; LEE; ROPER, 2004).

The first applications of products obtained through thiol-ene reactions were implemented in the construction of materials for simple applications, such as films, protective coatings, coatings for electronic materials and printed circuit boards (HOYLE; BOWMAN, 2010). The limited application of these materials was related to problems with the color of the coatings, caused by initiator residues in the product, allied to weathering. Benzophenone (photoinitiator) was used in large quantities in these systems, giving rise to significant light stability problems of the cured networks associated with colored byproducts formed upon exposure to interior and exterior light. The use of thiol-ene chemistry was severely reduced when new low-cost polymers of high purity became

available at prices that allowed for widespread applications (HOYLE; LEE; ROPER, 2004).

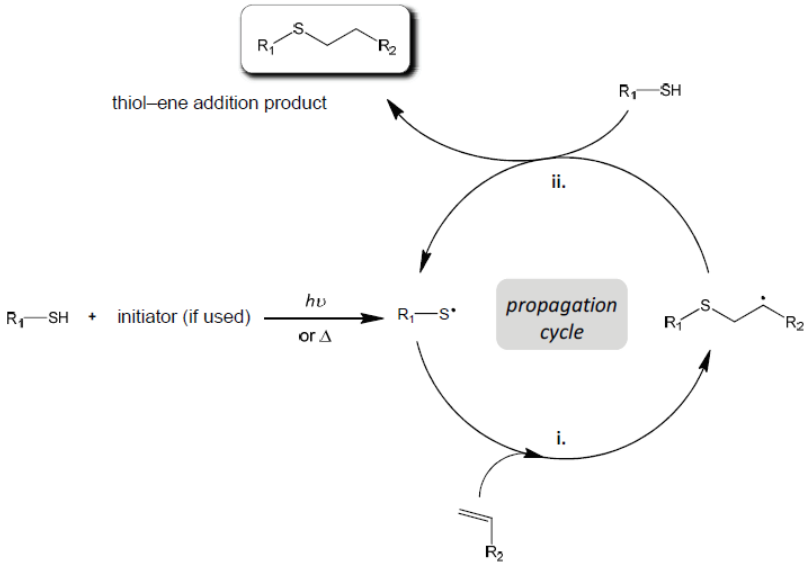
However, in recent years, thiol-ene addition reactions have been applied in the synthesis of new materials with high added value, being the surface modification, functionalization and crosslinking of polymers of great importance for applications in biomedical area, since a controlled polymer structure can be achieved by this method (HOYLE; BOWMAN, 2010; HOYLE; LEE; ROPER, 2004).

Thiol-ene reaction takes advantage of the easily abstractable hydrogen atom of the thiol group due to the relatively weak sulphur-hydrogen bond (FOSSEY; LEFFORT; SORBA, 1995). The cleavage of SH bonds can be promoted either by direct photolysis (or simply by thermolysis) or indirectly from heat (or light) generated nucleophilic alkyl radicals obtained from the cleavage of initiators. The resulting electrophilic thiyl radicals (RS•) are extremely reactive and can add to a wide variety of unsaturated compounds to form new carbon-carbon linkages. The RS• addition reaction to the double bond is exothermic and forms a strong σ C-C bond, (370 $kJ.mol^{-1}$), at the expense of the weaker π bond (235 $kJ.mol^{-1}$), which means this reaction is energetically favored (CLAUDINO, 2011).

Generally, thiol-ene reactions have been conducted under radical conditions which proceed through a typical chain process with initiation, propagation and termination steps (ATES, 2014; CLAUDINO, 2011). The characteristic two-step mechanism for the addition reaction of an isolated unsaturation is represented in Figure 2.8.

The thiol-ene reaction starts via initiation (UV or thermally induced), promoting the hydrogen transfer from the thiol (R-SH) to one of the initiating free-radicals generated (via photoinitiator or initiator cleavage), generating the thiyl radical (RS•). During the first propagation step, the generated thiyl radical adds across the C=C double-bond, yielding an intermediate β-thioether carbon centered radical. On the second propagation step occurs the chain transfer to a second thiol group, giving the final thiol–ene addition product, and regenerating RS• radical. The mechanism continues to generate thiyl radicals in this cyclic process, allowing the propagation of the reaction until the depletion of the thiol-ene species. Termination reactions are frequently considered unimportant if compared to the rates of propagation and usually involve bimolecular combination of the intervening radical species (β-carbon or thiyl radicals) (CLAUDINO, 2011).

Figure 2.8 - Two-step mechanism for free-radical thiol-ene reaction, alternating propagation (i) and chain transfer (ii).



Source: Claudino (2011).

2.7.2. Thiol-ene reactions in unsaturated polyesters

So far there are very few studies related to e-ROP from unsaturated macrolactones available in the open literature. The first study reported was developed by van der Meulen et al. (2008), which performed the synthesis of two unsaturated polymacrolactones, using globalide (15 carbons) and ambretolide (16 carbons) as monomers. The polymers obtained showed to be biocompatible and non-toxic (monomer derivative from hydroxy fatty acids), which is the primary requirement that all materials have to meet for applications in the body (VAN DER MEULEN et al., 2008). However, polyesters derived from macrolactones are very hydrophobic and semi-crystalline, which are undesired characteristics for applications were bioresorption is necessary, such as drug delivery devices or scaffolds. In their work van der Meulen et al. (2008) obtained polymacrolactones of molecular weight (M_n) around 24,000 Da. The degree of crystallinity obtained for polyambretolide e polyglobalide was

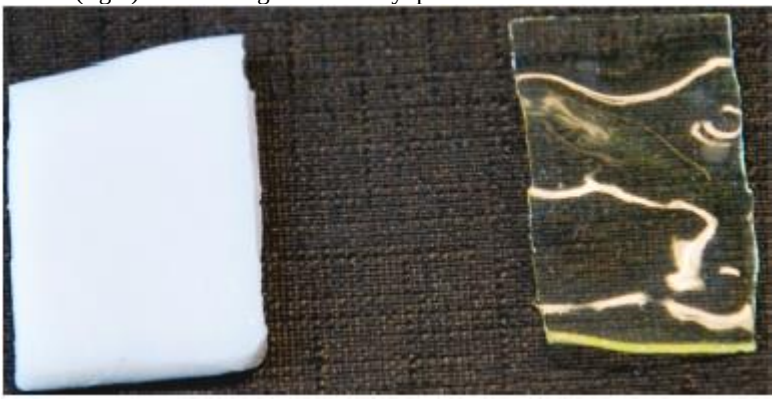
62 and 60%. It was not observed hydrolytic and enzymatic degradation after long monitoring periods.

Since then, this research group has studied the functionalization and crosslinking of polyesters obtained from the monomer globalide, in order to obtain materials susceptible to degradation for biomedical purposes. The globalide monomer is composed of 15 carbons and has an unsaturation in its chain (position 11 or 12 - mixture of two isomers). This chain unsaturation allows crosslinking or functionalization through thiol-ene reaction.

Ates, Thorton and Heise (2011) carried out the functionalization of polyglobalide via thiol-ene addition reactions using 6-mercapto-1-hexanol (MH) and N-acetylcysteamine (n-ACA). The authors obtained coupling efficiency of 95% for both MH and n-ACA, when using high concentrations of initiator (AIBN) and thiol in excess. The polymers formed showed branched chains and there was reduction in the crystallinity of the final material, resulting in totally amorphous materials. According to the authors, the introduction of these groups also potentially enabled further polymeric modification, to introduce functional amino acid groups (among others) that possess a wide range of chemical and physical properties.

Meulen et al. (2011) carried out the copolymerization of globalide and ambretolide with ε-caprolactone, 4-methyl caprolactone and 1,5-dioxepan-2-one, in order to increase the biodegradability of the material. The authors crosslinked the obtained copolymers with dicumyl peroxide. In the enzymatic degradation studies with *Pseudomonas cepacia* lipase, the authors observed a loss of mass of 30% and greater than 90% after approximately 100 days for the copolymers poly(Gl-co-4MeCl) - in the ratio 53/47 (Gl/4MeCL) - and poly(Am-co-DXO) - in ratio 25:75 (Am: DXO) -, respectively. Figure 2.9 shows the copolymer poly (Gl-co-4MeCl) before and after crosslinking with dicumyl peroxide. It is possible to observe the change in the color of the materials as a function of the transition from the initial crystalline state to the amorphous final state.

Figure 2.9 - Copolymer poly(globalide-co-4-methyl caprolactone), before (left) and after (right) crosslinking with dicumyl peroxide.

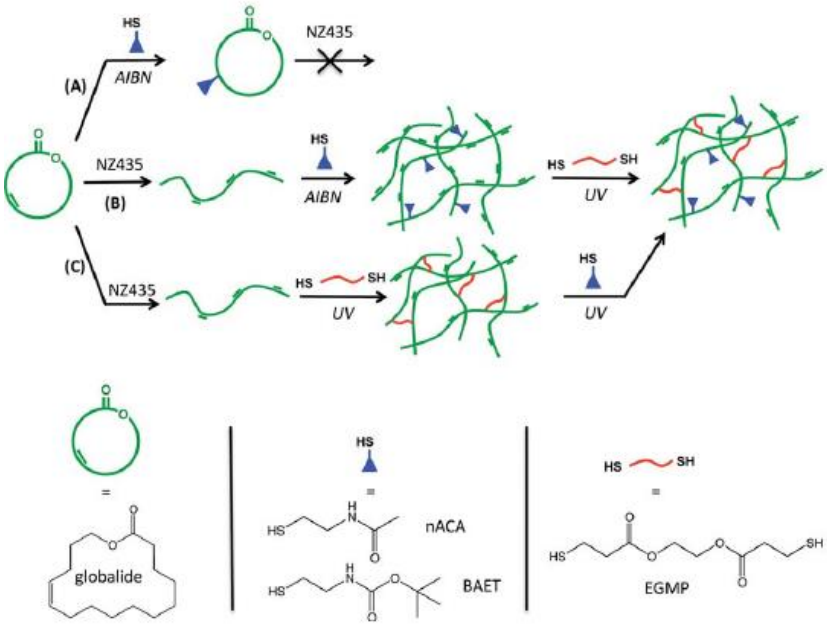


Source: van der Meulen et al. (2011).

Claudino et al. (2012) also synthesized copolymers of globalide and ε-caprolactone. The formed copolymer was crosslinked through the addition of a trithiol (trimercapto propionate - TMP) via photoinitiation (photoinitiator Irgacure 184) in the double bonds derived from the globalide units. The authors obtained high thiol–ene conversions (>80%) in all cases. For high globalide contents (high unsaturation density), crosslinked polymers lost all the crystallinity, becoming amorphous, since crosslinking inhibits chain mobility to crystallize. To lower contents of globalide, some crystallinity still remains in the sample.

After these studies, Ates and Heise (2014) evaluated different strategies to produce stable crosslinked surface functional films from polyglobalide, employing a dithiol crosslinker and N-acetylcysteamine (N-ACA) mono-thiol as a model compound. Three routes were tested, as shown in Figure 2.10. In route A, the authors tried to functionalize the monomer first, and then polymerize by e-ROP the functionalized monomer. In route B, first, the polymer was produced by e-ROP. After the polymerization, a fraction of the double bonds was functionalized with N-ACA. Lastly, the free double bonds of the functionalized polymer was crosslinked with the use of the dithiol. Route C was similar to route B, but performing the crosslinking step before the functionalization step.

Figure 2.10 - Synthetic routes to functional crosslinked films from macrolactone using thiol–ene chemistry.

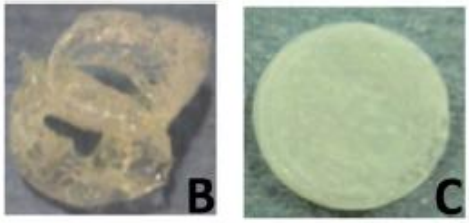


NZ435: Novozym 435; AIBN: azobisisobutyronitrile; N-ACA: N-acetylcysteamine; BAET: 2-(Boc-amino) ethanethiol; EGMP: ethylene glycol bis(3-mercapto propionate).
Source: (ATES; HEISE, 2014).

Routes A and B did not result in products with desirable qualities, leading to the formation of low yield polymers and molecular weights. The crosslinked polymers obtained by route B were tacky and of low mechanical integrity, irrespective of the crosslinker to polymer ratio, rendering this route unfeasible. Route C formed films with high molecular weights, 100% crosslinked, totally amorphous and transparent and presenting a 20% reduction in their mass when submitted to enzymatic degradation during 50 days. Figure 2.11 shows the difference between the films obtained by routes B and C. The authors explained that this difference might happen due to an incomplete network formation, which might be due to a too large distance between the double bonds preventing optimal formation of a three-dimensional network. This problem could be overcome by carrying out the geometrically more demanding

crosslinking reaction first, followed by N-ACA post-functionalization on the crosslinked film.

Figure 2.11 - Crosslinked functionalized PGl films prepared by Ates and Heise (2014) through routes B and C.

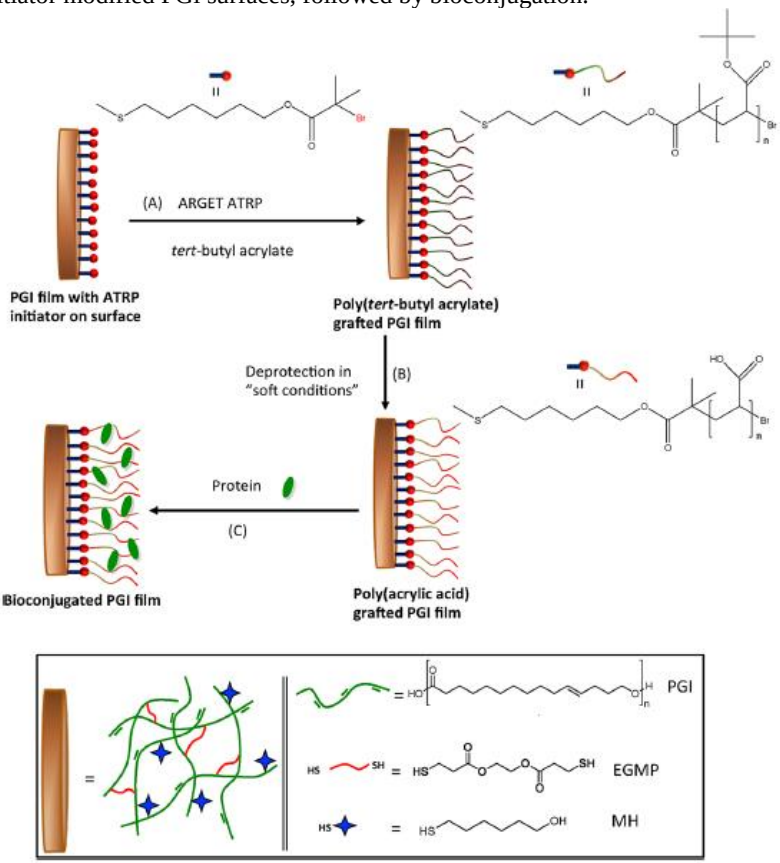


Source: Ates and Heise (2014).

After these series of studies, Ates et al. (2014) published a work putting together all the knowledge developed previously about the synthesis of functionalized crosslinked PGl films. Their idea was to synthesize a PGl film functionalized with chemical chains capable of bioconjugation, improving cell adhesion and enhancing cell growth. The sequence of reactions performed to obtain these films are represented in Figure 2.12.

The authors synthesized PGl films by e-ROP, the films were crosslinked and then functionalized with 6-mercapto-1-hexanol. This functionalized films, also called “hydroxyl decorated films” (PGl-OH), were converted into atom transfer radical polymerization (ATRP) initiators, through reaction of the hydroxy decorated film with α-bromoisobutyryl bromide, generating “initiator decorated films”. The initiator decorated films reacted to tert-butyl acrylate, forming a poly (tert-butyl acrylate) grafted PGl film. After deprotection of the tert-butyl ester groups, reactive carboxylic acid groups are generated, which makes the decorated PGl films able to bioconjugation. The authors successfully immobilized fluorescent protein and chitobase enzyme in the obtained grafted PGl film. This was the first work that reported the production of biofunctional films derived from natural macrolactones.

Figure 2.12 - Synthesis of poly (acrylic acid) grafted from crosslinked and ATRP initiator modified PGl surfaces, followed by bioconjugation.



Source: Ates et al. (2014).

2.7.3. Conjugation of nanoparticles and proteins

It is known that the composition of the protein corona, formed when the nanoparticles are in contact to a biological fluid, gives a biological identity to the nanoparticles and defines its interaction with cells (LYNCH; DAWSON, 2008; TREUEL et al., 2014). Engineering the surface of the nanoparticles is an excellent strategy to tune interfacial properties and produce tailored materials for specific cell targeting, enabling treatments to occur efficiently (DE et al., 2007; RANA; YEH; ROTELLO, 2010).

Polyethilene glycol (PEG) is a molecule widely used in order to functionalize nanoparticles, aiming to suppress cell uptake by defense cells through the composition control of the protein corona that surrounds nanoparticles. However, PEG is a non-biodegradable polyether, and its accumulation in the human body should be avoided, especially when NPs-PEG conjugates are administered in long-term treatments. In such cases, the accumulation of PEG in the human body can cause undesired side effects (SCHÖTTLER et al., 2016). In this context, the functionalization of nanoparticles directly with proteins rises as an alternative to the use of PEG, as a way to control the composition of the protein corona. Apolipoproteins, for example, when conjugated with nanoparticles, have the ability of inhibit its uptake by defense cells (CARACCIOLO et al., 2015; MONOPOLI et al., 2011; SCHÖTTLER; LANDFESTER; MAILÄNDER, 2016). Apolipoprotein J (Apo J), also known as clusterin, and apolipoproteins A4 and C3 (ApoA4 and ApoC3) presente high capacity to inhibit phagocytosis (RITZ et al., 2015; SCHÖTTLER et al., 2016).

When used in the nanoparticle pre coating (non-covalent), ApoA4 and ApoC3 significantly reduced non-specific cell uptake (RITZ et al., 2015). The non-covalent adsorption method is one of the simplest and most frequently employed procedures for the attachment of proteins onto particles(ARVIZO; DE; ROTELLO, 2007). The attachment occurs by hydrophobic or electrostatic interactions between the nanoparticle and the protein (ARVIZO; DE; ROTELLO, 2007; CARUSO, 2001). However, this method have a serious disadvantage, since conjugates produced by non-covalent methods are reversible. This means that proteins can adsorb and desorb to the surface of NPs, and it becomes very difficult to maintain the stability, uniformity and reproducibility of the conjugates, which are desired characteristics for biomedical applications. Under physiological conditions, proteins adsorbed to the NPs can desorb and be replaced by other proteins and biomolecules present in the local environment, making it difficult to predict the behavior and the composition of this conjugates for long times. (BOYER et al., 2011; CHEN; LIU LORETO MEGIDO; DÍEZ, 2018; PINO et al., 2014).

Covalent functionalization of proteins and nanoparticles is an interesting strategy to produce conjugates that are stable toward dissociation. The stability and irreversibility of covalent protein-NP conjugates are decisive factors that determine the biological response of cells and organisms. The stability of the conjugates is especially important for applications in complex biological media, where many interfering species can alter the composition of protein-NPs conjugates

and consequently change completely the targeting of nanoparticles over time (LESZCZYNSKI, 2010; RANA; YEH; ROTELLO, 2010).

2.7.3.1. Thiol-ene functionalization of proteins and nanoparticles

Thiol-ene click reactions are highly efficient and robust reactions and can be employed in the preparation of biologically active protein–nanoparticles conjugates, in spite of being still little reported in the literature to this purpose (DONDONI, 2008; KELEŞ; HAZER; CÖMERT, 2013; LOWE, 2009; TUNCA, 2014). Site-specific covalent conjugation strategies, using highly efficient orthogonal chemistries, such as thiol–ene click reactions, provide good control over the orientation of the conjugated proteins (BOYER et al., 2011), forming also very uniform and reproducible conjugates.

Thiol-ene reactions are especially interesting in the presence of free thiol groups in cysteine-containing proteins. The cysteine thiol group can be attached to double bonds on the surface of the nanoparticles, leading to site-selective and irreversible conjugations (BOYER et al., 2011).

In the work of Liu et al. (2017) a cysteine-selective ligand containing a double bond was designed and anchored to the surface of a hydrophobic nanoparticle surface. This way, any thiol-containing biomolecule can be conjugated on the surfaces of nanoparticles. In their work, a cysteine-containing horseradish peroxidase were selected for thiol-ene bioconjugation of colloidal nanoparticles. The success of the thiol-ene linkage occurrence was determined by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis).

It is important to highlight that is still a challenge to verify the covalent conjugation efficiency in systems involving nanoparticles and complex molecules such as proteins and peptides (ABBAS et al., 2017). In this cases, traditional analytical methods (spectroscopy techniques, nuclear magnetic resonance and chromatography, for example) are not always able to detect the formation of this covalent linkages, especially due to the complexity of proteins structure and difficulties in solubilizing the conjugates.

2.8. THE STATE OF ART

The arising of polymeric materials in the beginning of the 20th century has revolutionized the industry and the life of people. Due to its versatility and practicality, polymers have rapidly replaced traditional

materials such as metals, glass and wood in a wide range of applications, including the automotive, food, electronics and civil construction industries.

With the emergence of tissue engineering and nanomedicine, polymers also started to be intensely used in biomedical and pharmaceutical applications. The increasing demand for biocompatible materials for specific applications in biological environment is encouraging current and future scientists to work on researches towards designing bioresorbable polymers with enhanced properties and develop clean processes.

One of the most studied biocompatible/bioresorbable polymers is poly(ε-caprolactone). PCL is very attractive due to its excellent properties such as high flexibility, biodegradability and biocompatibility, as well as non-toxicity (PITT; MARKS; SCHINDLER, 1980). However, PCL has a high crystallinity, which makes its bioresorption in the organism very slow, which is not desired for some applications.

Polyglobalide, a polymer derivative from an unsaturated macrolactone, is a very little studied polymer, which is non-toxic and biocompatible and has very interesting mechanical properties (VAN DER MEULEN et al., 2008). Besides, PGl chain can easily be functionalized and/or crosslinked, due to the presence of unsaturation in its chain, through thiol-ene reactions. The functionalization is capable to reduce monomer crystallinity and hydrophobicity, as well as enhance the affinity of the polymer for different human cells/tissues.

The synthesis of copolymers based on ε-caprolactone and globalide come in order to unite the flexibility and the biodegradability features of PCL, to the good mechanical properties and the double bonds versatility from PGl, enabling the development of a material with tailored properties by tuning monomer ratios and/or using different functionalizing molecules.

The most interesting route for obtaining biocompatible and bioresorbable polymers with potential use in the biomedical and pharmaceutical areas, is the ring-opening polymerization catalyzed by enzymes (e-ROP). The use of enzymes avoids the use of toxic organometallic catalysts and is environmentally friendly. Supercritical carbon dioxide used as solvent also proved to be interesting from the environmental and health point of view, being easily separated from the polymer, and possible to be reused. The use of $scCO_2$, together with e-ROP have contributed to render the (co)polyesters production a clean process (COMIM ROSSO et al., 2013; SANTOS et al., 2012; THURECHT et al., 2006; VENERAL, 2014).

Post-polymerizations modifications rise as a strategy to enhance biomaterials performance. Thiol-ene click reactions are an efficient way to produce functionalized polymers for tissue engineering purposes, due to its high yields and formation of harmless byproducts. Also, it is one of the most interesting methods to produce nanoparticle-proteins covalent conjugates, yielding uniform and stable nanomaterials for treatments in specific targeted cells.

In the presented literature review, it was shown important results published in the scientific literature, which are directly related to the theme of this project. However, there are still a great number of challenges to be overcome, especially regarding the functionalization of polymers to proteins and its characterization, given the complexity of protein structures and its direct influence on protein-cell interactions.

Considering the actual scenario, there is a great demand for biocompatible/bioresorbable polymers, and there is wide accessibility for clean technologies. It is common to find in literature works that have been carried out individually on the topics here proposed: polymerization in supercritical fluids, post-polymerization functionalization by thiol-ene reactions and conjugation of proteins to nanoparticles. However, the innovation of this work consists in the connection among all these topics, leading to the construction of an engineered biomaterials platform based on PGlCL, free of toxic residues, and with enhanced properties for different biomedical applications. Researches in this area are fundamental for the development of clean processes and new biomedical devices, improving the quality of life of the population.

CHAPTER 3

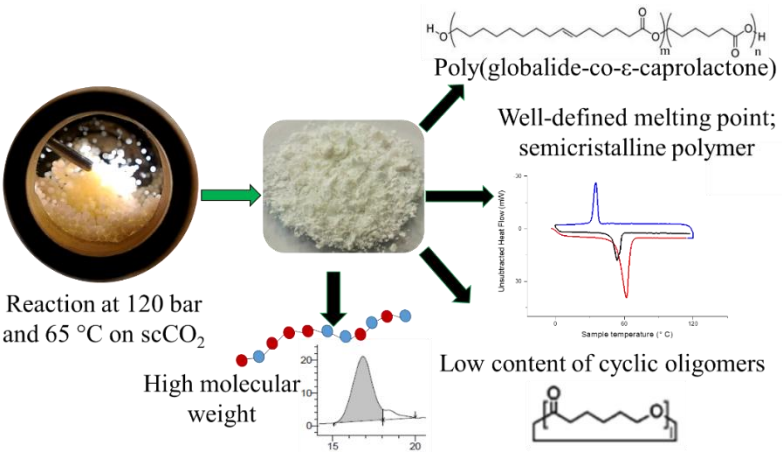
The use of $scCO_2$ and $scCO_2$ in mixture with DCM as solvents was studied by synthesizing PGlCL on different fed ratios between globalide and ε-caprolactone. It was evaluated the influence of the different solvents and monomers ratio on the molecular weight, thermal properties, formation of oligomeric cycles and ratio between globalide and ε-caprolactone repeating units present in the obtained copolymers. Through the understanding of the mass transfer behavior on e-ROP, it was possible to stablish relation between the variables evaluated and its effects on the polymer properties. The results presented in this chapter were published in The Journal of Supercritical Fluids (doi.org/10.1016/j.supflu.2017.06.008).

3. ENZYMATIC RING OPENING COPOLYMERIZATION OF GLOBALIDE AND E-CAPROLACTONE UNDER SUPERCRITICAL CONDITIONS

Abstract

The enzymatic ring opening copolymerization of globalide and ε-caprolactone under supercritical conditions was investigated. Samples were evaluated in terms of molecular weight, thermal properties, ratio between repeating units, and production of cyclic oligomers. For the first time, poly(globalide-co-ε-caprolactone) (PGlCL) was synthesized by e-ROP using supercritical carbon dioxide ($scCO_2$) and the mixture $scCO_2$+dichloromethane (DCM) as solvents. The feed ratio between globalide and ε-caprolactone was evaluated. PGlCL synthesized in $scCO_2$ showed the highest molecular weights, while the use of cosolvent caused a decrease on molecular weight values. For both $scCO_2$ and $scCO_2$+DCM, the increase on globalide content (relative to total monomers amount) increased molecular weight values. Thermal analysis indicated a non-isomorphic behavior, characteristic of random semi-crystalline copolymers, which is the case of the PGlCL produced. PGlCL synthesized on $scCO_2$+DCM presented double melting point, which is related to a greater presence of cyclic oligomers, in comparison to PGlCL synthesized using only $scCO_2$, being confirmed by MALDI-TOF analysis.

Graphical Abstract



3.1. INTRODUCTION

During the last decades, the development of biodegradable/bioresorbable polymeric materials for biomedical applications has advanced considerably, due to the interest in replacing traditional materials used as medical devices. In this context, the enzymatic synthesis of polymers for biomedical applications is very attractive, since enzymes do not leave toxic residues in the final product, are active under mild conditions of temperature, and provide very specific polymers (COMIM ROSSO et al., 2013).

The use of supercritical carbon dioxide ($scCO_2$) in substitution to organic solvents is an alternative on enzymatic ring-opening polymerization (e-ROP) reactions. $scCO_2$ is inexpensive, non-toxic, non-flammable (KUMAR; MADRAS; MODAK, 2004), and exhibits transport properties that can accelerate mass transfer in enzymatic reactions (OLIVEIRA; OLIVEIRA, 2000). Also, it can be easily separated from the final product by system depressurization and can therefore be reused in the process. In order to improve the system solubilization, it is also possible to use cosolvents, together with $scCO_2$. Dichloromethane (DCM) has been successfully used as solvent on e-ROP reactions, as well as on scaffolds without affect the cultivation and growth of different kinds of cell (CHEW et al., 2008; ELOMAA et al., 2011; POLLONI et al., 2017; VENERAL, 2014; XIE et al., 2009). Its low

boiling point (40 °C) (BROWN; STEIN, 2016) allows its easy separation from the final products, avoiding toxicity problems (JÉRÔME; LECOMTE, 2008).

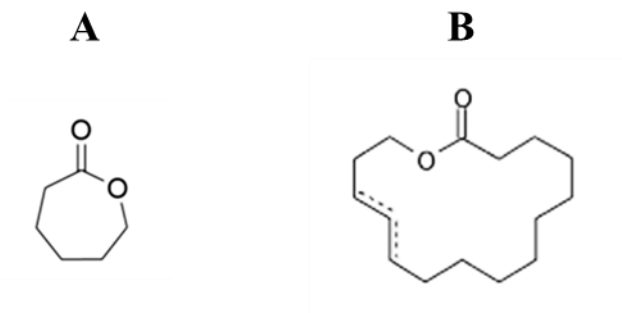
The synthesis of poly(ε-caprolactone) (PCL) by e-ROP using organic solvents has been extensively studied (CÓRDOVA et al., 1998; KUMAR; GROSS, 2000; KUNDU et al., 2011; MEI; KUMAR; GROSS, 2003; ZHANG et al., 2012a). PCL is one of the most attractive polymers for medical application, since it is biodegradable and bioresorbable, and presents mechanical properties proper for this application (ALBERTSSON; SRIVASTAVA, 2008). Some drug delivery devices made from PCL already have FDA approval and trademark (WOODRUFF; HUTMACHER, 2010).

Polyglobalide (PGl) is a biocompatible and non-toxic polymer, produced from the monomer globalide (VAN DER MEULEN et al., 2008). Globalide is an unsaturated 16-membered macrolactone, which contains a double bond. Typically, globalide is used in the fragrance industry due to its musky odor and its quality of losing its scent slowly (MICHROWSKA; WAWRZYNIAK; GRELA, 2004; WILLIAMS, 1999). In comparison to small lactones, the chemical polymerization of macrolactones is a challenge, since in general it proceeds slowly and gives low molecular weight polymers (FOCARETE et al., 2001; NOMURA; UENO; ENDO, 1994). However, the enzymatic ring-opening polymerization of macrolactones has shown to be very effective (FOCARETE et al., 2001; GEUS et al., 2010; POLLONI et al., 2017). Polymers obtained from macrolactones also have excellent mechanical properties (CAI et al., 2010; FOCARETE et al., 2001; GEUS et al., 2010). Besides, the double bonds in globalide structure lead to many possibilities of functionalizing the polymer, giving it the most diverse characteristics, making PGl very interesting for its application in medical devices, as it could increase biocompatibility or biodegradation.

The synthesis of copolymers of globalide (Gl) and ε-caprolactone (CL) is a versatile alternative for tissue engineering, since the ratio between the monomers may be tuned, producing copolymers with different molecular weights and melting temperatures, depending on the desired application. Besides, the presence of the double bond enables the change of other characteristics that cannot be changed by the simple variation on monomer ratio (increase on hydrophilicity, affinity for different human tissues, decrease on crystallinity index and increase on mechanical resistance for example) (ATES et al., 2014; ATES; HEISE, 2014; ATES; THORNTON; HEISE, 2011; CLAUDINO et al., 2012), by

functionalizing the copolymer. Figure 3.1 shows the structures of the monomers globalide (Gl) and ε-caprolactone (CL).

Figure 3.1 - Structure of the cyclic monomers (A) ε-caprolactone and (B) globalide. Globalide a mixture of two constitutional monomers with the double bond located either at the 11 or 12 position (dashed line).



The present work aimed to study the synthesis of poly(globalide-co-ε-caprolactone) (PGlCL) copolymers by e-ROP, using $scCO_2$ and the mixture $scCO_2$+DCM as solvents, being a subject which was not reported in literature until now. e-ROP of PGlCL using $scCO_2$ and $scCO_2$+DCM were performed under different feed ratios between globalide and ε-caprolactone. It were studied the effects of the different solvents and the globalide/ε-caprolactone feed ratio on copolymer properties, such as molecular weight, dispersity, melting temperature, crystallinity index and formation of cyclic oligomers.

In this way, the present work brings a series of unprecedented important results, which contributes to the understanding of the process phenomenology behind PGlCL e-ROP on $scCO_2$, being vital for future studies on PGlCL functionalization and its use for biomedical devices development.

3.2. MATERIAL AND METHODS

3.2.1. Material preparation

Dichloromethane P.A. 99.8% (DCM) and ethanol P.A. 99.8% (EtOH) was purchased from Vetec Química and used as received. Novozym 435 was kindly donated by Novozymes, Brazil, A/S (commercial lipase B from *Candida antarctica* immobilized on cross-linked polyacrylate beads, esterification activity 42 U/g, measured

according to a procedure adapted from literature (OLIVEIRA et al., 2006)). Enzymes were dried under vacuum (0.4 bar) and 70 °C, during 16 hours (VENERAL, 2014) and stored in a desiccator over silica and 4 Å molecular sieves. Globalide was a kind gift of Symrise. ε-caprolactone were purchased from Sigma-Aldrich. Both globalide and ε-caprolactone were dried under vacuum (0.1 bar) and 100 °C, during 24 hours (VENERAL, 2014) and also stored in a desiccator over silica and 4 Å molecular sieves. Carbon dioxide (99.9% purity) used as solvent was purchased from White Martins S/A, Brazil.

3.2.2. Enzymatic ring-opening polymerization in pressurized solvents

Polymerization experiments were carried out in a high-pressure variable-volume view cell, with a maximum internal volume of 27 mL, with two sapphire windows for visual observation, an absolute pressure transducer (Model LD 301, Smar, USA) and a syringe pump (260HP, Teledyne Isco, Lincoln, NE, USA). The cell contains a movable piston that allows the control of pressure inside the reactor. The variable-volume reactor allows an accurate control of pressure and temperature during reaction.

For polymerization experiments, the enzyme (Novozym-435), the monomer (globalide and ε-caprolactone) and DCM (when used) were weighed on a precision scale balance (Shimadzu AUY220, Philippines with 0.0001 g accuracy) and placed inside the reactor, which was immediately closed. A known amount of solvent, CO_2, was loaded into the reactor using the syringe pump until the desired composition was achieved, meeting the mass ratio specified conditions. The system pressure was increased until the desired pressure was achieved. A metallic jacket surrounds the reactor, and water from a thermostatic bath was used as heating/cooling fluid, keeping the cell at the desired temperature. Once the desired temperature was reached, the reaction time started. The cell content was kept under constant stirring with the use of a magnetic stirrer and a Teflon^{TM}-coated stirring bar.

To understand the influence of the different globalide to ε-caprolactone proportions on enzymatic synthesis of poly(globalide-co-ε-caprolactone) in $scCO_2$ and $scCO_2$ with the use of cosolvent, a series of assays were performed, varying the feed mass ratio of globalide/ε-caprolactone (10/90, 25/75, 50/50, 75/25 and 90/10). These ratios may also be expressed in percentage of globalide relative to the total monomer amount. With the aim of improving the mass transfer (increasing the

solubility of the system), DCM was used as cosolvent. DCM has an easy separation from final products due to its low boiling point (JÉRÔME; LECOMTE, 2008), and promotes good solubilization of globalide and ε-caprolactone (BORDES et al., 2010; VAN DER MEULEN et al., 2008).

The pressure and temperature conditions of the system were maintained constant at 120 bar and 65 °C, respecting the temperature range of higher enzyme activity (OLIVEIRA et al., 2004; RICHETTI et al., 2010; ROSA et al., 2008). Enzyme content was fixed at 5 wt% (relative to the total monomer amount). For assays which used only $scCO_2$ as solvent, the CO_2:monomers mass ratio was fixed at 1:2. For assays using $scCO_2$, and DCM as cosolvent ($scCO_2$+DCM), DCM:monomers mass ratio was maintained constant at 1:2 for all assays, while the mass ratio CO_2:MIX were 1:2 and 2:1 (where MIX = DCM+monomers).

Also, a reaction using only DCM as solvent was performed, using a feed globalide/ε-caprolactone mass ratio of 10/90, at exactly the same conditions used in reactions on $scCO_2$, fixing a DCM:monomers mass ratio of 1:2. The reaction was performed at this condition in order to compare the formation of cyclic oligomers by the use of different solvents through MALDI-TOF analysis.

The reaction conditions were chosen based on previous works (COMIM ROSSO et al., 2013, 2015; POLLONI et al., 2017) of e-ROP homopolymerization of ε-caprolactone and ω-pentadecalactone, using $scCO_2$ as solvent, that led to high values of yield and homopolymer molecular weight. The reaction time was set at 2 h. After polymerization, the material was purified through solubilization in DCM, followed by the separation of the enzymes and precipitation in cold EtOH. DCM and EtOH were used at the volumetric proportion of 1:6. The polymeric suspension was filtered and dried at room temperature in vacuum, up to constant mass.

3.2.3. Characterization of the copolymer

Gel Permeation Chromatography - GPC: Number average molecular weight (M_n), weight average molecular weight (M_w) and dispersity (Đ) were determined by Gel Permeation Chromatography (GPC). For the analysis, 0.02 g of the copolymer was dissolved in 4 mL of tetrahydrofuran (THF). The obtained solution was filtered through a nylon syringe filter, pore: 0.45 µm, diameter: 33 mm. The molecular weight distributions were obtained using a high-performance liquid chromatography equipment (HPLC, model LC 20-A, Shimadzu) and Shim Pack GPC800 Series columns (GPC 801, GPC 804 e GPC 807),

also from Shimadzu. THF was used as eluent with volumetric flow rate of 1 mL·min^{-1} at 40 °C. The calibration was performed using polystyrene standards with molecular weight ranging from 580 to 9.225 x 10^6 g.mol^{-1}.

Differential Scanning Calorimetry - DSC: Samples of approximately 5 mg of dried copolymer were analyzed using a Perkin-Elmer JadeDSC, under inert atmosphere (20 mL·min^{-1}), from 0 to 120 °C at a heating rate of 10 °C/min. The thermal history was removed prior to the analyses at a heating rate of 20 °C/min and cooling rate of 10 °C/min. Melting and crystallization temperatures were determined from the second heating run and the first cooling from the melt, respectively. The material crystallinity was determined using the fusion enthalpy (ΔH_m) from the second heating run.

Nuclear Magnetic Resonance - NMR: ^{1}H NMR and ^{13}C NMR spectroscopy were performed on a Bruker AC-200F NMR, operating at 200 MHz for ^{1}H NMR and 50 MHz for ^{13}C NMR. Chemical shifts are reported in ppm relative to tetramethylsilane (TMS) 0.01% (v/v) (δ=0.00). All samples were solubilized in $CDCl_3$ (δ = 7.27 for ^{1}H NMR, and δ = 77.0 for ^{13}C NMR).

Poly(globalide-co-ε-caprolactone) ^{1}H NMR ($CDCl_3$ 200 MHz): δ (ppm) 5.49-5.32 (m, C***H***=C***H***), 4.10-4.04 (m, C$\boldsymbol{H_2}$O(C=O)); 2.35-2.26 (m, C$\boldsymbol{H_2}$(C=O)O); 2.07-1.97 (m, C$\boldsymbol{H_2}$(CH=CH)); 1.71-1.62, 1.29 (m, C$\boldsymbol{H_2}$).

Poly(globalide-co-ε-caprolactone) ^{13}C NMR ($CDCl_3$ 50 MHz): δ (ppm) 173.9 (***C***=O), 133.5-125.0 (***C***H=***C***H), 64.1-63.7 (***C***H_2O(C=O)), 34.4-32.0 (***C***H_2(C=O)O), 29.5-23.6 (***C***H_2).

Degree of randomness - R: The degree of randomness of the copolymer was calculated from ^{13}C NMR data through the following equations (JANSEN, 2005; VAN DER MEULEN et al., 2011; YAMADERA; MURANO, 1967):

$$P_{G/C} = P_{GC} + P_{CG} \tag{3.1}$$

$$P_{G\,total} = \frac{P_{G/C}}{2} + P_{GG} \tag{3.2}$$

$$P_{C\,total} = \frac{P_{G/C}}{2} + P_{CC} \tag{3.3}$$

$$R = \frac{P_{G/C}}{2P_{G\,total}P_{C\,total}} \tag{3.4}$$

where P_{GG}, P_{CC}, P_{CG} and P_{GC} represents the integral value of the GG, CC, CG and GC diads, respectively, denoting the mol fraction of each diad.

$P_{G/C}$ denotes the total integral of the mixed diads (GC and CG) and $P_{G\ total}$ and $P_{C\ total}$ denotes the total mol fraction of G and C units respectively.

MALDI - TOF mass spectrometry: Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) was carried out in a Bruker Autoflex III smartbeam spectrometer. 2,5-dihydroxybenzoic acid (DHB) was used as matrix, being dissolved in a methanol/water solution (70% v/v methanol) in a concentration of 20 mg/mL. Samples were solubilized in chloroform and then mixed to the matrix solution in a 1:1 volume ratio. To semi quantify the cyclic chains present in the copolymer, the area under the cyclic peaks was compared to the area under the linear peaks. Analysis were performed in both reflector positive mode and linear positive mode.

3.3. RESULTS AND DISCUSSION

3.3.1. Synthesis and composition of PGlCL

The e-ROP reaction of the monomers globalide (Gl) and ε-caprolactone (CL) were carried out in the following systems: with $scCO_2$ in a mass ratio of 1:2 (CO_2:monomers); with $scCO_2$ + DCM in a mass ratio of 1:2 (CO_2:MIX); and with $scCO_2$ + DCM in a mass ratio of 2:1 (CO_2:MIX). As described in section 3.2.2, MIX = DCM+monomers, and the mass ratio of DCM:monomers is fixed in 1:2. The feed mass ratio of Gl/CL was varied (10/90, 25/75, 50/50, 75/25 and 90/10), while other parameters like pressure, temperature and enzyme content were maintained constant (section 3.2.2.). Reaction yield values varied from 45 to 60%.

Table 3.1 shows ^{1}H NMR spectroscopy data for samples obtained with the use of $scCO_2$ and $scCO_2$ + DCM. Differences between Gl/CL feed ratio and Gl/CL copolymer composition were not relevant for both solvents used ($scCO_2$ and $scCO_2$+DCM), which means that none of the monomers reacted preferentially.

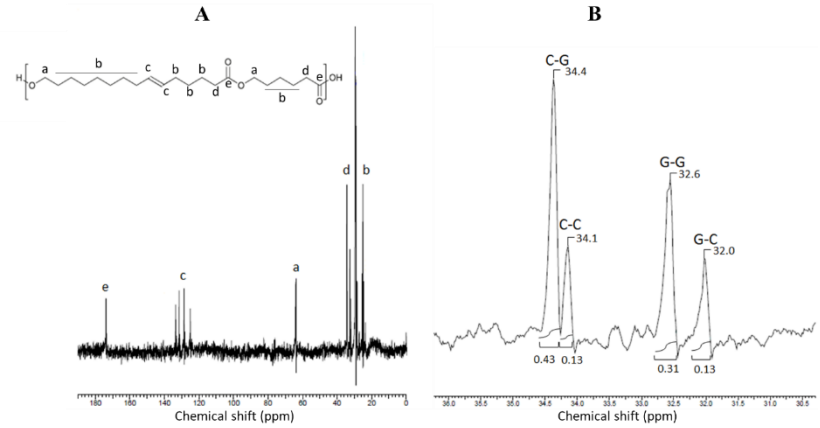
Table 3.1 - ^{1}H NMR data of PGlCL copolymer composition obtained using different solvents ($scCO_2$ and $scCO_2$+DCM) and different globalide/ε-caprolactone feed ratios.

Solvent	CO_2:monomers or CO_2:MIX	Feed Gl/CL (mass ratio)	Feed Gl/CL (mol ratio)	NMR Gl/CL copolymer composition (mol ratio)
$scCO_2$	1:2	10/90	5/95	2/98
		50/50	33/67	27/73
		75/25	59/41	62/38
		90/10	81/19	77/23
$scCO_2$ + DCM	1:2	10/90	5/95	2/98
		50/50	33/67	37/63
		75/25	59/41	63/37
		90/10	81/19	84/16

All spectra obtained are in good agreement to the spectra of polyglobalide and poly(ε-caprolactone) reported in literature (ATES; THORNTON; HEISE, 2011; BRANDOLINI; HILLS, 2000; VAN DER MEULEN et al., 2008).

The degree of randomness (R) was calculated from Equations (3.1) to (3.4) presented on item 3.2.3., in order to know the distribution of the repeating units in the copolymer chains. For this calculation it were used values determined by ^{13}C NMR diads peak integration of the signal from the carbon next to the carbonyl (***C***H_2(C=O)O), between 32 and 35 ppm. PGlCL synthesized using pure $scCO_2$, with 75/25 Gl/CL feed ratio (mass), was selected for the randomness determination. As shown in Figure 3.2, signals from Gl-Gl repeating units (GG, 32.6 ppm) can be found next to the signals from CL-CL repeating units (CC, 34.1 ppm) in the spectrum. Gl-CL (GC, 32.0 ppm) and CL-Gl (CG, 34.4 ppm) diads can be found to be adjacent to the Gl-Gl and CL-CL peaks.

Figure 3.2 - (A) ^{13}C NMR spectrum of PGlCL 75/25 (Gl/CL feed mass ratio). (B) Expanded **C**H_2(C=O)O region of ^{13}C NMR spectra, with the peaks of each respective diad.



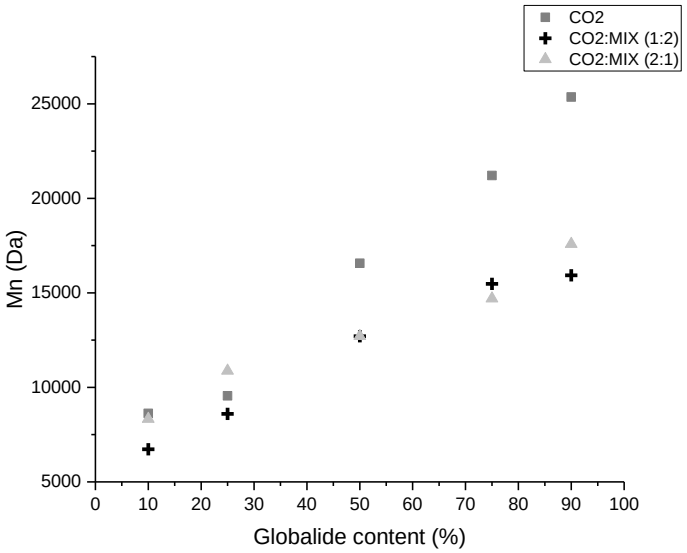
The diads values calculated by integration of the peaks were: GG=0.31, CC=0.13, GC=0.13; CG=0.43, resulting in R=1.1, indicating that PGlCL has a random distribution, which is in accordance to the results obtained in other copolymerization studies between lactones and macrolactones (CECCORULLI et al., 2005; CLAUDINO et al., 2012; KUMAR et al., 2000; VAN DER MEULEN et al., 2011). For R=1, units

take a random distribution, and the probability of finding a copolymer unit belongs to Bernoulli statistics. If R < 1 the units tend to cluster in blocks of each units, R = 0 for homopolymers, whereas for R > 1 the sequence length becomes shorter, and finally, R = 2 for alternating copolymers (VAN DER MEULEN et al., 2011; YAMADERA; MURANO, 1967). The spectra obtained are in good agreement to spectra obtained in literature (JACOBS et al., 1991; KUMAR et al., 2000; VAN DER MEULEN et al., 2011).

3.3.2. Molecular weight and dispersity

Figure 3.3 presents M_n as function of the feed globalide content (relative to the total monomer amount) for reactions carried out in systems with: $scCO_2$ in a mass ratio of 1:2 (CO_2:monomers); $scCO_2$ + DCM in a mass ratio of 1:2 (CO_2:MIX); and $scCO_2$ + DCM in a mass ratio of 2:1 (CO_2:MIX).

Figure 3.3 - Number average molecular weight (Mn) as function of feed globalide content (relative to total monomer amount) using $scCO_2$ and $scCO_2$+DCM as solvent.



As reported in literature (KUNDU et al., 2011; POLLONI et al., 2017; THURECHT et al., 2006; VENERAL, 2014), polyesters

synthesized by e-ROP does not generate symmetrical and monomodal peaks, as typically obtained when chemical catalysts are used. In this work, similarly to the literature, e-ROP synthesized polymers which chromatogram presents a broad polymer peak, followed by a long tail (bimodal distribution). Kundu et al. (KUNDU et al., 2011) also observed this behavior for e-ROP of PCL, and analyzed by mass spectrometry the fraction correspondent to the tail in the chromatograms, founding that they are relative mainly to cyclic oligomers. To obtain self-consistent results for all experimental conditions studied in this work, it was adopted as integration criterion the use of the inflection point of the broad polymer peak as the endpoint for integration procedures. This way, the peaks of higher molecular weights were used for integration, since these peaks are more representative (always above 90 % of the chromatogram area), and the oligomeric tail was not considered. The same integration criterion was used in literature (KUNDU et al., 2011) and enables valid data comparisons, maintaining the general trend in this data consistent with other studies (COMIM ROSSO et al., 2013; VAN DER MEULEN et al., 2008, 2011).

For all reaction systems evaluated, M_n increased with the increase of globalide content. Globalide is a large molecule, of molecular weight twice higher than ε-caprolactone. This way, knowing that the ratio of Gl and CL repeating units in the copolymer is in accordance to the monomer feed ratio (section 3.3.1), it is natural that the higher is the content of repeating units derived from globalide, higher is M_n values.

The systems with $scCO_2$, in comparison to $scCO_2$+DCM systems, provided higher M_n values. Since the experiments were carried on in a reactor with a sapphire window, it was possible to visualize that when e-ROP takes place using $scCO_2$ as solvent, the reaction media stirring was impaired and practically stops during the reaction, due to the formation of large copolymer chains, and consequent increase of the media viscosity. With a poor convection mass transfer, the monomer molecules which were in contact to the enzyme pellets can react subsequently with the active sites of the enzymes, generating polymer chains of high molecular weights. The high concentration of monomers surrounding the enzymes, lead to a greater growth of the copolymer chains. The use of DCM as cosolvent diluted the reaction media and improved convection mass transfer, in comparison to the use of only $scCO_2$. This way, there is a decrease in the monomer concentration in the surrounding of the enzyme pellets, which means that the copolymer chains grew less. Córdova et al. (1998), Thurecht et al. (2006), Comim Rosso et al. (2013) and Loeker et al. (2004a) also observed that the monomer concentration

has a strong influence on the polymer molecular weight. Many low molecular weight oligomeric chains may be formed in diluted systems, due to the greater difficulty of the monomer molecules to find the enzyme active sites. The formation of low molecular weight cyclic oligomers also occurs more frequently, since intermolecular reactions are not favored by the dilution of the reaction medium.

The use of $scCO_2$ + DCM in the two proportions of CO_2 studied (1:2 and 2:1 CO_2:MIX) provided M_n values very similar. In the beginning of the reaction, the system with composition 2:1 (CO_2:MIX) is formed by two phases (liquid and vapor), while the 1:2 (CO_2:MIX) system is fully miscible (one liquid phase). This behavior also could be observed during our assays by visualization through the sapphire window, and also by other authors (COMIM ROSSO et al., 2013; XU; WAGNER; DAHMEN, 2003). It was observed that few minutes after the reaction starts, a vapor phase (basically composed of CO_2 (XU; WAGNER; DAHMEN, 2003)) appears in the system 1:2, due to exceeding the solubility limit, which were also observed by Comim Rosso et al. (2013). It suggests that the amount of CO_2 fed in 1:2 system in the beginning of the reaction was already very close to the solubility limit. After this limit is reached, the concentration of the liquid phase is the same for both 1:2 and 2:1 systems (CO_2 saturation concentration). Since the reaction occurs in the liquid phase (composed by monomers, enzyme pellets, DCM and CO_2), it explains the similarity between molecular weight data of 1:2 and 2:1 studied systems.

Dispersity (Đ) values varied from 1.3 to 1.6 for samples obtained in $scCO_2$, while for samples obtained by the use of $scCO_2$ + DCM (1:2 and 2:1 CO_2:MIX ratio), values varied from 1.3 to 1.7 (oligomeric tail not considered). Đ values are low, and keep practically constant for all systems evaluated, indicating an uniform polymer chain size distribution.

3.3.3. Thermal analysis data

PGlCL samples obtained on different globalide/ε-caprolactone feed ratios, using $scCO_2$ and $scCO_2$ + DCM as solvents, were evaluated by DSC. Results of crystallization temperature (T_c), melting temperature (T_m), melting enthalpy (ΔH_m) and degree of crystallinity are presented in Table 3.2. The degree of crystallinity was calculated through the relation between ΔH_m of each sample, and the theoretical value of a 100% crystalline PCL sample, obtained from literature (CRESCENZI et al., 1972).

For the samples obtained by using only $scCO_2$ as solvent, the relation between the copolymer composition and T_m values presented an eutectic trend. At first, T_m values decrease as the globalide content increase, reaching a minimum value around 50% globalide content. After this point, T_m values begin to increase. For copolymers, this behavior known as "melting point depression", and is characteristic of random semi-crystalline copolymers constituted of very different monomers, in terms of size and structure (non-isomorphic) (CANEVAROLO JR., 2006), which is the case of globalide and ε-caprolactone. Each globalide repeating units is 16-membered an has an unsaturation, while each ε-caprolactone repeating unit is 7-membered and do not present any unsaturation. In this case, monomers do not substitute each other within the unit cell, and it is not possible to follow an additive rule for the determination of the T_m of the copolymers (CANEVAROLO JR., 2006). According to Wendling and Suter (1998), considering a copolymer of two monomers A and B crystallizing in the crystal lattice of A, the monomers B may either be excluded from the crystals or act as defects in the crystal. In both cases the Gibbs energy of the crystal will increase and the melting temperature decrease. Well-known equations (BAUR, 1966; FLORY, 1953; WUNDERLICH, 1980) can be applied to describe the observed melting temperature depression. Claudino et al. (2012) evaluated thermal properties of poly(globalide-co-ε-caprolactone) and observed the same behavior for melting temperature.

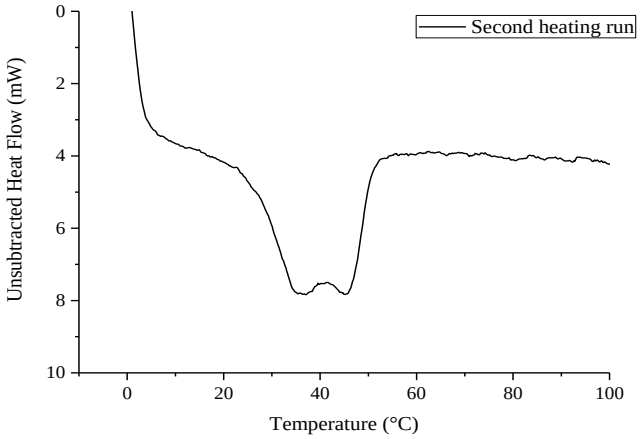
Table 3.2 - Thermal properties determined by DSC for PGlCL obtained using different solvents ($scCO_2$ and $scCO_2$+DCM) for different feed globalide/ε-caprolactone ratios.

Feed Gl/CL (mass ratio)	Solvent	CO_2:monomers or CO_2:MIX	Tc (°C)	T_m 1 (°C)	T_m 2 (°C)	ΔH_m (J/g)	X_c (%)
PCL	$scCO_2$	1:2	38	56	-	97.0	72
10/90			31	51	-	80.9	60
25/75			35	54	-	85.7	63
50/50			20	39	-	92.1	68
75/25			30	45	-	85.8	63
90/10			33	48	-	74.0	55
PGl			36	51	-	95.3	70
50/50	$scCO_2$ + DCM	1:2	22	36	-	96.6	71
75/25			26	-	44	85.7	63
50/50	$scCO_2$ + DCM	2:1	20	34	-	79.0	58
75/25			31	36	45	91.4	68

T_c: Crystallization temperature; T_m 1: First peak melting temperature; T_m 2: Second peak melting temperature; ΔH_m: Heat of fusion; X_c: Degree of crystallinity, calculated from the fusion enthalpy value of a PCL 100% crystalline sample (CRESCENZI et al., 1972).

An interesting point to note is that, for samples obtained with the use of cosolvent, broad melting peaks were obtained, or even double peaks (double melting point) (Figure 3.4), while samples obtained by using only $scCO_2$ as solvent presented a single well-defined melting peak. Multiple melting behavior (which includes double melting behavior) has been observed to semi-crystalline polymers, including biodegradable polymers such as poly(ethylene succinate) (AL-RAHEIL; QUDAH, 1995), poly(butylene succinate) (PBSu) (MIYATA; MASUKO, 1998; YASUNIWA; SATOU, 2002) and poly (L-acid lactic) (YASUNIWA et al., 2004). This double melting behavior has been explained with a melt-recrystallization model (WUNDERLICH, 1980). The model suggests that small and imperfect crystals change successively into more stable crystals through the melt-recrystallization mechanism, generating a competition between melting and recrystallization during the heating run. Since recrystallization is a slow process, and the heat is continuously provided by the system, the recrystallized chains and original chains which remained in the crystal lattice finally melt. Then, the second melting peak appears.

Figure 3.4 - Presence of double melting point behavior: DSC second heating run for PGlCL 25/75 (Gl/CL feed mass ratio) obtained with $scCO_2$+DCM, using CO_2:MIX = 2:1.



According to the results presented by Córdova et al. (1998) and Thurecht et al. (2006), when the reaction system is diluted by a solvent,

the formation of cyclic compounds is favored (in comparison to bulk reactions). The use of DCM as cosolvent in comparison to the use of only $scCO_2$, diluted the reaction media. Besides, the fact that DCM solvates better the monomers/oligomers/polymers molecules in all reaction media tested in this work, makes molecules more flexible and favors intramolecular reactions (cyclic oligomers formation). These oligomeric cycles may be responsible for the formation of less stable regions in the crystalline arrangement, which causes the melting - recrystallization behavior during the second heating run. T_m for samples obtained by using cosolvent were lower than T_m of samples obtained by using only $scCO_2$, probably due to a higher formation of cyclic oligomers, which may decrease the stability of the crystalline arrangement.

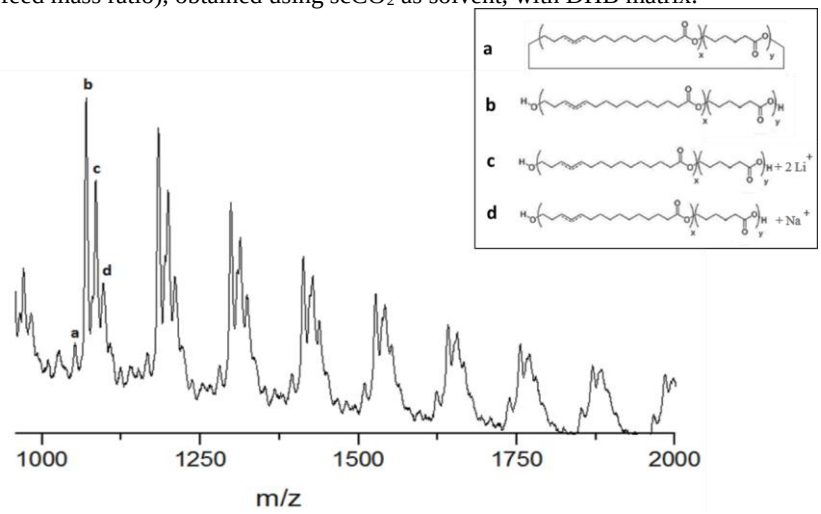
For all conditions evaluated, the melting enthalpy, which is related to the amount of crystalline regions, showed little variation, which reflects on the calculated degree of crystallinity values. All conditions evaluated formed semi-crystalline copolymers, being consistent to literature data for PGlCL, PCL and PGl (CLAUDINO et al., 2012; SINHA et al., 2004; VAN DER MEULEN et al., 2008).

3.3.4. Effect of dichloromethane on cyclic oligomers synthesis

The formation of cyclic oligomeric chains was evaluated through MALDI-TOF mass spectrometry. It were analyzed three samples of copolymer, synthesized in the same conditions, but using different solvents, with the objective of observing the formation of cyclic oligomeric chains, under the use of different solvents/cosolvent.

The ratio between cyclic and linear compounds were obtained by relating the area of the MALDI-TOF spectrum peaks which correspond to these compounds. The calculations were based on cyclic species with molecular weights greater than 1000 Da (background signal difficult the peaks identification bellow 1000 Da). Figure 3.5 presents a MALDI-TOF spectra in the region of 1000 to 2000 Da, were: peak (a) represent cyclic PGlCL chains (PGlCL without end groups); peak (b) represent linear PGlCL chains; and peaks (c) and (d) are probably related to PGlCL linear species (doubly-lithiated species and sodiated species, respectively).

Figure 3.5 - Expanded view of MALDI-TOF spectrum of PGlCL 10/90 (Gl/CL feed mass ratio), obtained using $scCO_2$ as solvent, with DHB matrix.



(a) cyclic PGlCL chains; (b) linear PGlCL chains; (c) and (d) probably related to PGlCL linear species (doubly-lithiated species and sodiated species, respectively).

In MALDI-TOF analysis, cationization can occur through the attachment of a cation to the sample. Due to their natural abundance, Li^+, Na^+ and K^+ ions are frequently attached to the sample molecules (PASCH; WOLFGANG, 2003). These peaks series are present in spectra obtained for all samples and are similar to those obtained by Córdova et al. (1998) and Thurecht et al. (2006) for PCL.

Samples evaluated are listed on Table 3.3, as well as the ratio between cyclic and linear chains, obtained by MALDI-TOF.

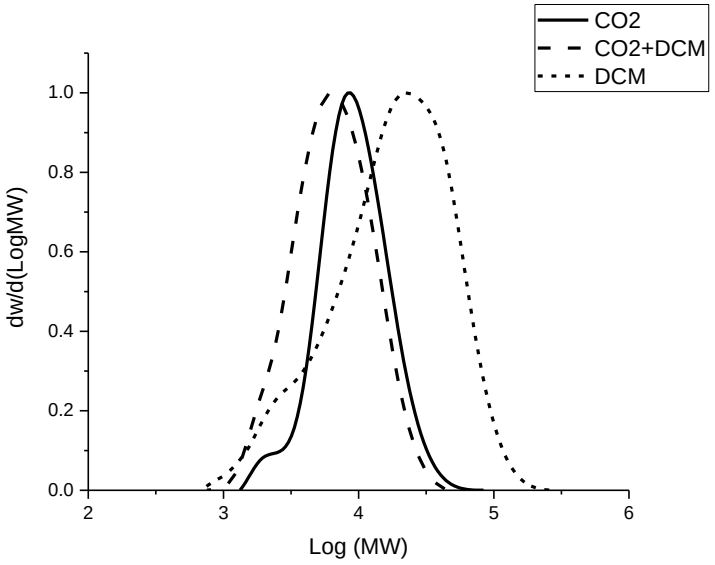
Table 3.3 - Relation between cyclic and linear chains obtained on e-ROP under the use of different solvents.

Solvent	CO_2: monomers	CO_2: MIX	DCM: monomers	Feed Gl/CL (mass ratio)	Cyclic:Linear ratio
$scCO_2$	1:2	-	-	10/90	1:6
$scCO_2$ + DCM	-	1:2	-	10/90	1:4
DCM	-	-	1:2	10/90	1:3

As expected, the sample synthesized using only $scCO_2$ as solvent, showed the lowest content of cyclic compounds, while the sample synthesized using only DCM, presented the highest content (twice higher than $scCO_2$). The use of DCM as cosolvent, caused an increase in the content of cycles, in comparison to the use of only $scCO_2$ as solvent, but it is still lower than the cycles content obtained by pure DCM. These results strongly suggest that the use of DCM favors the formation of cyclic oligomers, helping to explain the double melting point behavior (item 3.3.3), observed in samples synthesized in the presence of DCM as cosolvent. These samples presented higher cyclic oligomers content, in comparison to those obtained in $scCO_2$. The presence of cyclic oligomers among linear chains generate less stable crystalline arrangements, conferring double melting points to the copolymer samples, as observed in the thermal analysis.

Besides, the higher formation of cyclic oligomers also provides a wider molecular weight distribution. The cyclic oligomers detected by MALDI-TOF compose the oligomeric tail detected in the chromatograms of item 3.3.2. Figure 3.6 shows the molecular weight distribution of the same samples analyzed by MALDI-TOF.

Figure 3.6 - PGlCL molecular weight distribution of PGlCL 10/90 (Gl/CL feed mass ratio) by the use of different solvents



The samples synthesized using only $scCO_2$ showed a narrower molecular weight distribution, while the use of DCM generated samples with wider molecular weight distribution. As explained in item 3.3.2, when DCM is added as cosolvent, the concentration of monomers in the media decreases. This way, when only DCM is used, monomer concentration decreases even more, since all solvent and reactants are present in the liquid phase (no vapor phase formation). As explained in literature (CÓRDOVA et al., 1998; THURECHT et al., 2006) a diluted medium does not favor the incidence of intermolecular reactions. Besides, DCM has a greater ability to solubilize the monomers/oligomers/polymers, giving more flexibility to the chains, facilitating intramolecular backbiting.

3.4. CONCLUSIONS

This work brought important results about the enzymatic synthesis of poly(globalide-co-ε-caprolactone) which was until now poorly reported. Reactions were performed in $scCO_2$ and $scCO_2$+DCM, under different globalide/ε-caprolactone feed ratios, consisting of an unpublished study. The synthesis of PGlCL by e-ROP was successful, especially when conducted under the use of only $scCO_2$ as solvent. Copolymers of M_n up to 25,000 Da were obtained, with a well-defined melting point, random repeating units distribution, allied to low formation of oligomeric products. Thermal analysis of PGlCL showed a non-isomorphic behavior, which is typical for random semi-crystalline copolymers, composed by comonomers with very different structures. The use of DCM as cosolvent generated higher formation of cyclic oligomers (in comparison to only $scCO_2$), conferring to PGlCL double melting point and wider molecular weight distribution.

CHAPTER 4

After the study performed in Chapter 3, it was possible to understand the phenomena that occur during poly(globalide-co-ε-caprolactone) enzymatic synthesis in $scCO_2$. Besides, it was also possible to find the process conditions that yield copolymers with the most interesting characteristics in terms of biomedical applications, such as high molecular weight, well-defined melting point and small content of cyclic oligomers. In the present chapter, PGlCL samples synthesized using pure $scCO_2$ in different Gl/CL ratios were selected to be functionalized with N-acetylcysteine by thiol-ene coupling reaction. The functionalization of PGlCL with NAC was performed in order to make the polymer more amorphous and less hydrophobic, aiming to favor future applications where a faster bioresorption is required. The resulting materials were evaluated regarding its final composition, thermal properties (melting temperature, degree of crystallinity and heat of fusion), hydrophobicity and antioxidant potential. By varying the ratio between the copolymer repeating units it was possible to tune the properties of the functionalized polymer, increasing its range of applications. The results presented in this chapter were published in the journal Materials Science and Engineering: C (doi.org/10.1016/j.msec.2018.09.060).

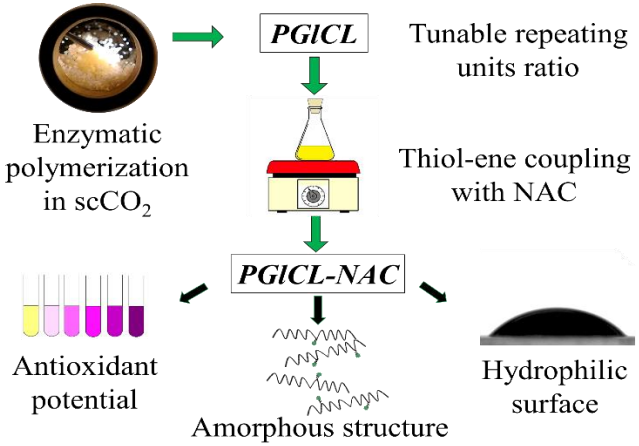
4. N-ACETYLCYSTEINE SIDE-CHAIN FUNCTIONALIZATION OF POLY(GLOBALIDE-CO-E-CAPROLACTONE) THROUGH THIOL-ENE REACTION

Abstract

N-Acetylcysteine (NAC) is a drug well known for its antimucolytic action, antioxidant activity and ability to protect cells from oxidative stress. Conjugation of NAC with double bonds in the main polymer chain of poly(globalide-co-ε-caprolactone) (PGlCL) through thiol-ene reaction is reported. Different globalide (Gl) (an unsaturated macrolactone) to ε-caprolactone (CL) ratios were employed for PGlCL synthesis. The polymeric materials (PGlCL-NAC) were evaluated in terms of the number of functionalized double bonds, thermal properties, affinity for water and antioxidant potential. PGlCL-NAC containing more globalide repeating units presented higher degree of functionalization, due to the higher number of double bonds available to react through thiol-ene coupling. For high globalide contents (Gl/CL ratios above 50/50), NAC coupling in PGlCL chains resulted in completely amorphous copolymers

with a more hydrophilic character, which should enhance bioresorption and cell adhesion characteristics. Functionalization also gave rise to a thioether linkage, conferring to PGlCL-NAC an antioxidant character, important for biomedical applications, where the material could combat cellular oxidative-stress.

Graphical abstract



4.1. INTRODUCTION

Over the last decades, biodegradable/bioresorbable polymeric materials has received significant attention, and its development has advanced considerably, focusing specially in their use as degradable drug delivery vehicles and in tissue engineering (ALBERTSSON; VARMA, 2003). In this context, many efforts have been concentrated in material modification studies, which allows specific interactions within biological systems. These modifications should significantly improve biomaterial performance, making it very dynamic for a series of applications.

Thiol-ene reactions consist of a simple and adaptable methodology to prepare functionalized polymers and polymer networks using combinations of multi-functional alkenes and thiols (HOYLE; LEE; ROPER, 2004; KELEŞ; HAZER; CÖMERT, 2013). Thiol-ene coupling have high yields, fast reaction rates and form harmless byproducts, being considered "*click*" reactions (HOYLE; BOWMAN, 2010; KOLB; FINN; SHARPLESS, 2001). These reactions may be employed as a post-polymerization modification in unsaturated polymers, even as multiple *click* reactions, allowing the side-chain functionalization of simple and

complex structures with different chemical groups, enabling the formation of biofunctional materials (DONDONI, 2008; KELEŞ; HAZER; CÖMERT, 2013; LOWE, 2009; TUNCA, 2014).

Poly(ε-caprolactone) (PCL) is one of the most attractive polymers for medical application, since it is biodegradable and bioresorbable, and presents mechanical properties proper for this application (ALBERTSSON; SRIVASTAVA, 2008). Some drug delivery devices made from PCL have already FDA approval and trademark (WOODRUFF; HUTMACHER, 2010). However, the high crystallinity of PCL reduces water permeability within the polymer matrix and make the bioresorption process very slow (2-4 years) (WOODRUFF; HUTMACHER, 2010). Besides, PCL is very hydrophobic, which in addition to hinder its bioresorption, also difficult the cell adhesion and proliferation limiting its potential applications (JEON; LEE; KIM, 2014).

Polyglobalide (PGl) is a biocompatible and non-toxic polyester with mechanical properties suitable for biomedical applications (CAI et al., 2010; FOCARETE et al., 2001; GEUS et al., 2010) obtained from ring-opening polymerization (ROP) of globalide, an unsaturated 16-membered macrolactone (VAN DER MEULEN et al., 2008). After ROP, the double bonds remain in the main polymer chain of PGl enabling the functionalization of the polymer chain by thiol-ene coupling reaction.

The development of copolymers composed by ε-caprolactone and globalide tends to be a very versatile alternative for biomedical applications, contributing to add new properties to the well-known PCL through the addition of globalide units, which can be further functionalized. The ratio between the monomers and the type of functionalizing group may be tuned depending on the desired application, producing copolymers with different characteristics such as different melting temperatures, degrees of crystallinity, hydrophilicity, affinity for different human tissues, crosslinking and mechanical properties, for example.

N-acetylcysteine (NAC) is a drug well known for its antimucolytic action, besides being used in a wide range of neuropsychiatric disorders (BERK et al., 2013; DEAN; GIORLANDO; BERK, 2011). NAC presents antioxidant activity (ANDRADE; MOURA; MARQUES, 2015; CAZZOLA et al., 2015; DHOUIB et al., 2016) and has the ability to protect cells from oxidative stress. NAC is a hydrophilic molecule containing a thiol group (OSOL, 1970) that enables its conjugation by thiol-ene reaction. The incorporation of NAC molecules covalently bonded to PGlCL copolymer (PGlCL-NAC) by thio-ether linkages could increase the affinity of the polymeric material for water and reduce its

degree of crystallinity. Besides, it is expected that the final material presents an antioxidant potential, that is an important tool in the cellular oxidative stress combat (VAN LITH et al., 2014; WANG et al., 2006).

In this sense, the present study discusses the side-chain functionalization of the copolymer poly(globalide-co-ε-caprolactone) (PGlCL) with N-acetylcysteine, through thiol-ene reactions, aiming to produce modified PGlCL copolymers with enhanced characteristics for future biomedical applications. Here, PGlCL was synthetized by enzymatic ring-opening polymerization (e-ROP) using supercritical carbon dioxide ($scCO_2$) as solvent. After polymerization, PGlCL with different globalide/ε-caprolactone repeating unit ratios were functionalized with NAC, and the materials were evaluated in terms of the number of functionalized double bonds, melting temperature, degree of crystallinity, contact angle with water and antioxidant potential. To our knowledge these are the first studies on poly(globalide-co-ε-caprolactone) (PGlCL) side-chain functionalization with N-acetylcysteine where the functionalized polymer reduced crystallinity, increased hydrophilicity and improved antioxidant activity.

4.2. MATERIAL AND METHODS

4.2.1. Materials

Dichloromethane P.A. 99.8% (DCM), ethanol P.A. 99.8% (EtOH), chloroform P.A. 99.8%, glacial acetic acid P.A. 99.8%, and the free radical initiator azobisisobutyronitrile 98% (AIBN) were purchased from Vetec Química (Brazil). Carbon dioxide (99.9% purity) used as solvent was purchased from White Martins A/S, Brazil. N-acetylcysteine 99.8% (NAC) was purchased from Gemini (Brazil). Novozym 435 was kindly donated by Novozymes, Brazil, A/S (commercial lipase B from *Candida antarctica* immobilized on cross-linked polyacrylate beads, esterification activity 42 U g^{-1}, measured according to a procedure adapted from literature) (OLIVEIRA et al., 2006). Enzymes were dried under vacuum (0.4 bar) and 70 °C, during 16 hours (COMIM ROSSO et al., 2015) and stored in a desiccator over silica and 4 Å molecular sieves. Globalide (Gl) was a kind gift of Symrise. ε-caprolactone were purchased from Sigma-Aldrich. Both globalide and ε-caprolactone (CL) were dried under vacuum (0.1 bar) and 100 °C, during 24 hours (COMIM ROSSO et al., 2015) and also stored in a desiccator over silica and 4 Å molecular sieves.

4.2.2. Poly(globalide-co-ε-caprolactone) synthesis using supercritical carbon dioxide

Polymerization experiments were carried out as previously described by Guindani et al. (2017) using supercritical carbon dioxide ($scCO_2$) as solvent. The pressure and temperature of the system were maintained constant at 120 bar and 65 °C, and the monomers were allowed to react for 2 hours. Enzyme content was fixed at 5 wt% (relative to the total monomer amount), and the CO_2 to monomers mass ratio was fixed at 1:2. Experiments were performed varying the globalide/ε-caprolactone (Gl/CL) mass ratio (10/90, 25/75, 50/50, 75/25 and 90/10). After polymerization, the material was purified through solubilization in DCM, followed by the separation of the enzymes and precipitation of the polymer in cold EtOH. The polymeric suspension was filtered and dried at room temperature in vacuum, up to constant weight.

4.2.3. Thiol-ene functionalization of poly(globalide-co-ε-caprolactone) with N-acetylcysteine

PGlCL copolymers produced in $scCO_2$, with different Gl/CL ratios (10/90, 25/75, 50/50, 75/25 and 90/10) were functionalized through thiol-ene reactions, aiming to understand the influence of the different globalide/ε-caprolactone repeating unit ratio in the final properties of the functionalized copolymer. NAC was chosen as a thiol compound suitable for PGlCL functionalization. PGlCL and NAC were placed in a flask together with the free radical initiator AIBN and a mixture of solvents, under nitrogen atmosphere. During the reactions, the flask was kept immersed in an oil bath at 80 °C, for 24 hours, under continuous magnetic stirring. The amount of NAC used was established as being twice the minimum amount of NAC required to functionalize all double bonds. AIBN content was fixed in 5% (mol), relative to NAC amount. A mixture of acetic acid and chloroform in a volumetric proportion of 3:1 (acetic acid:chloroform) was used as solvent. In a typical reaction, 300 mg of PGlCL is dissolved in 4 mL of solvent.

After polymerization, the solvents were removed in air circulation oven. The dried material was washed in cold water, for the free NAC removal, followed by a second wash in methanol for AIBN removal. PGlCL functionalized with NAC (PGlCL-NAC) was then dried under vacuum at room temperature, up to constant weight. Thiol-ene conversions were determined through proton nuclear magnetic resonance

(^{1}H NMR), by comparing the integral values of the peaks related to the double bonds before and after thiol-ene reaction.

4.2.4. Characterization of the copolymer

Differential Scanning Calorimetry - DSC: Samples of approximately 5 mg of dried copolymer were analyzed using a Perkin-Elmer Jade DSC, under inert atmosphere (20 mL min^{-1}), from 0 to 150 °C at a heating rate of 10 °C min^{-1}. The thermal history was removed prior to the analyses at a heating rate of 20 °C min^{-1} and cooling rate of 10 °C min^{-1}. Melting temperatures were determined from the second heating run. The material crystallinity was determined using the fusion enthalpy (ΔH_m) from the second heating run.

Nuclear Magnetic Resonance - NMR: ^{1}H NMR spectroscopy were performed on a Bruker AC-200F NMR, operating at 300 MHz. Chemical shifts are reported in ppm relative to tetramethylsilane (TMS) 0.01% (vol%) (δ=0.00). All samples were solubilized in $CDCl_3$ (δ = 7.27 for ^{1}H NMR).

Poly(globalide-co-ε-caprolactone) ^{1}H NMR ($CDCl_3$ 300 MHz): δ (ppm) 5.49-5.32 (m, C***H***=C***H***); 4.10-4.04 (m, C***H₂***O(C=O)); 2.35-2.26 (m, C***H₂***(C=O)O); 2.07-1.97 (m, C***H₂***(CH=CH)); 1.71-1.62, 1.29 (m, C***H₂***).

Poly(globalide-co-ε-caprolactone)- NAC ^{1}H NMR ($CDCl_3$ 300 MHz): δ (ppm) 6.70-6.40 (d, N***H***); 5.49-5.32 (m, C***H***=C***H***); 4.91-4.79 (m, C***H***-N); 4.12-4.05 (m, C***H₂***O(C=O)); 3.81-3.70 (t, C***H***-S); 3.20-2.99 (m, S-C***H₂***); 2.41-2.28 (m, C***H₂***(C=O)O); 2.11-1.94 (m, C***H₂***(CH=CH); 1.78-1.88 (t, C***H₃***(C=O)); 1.73-1.56, 1.27 (m, C***H₂***).

Free radical scavenging activity - DPPH: NAC, PGlCL and PGlCL-NAC free radical scavenging were evaluated using 1,1-diphenyl-2-picrylhydrazil (DPPH) as described by Mensor et al. (MENSOR et al., 2001) Different sample concentrations were evaluated by mixing in Eppendorf 710 µL of DPPH diluted solution and 290 µL of sample solution, and the reaction was kept away from the light, at room temperature. After 30 min, the absorbance values were measured at 517 nm in spectrophotometer and converted into percentage of antioxidant activity (AA%), and in EC_{50}, i.e., concentration of test solution required to decrease 50% absorbance compared to a blank solution and expressed in µg of sample mL^{-1}. EC_{50} values were calculated from the linear

regression of the AA% curves obtained for all sample concentrations and considering as the mean value of triplicate assays.

Free radical scavenging activity - ABTS: NAC, PGlCL and PGlCL-NAC free radical scavenging were evaluated using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), based on the procedure described by Re et al. (RE et al., 1999) $ABTS^{\bullet+}$ radical was produced by reacting 7 mM ABTS and 2.45 mM potassium persulfate in the dark at room temperature during 16 h before use. The aqueous $ABTS^{\bullet+}$ solution was diluted with 5 mM phosphate buffer (pH 7.4) to an absorbance of 0.7 (±0.02) at 734 nm. Later on, 20 µL of sample solution (5 different concentrations) and 980 µL of $ABTS^{\bullet+}$ solution was mixed in an Eppendorf vial and the reaction was kept away from the light, at room temperature. After 45 min of reaction, the absorbance was measured at 734 nm in spectrophotometer. These values were obtained from the different concentrations of each sample tested in the assay and converted into percentage of antioxidant activity (AA%), and in EC_{50}, i.e., concentration of test solution required to decrease 50% absorbance compared to a blank solution, and expressed in µg of sample mL^{-1}. EC_{50} values were calculated from the linear regression of the AA% curves obtained for all sample concentrations and considering as the mean value of triplicate assays.

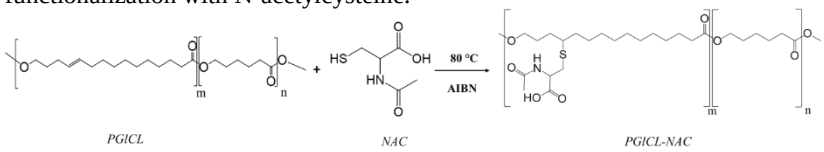
Contact angle assay: PGlCL and PGlCL-NAC films were obtained by solvent casting in microscope slides, and the contact angle between the polymeric films and water droplets were measured in a goniometer (Ramé-Hart Instrument Co. - Ramé-Hart 250). All measurements were performed in triplicate at room temperature with a drop volume of 10 µL.

4.3. RESULTS AND DISCUSSION

4.3.1. Synthesis of PGlCL-NAC by thiol-ene reaction

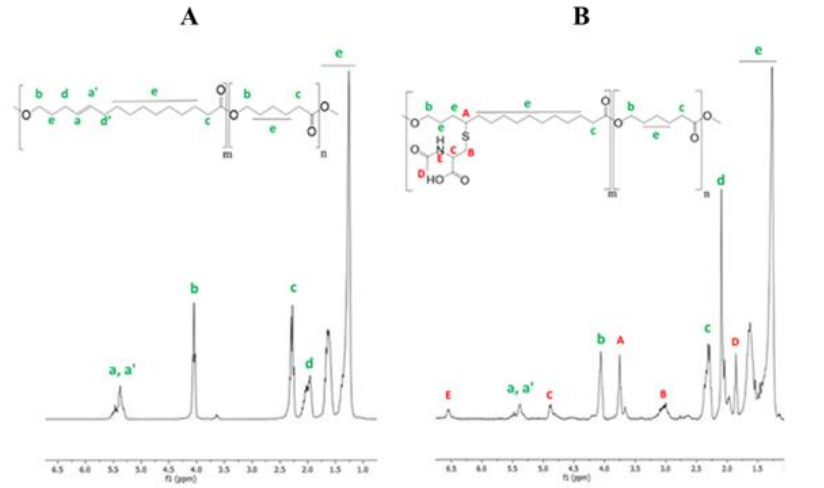
The synthesis of PGlCL-NAC was carried out through post-polymerization thiol-ene reaction of PGlCL with NAC. PGlCl on the other hand, was synthesized by e-ROP using $scCO_2$ as solvent in a fixed mass ratio of 1:2 (CO_2:monomers) as described previously. After its synthesis, PGlCL with different Gl/CL ratios were submitted to thiol-ene reaction with NAC, generating the functionalized copolymer PGlCL-NAC, as can be seen in Figure 4.1.

Figure 4.1- Poly(globalide-co-ε-caprolactone) side-chain thiol-ene functionalization with N-acetylcysteine.



^{1}H NMR analysis was performed to quantify the degree of NAC coupling to PGlCL, related to the number of unsaturations at the polymer chain. All spectra obtained for PGlCL and PGlCL-NAC are in good agreement to the spectra obtained in the literature for polyglobalide, poly(ε-caprolactone), N-acetylcysteine and other thiol-ene functionalized polymers (ATES; THORNTON; HEISE, 2011; BRANDOLINI; HILLS, 2000; HMDB, 2017; VAN DER MEULEN et al., 2008). Figure 4.2 presents PGlCL and PGlCL-NAC ^{1}H NMR spectra for Gl/CL 75/25.

Figure 4.2 - ^{1}H NMR spectra of (A) PGlCL and (B) PGlCL-NAC (75/25 Gl/CL ratio) and its respective peak assignments to the chemical structure of the polymers.



Conversion values of the functionalization reactions were determined through ^{1}H NMR spectroscopy, by comparing the integral values of the double bond peaks of PGlCL and PGlCL-NAC. For all Gl/CL ratios evaluated, it was possible to observe a reduction in the area of double bond peaks, besides the appearance of other new peaks related

to NAC, indicating the occurrence of thiol-ene coupling. Comparing some peaks that appear after functionalization with NAC and peaks that remain from the original PGlCL chain, it is possible to notice the areas are not proportional to the occurrence to the protons relative to each group**.** Usually, NMR spectrum of polymers present broader peaks. In such cases, the differences in chemical shifts of various ^{1}H nuclei, which are in different stereochemical and compositional environment, are usually small, causing broad and overlapping signals (BRAR; GOYAL; HOODA, 2009).

Also, in spite the processes employed to remove the solvents used during functionalization of purification, it is possible that some residues still remain in the material, causing peaks overlap. This way, more than one peak may be appearing together as single peak in the spectra, presenting then an area larger than the expected.

The number of functionalized globalide units are presented in Table 4.1. Analyzing these data, it is possible to see that the increase in the amount of globalide units in PGlCL causes an increase in the number of functionalized globalide units per polymer chain. When a PGlCL sample containing a smaller amount of globalide units undergo thiol-ene reaction with NAC, there are less double bonds available for functionalization, in comparison to samples containing higher amounts of globalide units.

Table 4.1 - Thiol-ene reaction conversion and number of functionalized Gl units per PGlCL-NAC molecule, calculated through ^{1}H NMR spectroscopy data.

Gl/CL (mass) [a]	Number of Gl units per PGlCL chain	Conversion (%) [b]	Number of functionalized Gl units per PGlCL-NAC chain
10/90	4	80	3
25/75	17	71	12
50/50	35	53	19
75/25	67	51	34
90/10	96	42	40

[a] Gl/CL: feed mass ratio of the monomers globalide (Gl) and ε-caprolactone (CL).
[b] Conversion values calculated based on the consumption of the double bonds present in PGlCL chains.

As a result, the PGlCL-NAC containing higher amounts of globalide are much more prone to functionalization implying in more intense changes in the material characteristic.

4.3.2. Effect of functionalization on crystallinity and melting temperature

Samples of PGlCL with different Gl/CL repeating unit ratios functionalized with NAC were evaluated by DSC. Results of melting temperature (T_m), melting enthalpy (ΔH_m) and degree of crystallinity (X_c) are presented in Table 4.2.

The degree of crystallinity was calculated through the relation between ΔH_m of each sample, and the theoretical value of a 100% crystalline PCL sample, obtained from literature (CRESCENZI et al., 1972). It was not possible to obtain molecular weight data for PGlCL-NAC, due to the low solubility of the functionalized polymer in solvents traditionally used in GPC. After functionalization, PGlCL is soluble in mixtures of solvents with different polarities. The data obtained for PGlCL-NAC were compared to the data obtained by Guindani et al. (2017) for non-functionalized PGlCL samples.

Table 4.2 - Properties of synthesized PGlCL functionalized with NAC for different globalide/ε-caprolactone repeating units ratio.

	PGlCL[a]				PGlCL-NAC			
Gl/CL (mass)	T_m (°C)	ΔH_m (J g^{-1})	X_c (%)	M_n (Da)	T_m (°C)	ΔH_m (J g^{-1})	X_c (%)	Mn (Da)
10/90	51	80.9	60	8,623	43	84.5	62	ND
25/75	54	85.7	63	9,552	39	63.1	47	ND
50/50	39	92.1	68	16,568	-	-	-	ND
75/25	45	85.8	63	21,206	-	-	-	ND
90/10	48	74.0	55	25,360	-	-	-	ND

[a] PGlCL properties previously reported by Guindani et al. (2017). T_m, ΔH_m and X_c determined by DSC, M_n determined by gel permeation chromatography (GPC). T_c: Crystallization temperature; T_m: Melting temperature; ΔH_m: Heat of fusion, X_c: Degree of crystallinity, calculated from the fusion enthalpy value of a PCL 100% crystalline sample (CRESCENZI et al., 1972), M_n: Number average molecular weight, ND: not determined.

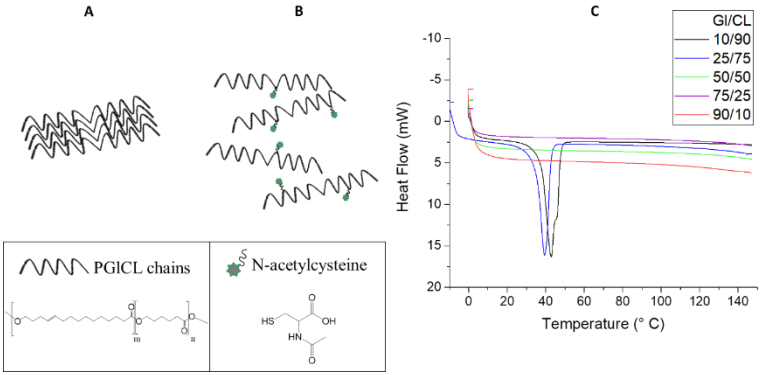
Typically, for polylactones, when the molecular weight increases, the degree of crystallinity presents a tendency of decreasing (TUBA; OLÁH; NAGY, 2014). On the other hand, T_m is reported to not suffer

significant changes with molecular weight increase (TIPTIPAKORN et al., 2015; TUBA; OLÁH; NAGY, 2014). However, for copolymers, the molecular weight is a direct consequence of the composition of repeating units in the copolymer and the level of energy required for the copolymer chains to acquire some mobility will be the result of the contribution of each constituent (CANEVAROLO JR., 2006). This way, the molecular weight itself do not exert a strong influence in T_m and ΔH_m, but this values are influenced by the composition of the copolymer.

The DSC second heating run of each sample is presented in Figure 4.3C. Guindani et al. (2017) obtained semi-crystalline polymers, with a degree of crystallinity ranging between 55-68%. Generally, semi-crystalline polymers exhibit a lower degradation rate than amorphous polymers (CASALINI, 2017), limiting applications where a faster degradation is necessary.

The functionalization of PGlCL containing a 10/90 Gl/CL ratio resulted in PGlCL-NAC samples with lower T_m, in comparison to non-functionalized PGlCL, while the degree of crystallinity (X_c) remained practically the same after functionalization with NAC. The decrease of the melting temperature after functionalization is an indicative that the intermolecular forces in the crystalline arrangement of the material became weaker, and its structure became easier to be undone. The presence of branches implies in a free volume increase, which leads to an easier chain movement of the chains, reducing the energetic level necessary to overcome the secondary intermolecular forces between the chains of the crystalline phase, destroying the regular structure of packaging (CANEVAROLO JR., 2006).

Figure 4.3 - Schematic representation of the (A) PGlCL chains packaged arrangement, forming crystalline regions, and (B) PGlCL-NAC chains disordered arrangement, forming amorphous regions due to the presence of NAC side-chains. (C) DSC second heating run curves



This way, the addition of NAC side-chains in the copolymer structure in a small amount (10/90 Gl/CL, Table 4.1), despite not changing the amount of crystalline arrangements (practically unchanged ΔH_m and X_c), was enough to reduce the amount of energy necessary to undo these arrangements (T_m decreases).

PGlCL-NAC samples containing 25/75 Gl/CL presented a decrease in X_c and T_m values, in comparison to non-functionalized PGlCL (25/75 Gl/CL) and to PGlCL-NAC (10/90 Gl/CL).

Finally, for PGlCL-NAC containing 50/50, 75/25, and 90/10 (Gl/CL), the higher density of NAC side-chains prevented the formation of crystalline arrangements, resulting in completely amorphous polymers. As shown in ^{1}H NMR results (Table 4.1), the higher is the globalide content in the copolymers (and consequently the number of double bonds available to be functionalized), the higher is the presence of NAC side-chains, which leads to materials with more amorphous characteristics.

4.3.3. Effect of functionalization on the surface characteristics of the polymer

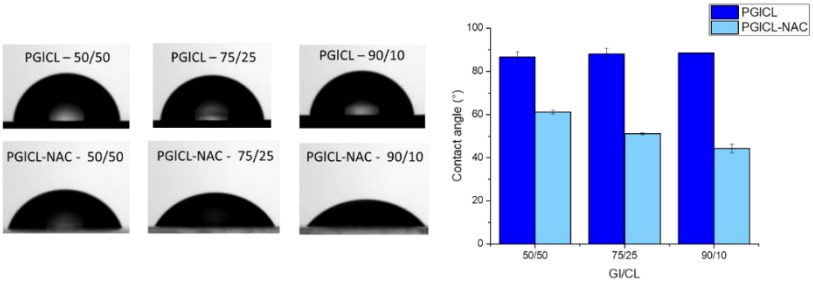
Samples that presented a completely amorphous behavior (DSC assay), and are consequently interesting candidates for biomedical applications (CASALINI, 2017; FERRARI et al., 2013), were evaluated in terms of its affinity for water, through contact angle assay. Figure 4.4

compares the contact angle of PGlCL and PGlCL-NAC films with 50/50, 75/25 and 90/10 (Gl/CL) ratios.

PGlCL with different Gl/CL ratios presented contact angle values around 88°, and may be considered hydrophobic materials (ZHENG et al., 2005). The surface hydrophilicity/hydrophobicity influences the adsorption of protein onto the polymer surface. Hydrophobic polymers are usually not desirable for biomedical applications, since they are unsuitable for cell attachment (DOWLING et al., 2011; SYROMOTINA et al., 2016).The surface property most frequently correlated with adhesion is surface-free energy, a measure of the capacity of a surface to interact spontaneously with other materials by forming new bonds (BECKA; LOEB, 1984; CALLOW et al., 2005). At lower surface energy surfaces (hydrophobic, high contact angle) on rigid surfaces, cell attachment is generally poor, in comparison to high surface energy (more hydrophilic, low contact angle) surfaces (GUELCHER; HOLLINGER, 2006; SYROMOTINA et al., 2016).

Bioresorption is another property affected by polymer hydrophobicity/hydrophilicity, since it occurs initially through the hydrolysis of the ester bonds. The high hydrophobicity of the polymer limits water uptake, which decrease the polymer degradation rate (CASALINI, 2017; GUALANDI et al., 2010; SAHA; TSUJI, 2006).

Figure 4.4 - Contact angle values of PGlCL and PGlCL-NAC samples as function of Gl/CL ratio.



The results obtained for PGlCL-NAC, on the other hand, presented lower contact angle values in comparison to PGlCL, for each respective Gl/CL ratio evaluated. This result, together with ^{1}H NMR and DSC results, strengthen the idea that the NAC thiol-ene functionalization was successful. As expected, the higher is the globalide content of repeating units (higher number of NAC functionalization - Table 4.1), the lower is

contact angle values. NAC molecules were capable of giving a hydrophilic character to the material, yielding PGlCL-NAC with contact angle values varying from ~60° to ~47°. These values are within the range of optimal contact angle for good cell attachment, reported in previous studies as being between 50° and 70° for various polymers and cells (BERNHARDT et al., 2009; KIM; PARK; PH, 2006; SELVAKUMAR et al., 2015; TORRES et al., 2017; XU; SIEDLECKI, 2007). Increasing the surface hydrophilicity induces the attachment of proteins like fibronectin and cells like osteoblast and fibroblasts (SELVAKUMAR et al., 2015; TORRES et al., 2017; YOSHINARI et al., 2010). Besides, a more hydrophilic character is also interesting for applications in drug delivery systems, were nanocarriers coated with a hydrophilic surface (stealth nanocarriers) has a prolonged permanence in the bloodstream and allows specific targeting (GUNASEELAN et al., 2010; MOGHIMI et al., 1993; SALMASO; CALICETI, 2013; SCHÖTTLER et al., 2016).

4.3.4. Antioxidant potential of the material

N-acetylcysteine is known for its antioxidant activity. After functionalization, NAC is covalently bonded to the polymer through a thioether linkage, which also presents antioxidant activity, and is able to induce the decomposition of hydroperoxides in inert products, being considered a secondary antioxidant (AGNELLI; CHINELATTO, 1992; CHANDA; ROY, 2010). PGlCL-NAC with a Gl/CL ratio of 50/50 was evaluated in relation to its antioxidant potential through DPPH and ABTS assays. The results are presented in terms of EC_{50} and are shown in Table 4.3.

The antioxidant activity of non-functionalized PGlCL was evaluated and did not present antioxidant activity against DPPH• nor $ABTS^{\bullet+}$ radicals.

Table 4.3 - EC_{50} values of DPPH and ABTS assays for NAC, PGlCL, and PGlCL-NAC in a 50/50 Gl/CL ratio.

	DPPH assay	ABTS assay
Sample	EC_{50} ($\mu g\ mL^{-1}$)	EC_{50} ($\mu g\ mL^{-1}$)
PGlCL	ND	ND
NAC	4.31 ± 0.03	137 ± 3
PGlCL-NAC	4065 ± 157	1553 ± 22

NAC in its free form, as expected, presented a strong antioxidant activity in both DPPH (EC_{50} = 4.31 ± 0.03 μg mL^{-1}) and ABTS (EC_{50} = 137 ± 3 μg mL^{-1}) assays, being comparable to other for traditional antioxidant compounds such as BHT, eugenol and ascorbic acid, for example (BADANAI et al., 2015; PÉREZ-ROSÉS et al., 2016; TAYLOR et al., 2014; ZHANG et al., 2012b). The antioxidant potential of the functionalized polymer PGlCL-NAC, conferred by the thioether linkage, was evaluated through DPPH and ABTS assays. For DPPH assay, PGlCL-NAC presented EC_{50} = 4065 ± 157 μg mL^{-1}.

This value is similar to the values obtained by Chen et al. (2009) for eugenol-grafted chitosan nanoparticles (EC_{50} = 2600 μg mL^{-1}) and carvacrol-grafted chitosan nanoparticles (EC_{50} > 4000 μg mL^{-1}), with application focus in wound healing. Also, similar EC_{50} values were obtained by Salarbashi et al. (2013) for soybean polysaccharide incorporated with *Mentha pulegium* (EC_{50} = 5225.24 μg mL^{-1}) and *Zataria multiflora Boiss* (EC_{50} = 4188.60 μg mL^{-1}) essential oils, for food packaging applications.

In other work, Bishai et al. (2014) successfully performed a modification of polylactic acid with humic acid (a natural antioxidant), in order to obtain a material with enhanced functional properties ideal for tissue engineering applications. Bishai et al. (2014) evaluated polylactic acid modified with humic acid (PLA-HA) antioxidant activity through DPPH (EC_{50} = 22450 μg mL^{-1}) and ABTS assay (EC_{50} = 57500 μg mL^{-1}). For ABTS assay, PGlCL-NAC EC_{50} = 1553 ± 22 μg mL^{-1}. PGlCL-NAC presented lower EC_{50} values than PLA-HA against both DPPH• nor $ABTS^{\bullet+}$, indicating that PGlCL-NAC has a stronger antioxidant potential.

The importance of developing a functionalized copolymer for biomedical applications able to scavenge free radicals is something that should be highlighted. This kind of material that can locally and continuously (while the polymer is present) inhibit excessive reactive oxygen species generation may be a useful tool for treatments in many biomedical areas, allowing a normal function of the cells (VAN LITH et al., 2014).

4.4. CONCLUSIONS

Poly(globalide-co-ε-caprolactone) (PGlCL) was successfully functionalized with N-acetylcysteine (NAC) through thiol-ene reaction. Higher globalide contents in PGlCL led to higher incorporation of NAC to the polymer chain due to the higher number of double bonds available

to react through thiol-ene coupling. Increasing the amount of NAC to PGlCL reduced the melting temperature and amount of crystalline domains. For copolymers with high contents of globalide (Gl/CL ratios above 50/50), functionalization resulted in completely amorphous copolymers with a more hydrophilic character, which should favor biomedical applications where bioresorption and cell adhesion are required. Besides, the pendant thioether linkage formed through thiol-ene coupling conferred to the polymer an antioxidant character, very important for tissue engineering applications, where the material could locally combat oxidative-stress related to inflammatory responses.

CHAPTER 5

The study presented in this chapter was developed at the Max-Planck-Institut für Polymerforschung, in Mainz, Germany, under the supervision of Professor Katharina Landfester. This period abroad was part of a "sandwich-PhD" program, funded by CAPES/DAAD/CNPq. As mentioned before, through the study performed on chapter 3 it was possible to understand the mechanisms involved in the e-ROP of poly(globalide-co-ε-caprolactone) in $scCO_2$, and select conditions that yield PGlCL samples with the most interesting characteristics in terms of biomedical applications. On chapter 4, it was possible to functionalize PGlCL with NAC and confer it completely new properties after this chemical modification. In the present chapter, the work was expanded to the nanoparticles field, which have a huge range of applications in the biomedicine, such as in drug delivery, diagnostic imaging and hyperthermia treatments for cancer. PGlCL nanoparticles (NPs) were covalently conjugated with bovine serum albumin (BSA) by thiol-ene coupling reaction, aiming to obtain engineered nanoparticles that present enhanced internalization by cells. Uncoated particles and BSA conjugated NPs were characterized regarding size, stability and morphology. The success of the covalent conjugation is evaluated by fluorescence-activated cell sorting and fluorescence correlation spectroscopy. Transmission electron microscopy imaging was applied to the visualization of the protein layer surrounding the nanoparticles. Cell uptake assays with macrophage cells were also performed, comparing BSA-NPs conjugates and uncoated NP.

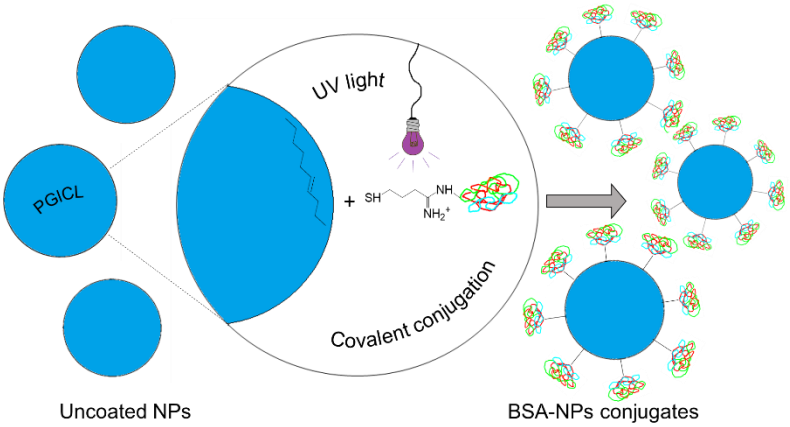
5. COVALENTLY BINDING OF PROTEINS TO POLYMERIC NANOPARTICLES

Abstract

When applied in biological fluids, proteins immediately adsorb onto nanoparticles surface, forming a protein corona, which gives the nanoparticle a new "identity" and determine its biological fate. Living systems usually interact better with protein-nanoparticle conjugates rather than with uncoated nanoparticles. Non-covalent conjugates are less stable and more susceptible to desorption in biological media, which makes the development of engineered nanoparticle surfaces by covalent attachment an interesting topic. In this work, the surface of poly(globalide-co-ε-caprolactone) (PGlCL) nanoparticles containing clickable groups is

covalently functionalized with bovine serum albumin (BSA) by thiol-ene chemistry, producing conjugates which are irreversibly attached. The successful formation of the covalent conjugates is confirmed by fluorescence-activated cell sorting (FACS) and fluorescence correlation spectroscopy (FCS). By help of transmission electron microscopy (TEM) it is possible to visualize the formation of the conjugates and observe the presence of a protein layer surrounding the NPs. After conjugation with BSA, nanoparticles present reduced cell uptake by immune cells, in comparison to uncoated nanoparticles. These are original results that demonstrate that it is possible to produce stable and irreversible conjugates by covalently binding BSA to PGlCL nanoparticles through thiol-ene reaction.

Graphical abstract



5.1. INTRODUCTION

The application of nanomaterials has attracted the attention of many scientific fields in the last decades. In the biomedical field, there is a special interest regarding the use of nanomaterials due to their capability to interact with cells and reach difficult access targets (MAHMOUDI et al., 2011). The scientific and medical community has now recognized that when nanoparticles (NPs) are exposed to a biological environment, its surface suffers modifications by the adsorption of proteins, forming the so-called protein corona. Nowadays, it is well-accepted that the presence

of the protein corona affects cellular responses to the NPs, determining its biological fate (LYNCH; DAWSON, 2008; TREUEL et al., 2014). In this context, engineering the surface of the NPs is an excellent way to tune its interfacial properties and create a wide material platform for specific biological and biomedical applications (DE et al., 2007; RANA; YEH; ROTELLO, 2010).

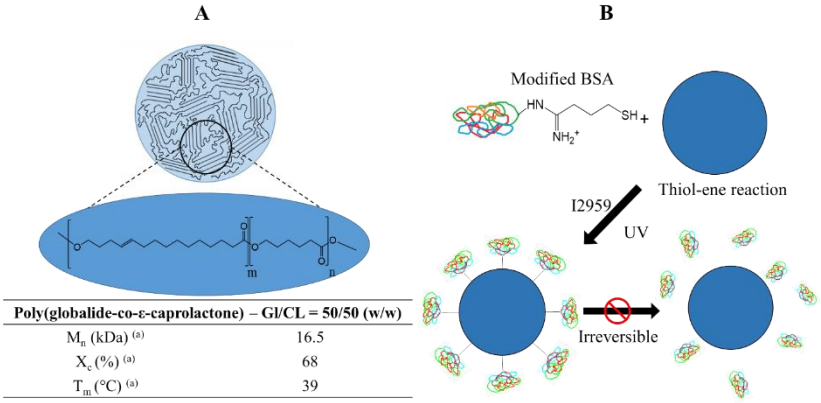
Nanoparticles with tailored surface properties are potentially useful in a broad range of applications. The non-covalent adsorption method is one of the most frequently employed procedures for the attachment of proteins onto particles (ARVIZO; DE; ROTELLO, 2007). This is the simplest way to produce NP-protein conjugates, and the attachment occurs by hydrophobic or electrostatic interactions between the NP and the protein (ARVIZO; DE; ROTELLO, 2007; CARUSO, 2001). However, conjugates produced by non-covalent methods are reversible, which means that proteins can adsorb and desorb to the surface of NPs, making it difficult to maintain its stability, uniformity and reproducibility. Under physiological conditions, proteins adsorbed to the NPs can desorb and be replaced by other proteins present in the local environment, and a long-term behavior of these conjugates may be difficult to predict (BOYER et al., 2011; CHEN; LIU LORETO MEGIDO; DÍEZ, 2018; PINO et al., 2014). In order to avoid these problems, covalent attachment of proteins to NPs becomes an alternative to produce conjugates that are stable toward dissociation. The stability and irreversibility of covalent protein-NP conjugates can be decisive factors governing the biological response of cells and organisms, making this strategy useful for applications in complex biological media with other interfering species (LESZCZYNSKI, 2010; RANA; YEH; ROTELLO, 2010).

Click reaction methods are an excellent approach to the preparation of biologically active protein–polymer NPs. Thiol-ene reactions are an example of a type of *click* reaction, consisting of a simple and adaptable methodology to prepare functionalized polymers using combinations of multi-functional alkenes and thiols (HOYLE; LEE; ROPER, 2004; KELEŞ; HAZER; CÖMERT, 2013). Thiol-ene coupling present high yields, fast reaction rates and form harmless byproducts (HOYLE; BOWMAN, 2010; KOLB; FINN; SHARPLESS, 2001). These reactions are frequently employed as a post-polymerization modification in unsaturated polymers, enabling the formation of biofunctional materials (DONDONI, 2008; KELEŞ; HAZER; CÖMERT, 2013; LOWE, 2009; TUNCA, 2014). Thiol-ene reactions are especially interesting in the presence of free thiol groups in cysteine-containing proteins, since this

allows a *click* reaction with accessible groups on NPs surface, leading to site-selective and irreversible conjugations (BOYER et al., 2011).

In the present study, we aimed to produce poly(globalide-co-ε-caprolactone) (PGlCL) nanoparticles and covalently conjugate them with bovine serum albumin (BSA) protein, in order to obtain irreversible and stable conjugates, suitable for biomedical applications. The unsaturated polyester PGlCL was synthetized by enzymatic ring-opening polymerization (e-ROP) using supercritical carbon dioxide ($scCO_2$) as solvent, and then PGlCL NPs were produced by the solvent evaporation method. The double bonds present on the surface of PGlCL NPs were then directly functionalized by thiol-ene reactions. At first, the nanoparticles were functionalized with a small molecule, N-acetylcysteine (NAC), in order to give us information about the feasibility of the surface functionalization. Then, BSA was modified to increase the amount of thiol units in its structure, and it was conjugated to PGlCL NPs (Figure 5.1).

Figure 5.1 - (A) Structure and properties of the copolymer PGlCL in a mass ratio Gl/CL = 50/50 (B) Scheme representing the conjugation of PGlCL NPs to modified BSA through thiol-ene reaction.



Poly(globalide-co-ε-caprolactone) – Gl/CL = 50/50 (w/w)	
M_n (kDa) (a)	16.5
X_c (%) (a)	68
T_m (°C) (a)	39

(a) M_n, X_c and T_m reported previously by Guindani et al., (2017) M_n: Number average molecular weight; X_c: Degree of crystallinity; T_m: Melting temperature; I2959: photoinitiator Irgacure 2959®

The success of the covalent conjugation of BSA to PGlCL NPs was evaluated through fluorescence-activated cell sorting (FACS) and fluorescence correlation spectroscopy (FCS) measurements. The conjugate visualization was possible through transmission electron

microscopy (TEM). Finally, cell uptake assays with macrophage cells were performed, comparing BSA-NPs conjugates and uncoated NPs. To the best of our knowledge these are the first studies on covalent conjugation of PGlCL NPs with BSA and we see our experiments as an important contribution for the development of engineered nanomaterial surfaces and for safer and more effective medical treatments.

5.2. MATERIAL AND METHODS

5.2.1. Materials

Solvents were purchased from Merck and Sigma Aldrich and used as received, unless otherwise stated. The thermal initiator potassium persulfate (KPS) was purchased from Thermo Fisher Scientific and the surfactant sodium dodecyl sulfate (SDS) was from Alpha Aesar. The photoinitiator Irgacure 2959® was purchased from BASF.

Carbon dioxide used as solvent was purchased from White Martins S/A. ε-caprolactone (CL), N-acetylcysteine (NAC), bovine serum albumin (BSA) and bovine serum albumin-fluorescein isothiocyanate conjugate (BSA-FITC) were purchased from Sigma Aldrich. Novozym 435 was kindly donated by Novozymes A/S (commercial lipase B from *Candida antarctica* immobilized on cross-linked polyacrylate beads, esterification activity 42 U g^{-1}, measured according to a procedure adapted from literature (OLIVEIRA et al., 2006)). Globalide (Gl) was a kind gift from Symrise. The enzymes and the monomers globalide and ε-caprolactone dried under vacuum (GUINDANI et al., 2017) and stored in a desiccator over silica and 4 Å molecular sieves. The proteins were used without further purification.

5.2.2. Poly(globalide-co-ε-caprolactone) synthesis using supercritical carbon dioxide as solvent

Polymerization experiments were carried out as described by Guindani et al. (2017), using supercritical carbon dioxide ($scCO_2$) as solvent. The pressure and temperature conditions of the system were maintained constant at 120 bar and 65 °C, in a fixed reaction time of 2 hours. Enzyme content was fixed at 5 wt% (relative to the total monomer amount), and the CO_2:monomers mass ratio was fixed at 1:2. The feed mass ratio of globalide/ε-caprolactone (Gl/CL) was fixed in 50/50. After polymerization, the material was purified through solubilization in dichloromethane (DCM), separation of the enzymes by filtration and

precipitation of the polymer in cold ethanol (EtOH). The polymeric suspension was then filtered and dried at room temperature in vacuum, up to constant mass.

5.2.3. Poly(globalide-co-ε-caprolactone) nanoparticles preparation

The NPs were prepared according to the solvent evaporation method. The aqueous phase was prepared mixing water (14 g) and the surfactant SDS (0.2 % w/w). The organic phase was prepared mixing the pre-synthesized PGlCL (100 mg), and DCM as solvent (3.5 g). The aqueous phase was added to the organic phase and the mixture was sonicated. Sonication occurred for 3 minutes (10 seconds pulse on, 10 seconds pulse off) with an amplitude of 70%. An ice bath was used to reduce the temperature increase during the sonication. The miniemulsion was left in a thermostatic bath with a temperature of 50 °C until complete solvent evaporation.

The purification of miniemulsions was carried out by centrifugation (12,000 rpm, 4 °C for 50 min). The supernatant containing the excess of surfactant was removed and the NPs re-solubilized in distilled water. This process was performed twice. Purified miniemulsions were stored in refrigerator (4 °C) until functionalization steps were performed.

5.2.4. Surface modification of poly(globalide-co-ε-caprolactone) nanoparticles

5.2.4.1. Functionalization with N-acetylcysteine (NAC)

The surface of PGlCL NPs was functionalized with N-acetylcysteine (NAC) through thiol-ene reactions. The miniemulsion containing PGlCL NPs was placed in a flask together with NAC and the water-soluble initiator KPS, under nitrogen atmosphere. During the reaction, the flask was kept immersed in an oil bath at 70 °C, during 4 hours, under continuous magnetic stirring. NAC was used in sufficient amount to functionalize 5% of the double bonds. Different KPS amounts were tested, varying from 0.625 to 5% (mol), relative to NAC amount.

After the reaction, the miniemulsion containing NAC functionalized NPs was purified by centrifugation (described in 5.2.3.) for NAC excess removal. The functionalized NPs were re-solubilized in distilled water. Purified miniemulsions were stored in refrigerator (4 °C).

5.2.4.2. Functionalization with Bovine Serum Albumin (BSA)

BSA modification - Before conjugation with nanoparticles, BSA was modified by reaction with Traut's reagent (2-Iminothiolane), aiming to introduce more thiol groups to the BSA structure, which should enhance its capacity of conjugation with PGlCL NPs through thiol-ene reaction. A BSA solution was prepared (10 mg BSA mL^{-1}) in sodium phosphate buffer 0.1 M, pH 8.0 containing 1mM EDTA. The 2-Iminothiolane solution was prepared in a 2 mg mL^{-1} concentration and was mixed to the BSA solution in a proportion of 1:50 (BSA:2-Iminothiolane) (v/v). The reaction was carried out for 1 h in a shaker, at room temperature. After reaction, the modified BSA was purified by dialysis for 48 hours and then stored in a freezer (-18 °C).

Covalent functionalization of the nanoparticles with modified BSA - The covalent conjugation of PGlCL nanoparticles with the modified BSA was carried out through thiol-ene reactions, directly in the polymeric chain double bonds present on the surface of the particle. The miniemulsion containing PGlCL NPs was placed in a flask together with modified BSA and the water-soluble initiator Irgacure 2959®, under nitrogen atmosphere. During the reactions, the flask was exposed to UV light (365 nm) for 4 hours, under continuous magnetic stirring. The amount of BSA used was established as 10^{-8} mol of BSA per mL of miniemulsion. This amount is theoretically enough to cover the surface of all nanoparticles. Irgacure 2959® content was fixed in 1% (mol), relative to the total amount of thiol groups from BSA present in the reaction media. After the reaction, the miniemulsion containing covalent BSA-NPs conjugates was purified by centrifugation (described in 5.2.3.) for BSA excess removal. The conjugates were re-solubilized in distilled water. Purified miniemulsions containing the conjugates were stored in refrigerator (4 °C).

Non-covalent functionalization of the nanoparticles with modified BSA - The non-covalent conjugation of PGlCL nanoparticles with the modified BSA was carried out by mixing in a flask the miniemulsion containing PGlCL NPs and the modified BSA, without any initiator. The mixture was incubated overnight in refrigerator. The amount of BSA used was 10^{-8} mol of BSA per mL of miniemulsion. After the reaction, the miniemulsion containing non-covalent BSA-NPs conjugates was purified by centrifugation (described in 5.2.3.) for BSA excess removal. The NPs were re-solubilized in distilled water. Purified miniemulsions containing the conjugates were stored in refrigerator (4 °C).

5.2.5. Proton nuclear magnetic resonance (^{1}H NMR)

^{1}H NMR spectroscopy was performed on a Bruker Avance 300, operating at 300 MHz. All spectra were referenced internally to residual proton signals of the deuterated solvent. Samples were solubilized in $CDCl_3$ (δ = 7.27 ppm for ^{1}H NMR).

5.2.6. Ellman's assay

The Ellman's assay was used to determine the final concentration of thiol groups present on modified BSA, as well as the amount of NAC non-reacted to the NPs. Ellman's reagent (5,5'-dithio-bis-[2-nitrobenzoic acid], DTNB) was solubilized in sodium phosphate buffer (0.1 M, pH 8.0, containing EDTA 1 mM), in a concentration of 4 mg mL^{-1}. The assay was carried out in triplicate, in a 96 well plate, mixing in each well 4 µL of Ellman's reagent, 200 µL of buffer and 20 µL of sample. The reaction occurs under constant stirring and protected from light, during 15 minutes. N-acetylcysteine (NAC) was used as standard. Absorbance was measured at 412 nm with a Tecan infinite plate reader.

5.2.7. Pierce assay

The protein concentration was determined by Pierce 660 nm Protein Assay according instructions of the manufacturers. Bovine serum albumin (BSA) was used as standard. Absorbance was measured with a Tecan infinite plate reader.

5.2.8. Dynamic light scattering (DLS)

Intensity particle average diameters of the uncoated nanoparticles and the conjugates (Dp) and the polydispersity indexes (PDI) were measured by dynamic light scattering (DLS —Malvern Instruments, Zetasizer Nano S). The latex samples were diluted approximately 1:15 with distilled water prior to DLS measurements.

5.2.9. Zeta potential

The zeta (ζ) potential of the uncoated nanoparticles and the conjugates (10 µL, 10 mg mL^{-1}) was measured in a 1 mM potassium

chloride solution (1 mL) with a Zeta Sizer Nano Series (Malvern Instruments).

5.2.10. Transmission electron microscopy (TEM)

Transmission electron microscopy was carried out with a FEI Tecnai F20 transmission electron microscope operated at an acceleration voltage of 200 kV. The NPs were first diluted with 1 mL water and then one droplet was placed on a carbon-coated copper grid and dried overnight.

In order to visualize the presence of the protein coating on the conjugates, the negative staining technique was used. NPs (uncoated and conjugates) were first diluted with water and then one was placed onto a lacey grid and let to dry. The samples were stained with 4% uranyl acetate + 1% trehalose solution (1:1) (v/v) according to the method from Kokkinopoulos et al. (2016). Images were taken with an Ultrascan 1000 (Gatan) charge-coupled device (CCD) camera.

5.2.11. Flow cytometry - Fluorescence-activated cell sorting (FACS) measurements

Flow cytometry measurements were performed for NPs covalently and non-covalently conjugated with BSA, and also for uncoated NPs. For these measurements, the conjugates were produced using fluorescein isothiocyanate (FITC) labeled BSA. BSA-FITC was modified before the conjugation reactions, in the same way as described in item 5.2.4.2. Measurements were performed using an Attune NxT Flow Cytometer (laser: 488 nm laser for FITC excitation; emission: 530 nm band pass filter). The fluorescent signal was expressed in a histogram and the median intensity was determined. Attune NxT software was used for data analysis.

5.2.12. Fluorescence correlation spectroscopy (FCS)

FCS measurements were performed for NPs covalently and non-covalently conjugated with BSA. For these measurements, the conjugates were produced using fluorescein isothiocyanate (FITC) labeled BSA. BSA-FITC was modified before the conjugation reactions, in the same way as described in item 5.2.4.2. The measurements were also performed for pure modified BSA-FITC.

FCS was carried out on a commercial setup (Carl Zeiss, Germany) consisting of the modules LSM510, ConfoCor 2 and an inverted microscope model Axiovert 200 with a C-Apochromat 40×, NA 1.2 water immersion objective. An argon ion laser (488 nm) was used for excitation and the emission was collected after filtering with a BP505-550 band pass filter. 8-well, polystyrene chambered cover glasses (Laboratory-Tek, Nalge Nunc International) were used as sample cells. For each sample series 10 measurements with a total duration of 10 s were performed. The time-dependent fluctuations of the fluorescence intensity δI(t) caused by the diffusion of the fluorescent species through the confocal observation volume were recorded and analyzed by an autocorrelation function $\mathrm{G}(t) = 1 + \langle \delta I(t) \cdot \delta I(t+\tau) \rangle / \langle \delta I(t) \rangle^2$. As has been shown theoretically, for an ensemble of identical freely diffusing fluorescence species, $\mathrm{G}(t)$ has the following analytical form:

$$\mathrm{G}(t) = 1 + \left[1 + \frac{f_T}{1-f_T} e^{-t/\tau_T}\right] \frac{1}{N} \frac{1}{\left[1+\frac{\tau}{\tau_D}\right] \sqrt{1+\frac{\tau}{S^2 \tau_D}}} \qquad (5.1)$$

Where N is the average number of diffusing fluorescence species in the observation volume, f_T and τ_T are the fraction and the decay time of the triplet state, τ_D is the diffusion time of the species, and S is the so-called structure parameter, $S = z_0/r_0$, where z_0 and r_0 represent the axial and radial dimensions of the confocal volume, respectively. The diffusion time τ_D is related to the respective diffusion coefficient D, through $D = {r_0}^2/4\tau_D$. The experimentally obtained $\mathrm{G}(t)$ can be fitted with Equation (5.1), giving as a result the diffusion times and the diffusion coefficients of the fluorescent species. The hydrodynamic radii R_h can be calculated (assuming spherical particles) using the Stokes-Einstein relation: $R_\mathrm{h} = k_B T/6\pi\eta D$, where k_B is the Boltzmann constant, T is the temperature, and η is the viscosity of the solution. As the radial dimension r_0 of the confocal probing volume is not known a priory it was determined by performing calibration experiments using a fluorophore with known diffusion coefficient in water, i.e. AlexaFluor488.

5.2.13. Cell culture

RAW264.7 were bought from DSMZ (Deutsche Sammlung für Mikroorganismen und Zellen, Braunschweig, Germany). The cells were maintained in Dulbecco´s modified eagle medium (DMEM)

supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/ml streptomycin and 2 mM glutamine (Thermo Fisher).

5.2.14. Cell uptake: Flow cytometry and confocal laser scanning microscopy

RAW 264.7 (100 000 cells/well) were seeded out in 24 well plates (200 µL) for flow cytometry analysis. After overnight incubation at 37 °C, cells were washed and serum-free culture medium was added. It were also tested the addition of cell culture medium containing 10% human serum or 10% fetal bovine serum (FBS). For nanoparticle uptake analysis, the fluorescent dye Coumarin-6 was first encapsulated in the NPs, and then the BSA-NPs covalent conjugates were produced. Uncoated nanoparticles and conjugates were applied to cells at a concentration of 37.5 µg mL^{-1} for 2 h and 24 h.

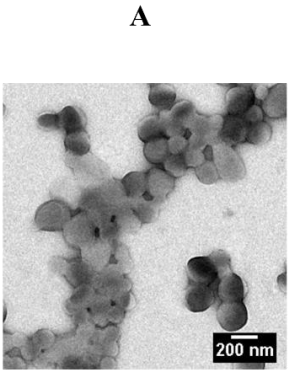
Afterwards, cells were collected and detached with 2.5 % trypsin from cell culture wells. Flow cytometry measurements were performed with Attune™ NxT Flow Cytometer (Invitrogen, USA). The fluorescent dye Coumarin-6 was excited with a 488 nm laser. Data analysis was performed using Attune™ NxT software (Invitrogen, USA). Values are expressed as percentage (%) of fluorescent positive cells as an average of at least two independent experiments.

5.3. RESULTS AND DISCUSSION

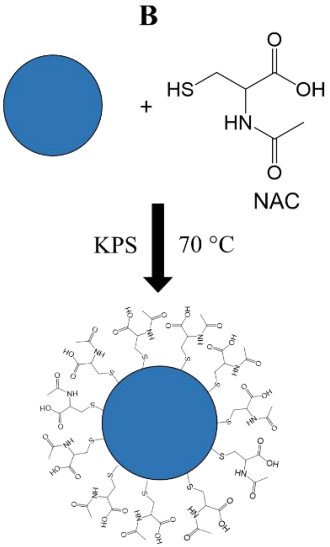
5.3.1. PGlCL nanoparticle formation and functionalization with NAC

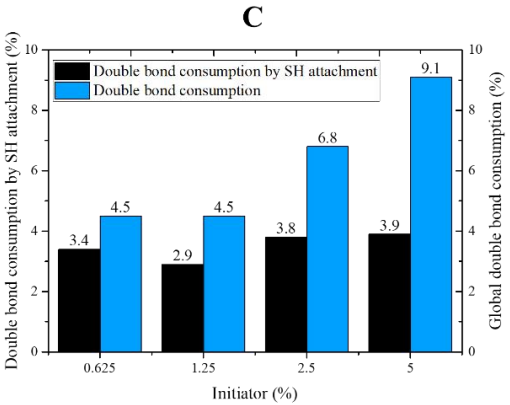
The formation of PGlCL NPs was carried out through the solvent evaporation method. At first, PGlCl was synthesized by e-ROP using $scCO_2$ as solvent, and then pre-synthesized PGlCL was used to prepare the NPs. In order to evaluate size, stability and morphology of the NPs, DLS and zeta potential measurements were performed, as well as TEM imaging (Figure 5.2A). The mean particle diameter was determined to be around 145 nm, with a low PDI value, indicating an uniform particle size distribution. On the other hand, zeta potential value was determined to be around -54.0 mV, revealing a good miniemulsion stability. The negative charge is associated to the presence of SDS (an anionic surfactant) adsorbed on the surface of the particles (LOOSLI; STOLL, 2017). TEM imaging corroborate with results obtained by DLS and confirm the spherical morphology of the NPs.

Figure 5.2 - (A) TEM image of PGlCL nanoparticles and information about particle size and zeta potential; (B) Scheme representing the surface functionalization of PGlCL nanoparticles with NAC through thiol-ene reaction; (C) Global double bond consumption and double



Poly(globalide-co-ε-caprolactone) NPs	
Dp (nm)	145
PDI	0.075
ζ potential (mV)	-54.0





D_p: Particle diameter; PDI: polydispersity index; ξ potential: zeta potential

After its preparation, the double bonds present on the surface of PGlCL NPs were functionalized through thiol-ene reaction with NAC, as can be seen in Figure 5.2B. The global consumption of the particle double

bonds was determined through ^{1}H-NMR measurements, by comparing the integral values of the double bond peaks before and after functionalization with NAC. Besides, the double bond consumption caused specifically by thiol (SH) groups attachment could be determined. After the functionalization reaction, the samples were purified and the supernatant containing excess of NAC was collected. The concentration of non-reacted NAC was determined by the Ellman's assay. Figure 5.2C compares the global double bond consumption and the double bond consumption caused by SH attachment.

It is possible to observe that the higher the amount of initiator used, the higher is the global double bond consumption, reaching around 9% for the highest initiator concentration tested. As described in 5.2.4.1, NAC was used in sufficient amount to consume 5% of the double bonds, which indicate that the double bond consumption is not happening only due to the NAC coupling. On the other hand, the double bond consumption caused by SH attachment remained almost constant at a value around 4%, which is consistent to the amount of NAC used. The consumption of the double bonds may happen not only due to the SH attachment by thiol-ene reaction, but also because of the occurrence of other side reactions, such as crosslinking reactions, addition of initiator derived radicals to the double bonds, head-to-head coupling of carbon-centered radicals, and cross-termination between carbon-centered and thiyl radicals (DERBOVEN et al., 2013). The incidence of these side reactions crucially depend on the applied initiator concentrations. The higher the initiator concentration applied is, the higher is the incidence of side reactions (DERBOVEN et al., 2013), and the data obtained in the present work endorse this behavior. It is possible to notice that small amounts of initiator are enough to promote effective SH attachment with a lower incidence of side reactions. This way, in the next steps of this work, the amount of initiator was fixed in 1 % (mol) relative to the amount of SH groups.

5.3.2. Covalent conjugation of PGlCL NPs with BSA

After confirming the feasibility of functionalizing the double bonds on the surface of PGlCL NPs through thiol-ene reaction (item 5.3.1.), the next step of this work was to use the same type of reaction to covalently attach cysteine derivative SH groups from BSA to the surface of the NPs. First, BSA was modified through reaction with the Traut's reagent, in order to introduce more SH groups in its structure. This modification is

necessary, since BSA has originally only one cysteine with a free thiol group (SUGIO et al., 1999). After modification, the final protein concentration was determined by the Pierce assay, and the final concentration of thiol groups present on modified BSA was determined by the Ellman's assay. This way, it was possible to calculate the final amount of SH groups per BSA molecule, which was on average 5 thiol groups for each protein molecule.

As shown in Figure 5.3, after purification, DLS, ξ potential and TEM imaging were performed in order to characterize the conjugates regarding its size, stability and morphology, respectively. FACS and FCS were performed to evaluate the success of the covalent conjugation. For FACS and FCS measurements, the conjugates were produced using the fluorescently labeled protein, BSA-FITC, which was also modified by Traut's reagent before conjugation reactions.

Figure 5.3A shows FACS results for uncoated NPs, non-covalent and covalent conjugates. FACS measurements give information about the amount of particles that emitted fluorescent light after excitation by a 488 nm laser in each evaluated sample. For non-covalent conjugates, around 26% of the nanoparticles emitted fluorescence, while for covalent conjugates, this value was around 36%. For uncoated NPs, a very small amount of particles presented fluorescence (around 2%), as expected. As FACS results shows, after purification, covalent conjugates remained with a higher amount of fluorescent particles, in comparison to the non-covalent conjugates. Since the fluorescence of the particle is conferred by the presence of fluorescent protein on its surface, this is a strong indicative that BSA-FITC was covalently bonded to the nanoparticles, forming more resistant and stable conjugates.

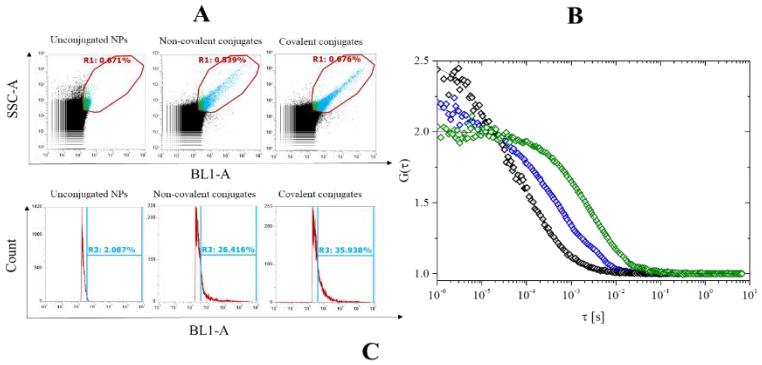
FCS measurements were also performed, in order to investigate the covalent attachment of BSA-FITC to the NPs. Figure 5.3B shows the FCS autocorrelation curves for pure modified BSA-FITC, BSA-FITC non-covalent conjugates and BSA-FITC covalent conjugates, measured at excitation wave length of 488 nm. All correlation curves were fitted with Equation 5.1 (item 5.2.12), and the hydrodynamic radii of the corresponding fluorescent species were obtained with an experimental error of ±10%, typical for FCS studies.(WINZEN et al., 2016) For the pure BSA-FITC sample, a hydrodynamic radius of $R_h = 3.3 \pm 0.3$ nm was obtained, which matches the values reported for BSA in the literature (BOHIDAR, 1989). If BSA-FITC molecules get attached to the NPs, forming conjugates, this attachment should result in a shift of the autocorrelation curve, since conjugates are large species that diffuses slower in comparison to small molecules such as BSA, leading to longer

diffusion times. Comparing pure BSA-FITC and BSA-FITC conjugates autocorrelation curves, it is possible to clearly see these shifts, indicating that BSA-FITC is binding to the NPs and forming conjugates. BSA-FITC covalent conjugates curve presented a strong shift in relation to the pure BSA-FITC curve. For BSA-FITC non-covalent conjugates curve, this shift also occurred, but in a less intense way. This behavior suggests that in fact, the interactions between the protein and the particles are stronger for BSA-FITC covalent conjugates, since they showed to be larger and slower, which means that more proteins were kept attached to the NPs surface.

The fitting of the conjugates autocorrelation curves also revealed that for both covalent and non-covalent conjugates samples, there are two species responsible for emitting fluorescence: free BSA-FITC, and the BSA-FITC nanoparticle conjugates, which are the larger and slower species. In the case of the covalent conjugates, free BSA-FITC is responsible for the emission of 10% of the detected fluorescence, while 90% of the fluorescence is emitted by the conjugates. On the other hand, for the non-covalent conjugates, free BSA-FITC is responsible for the emission of 40% of the detected fluorescence, while 60% of the fluorescence is emitted by the conjugates. Here we can clearly see that, in fact, proteins and NPs are interacting much more strongly when BSA-FITC is covalently bonded to the NPs, forming conjugates that are more resistant to dissociation.

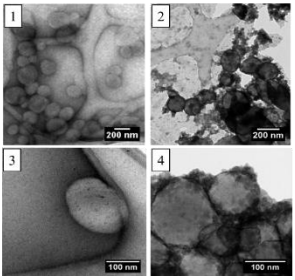
As shown in Figure 5.3C, after covalent functionalization, the conjugates presented a very subtle increase in the particle diameter, as expected, from 145 nm (uncoated NPs - Figure 5.2A), to 152 nm, probably caused by the formation of the protein layer around the NPs. ξ potential values also suffered changes after NPs conjugation with BSA, slightly decreasing from -54 mV (uncoated NPs - Figure 5.2A) to -61.0 mV. This behavior was also observed by other authors (SIMON et al., 2018) and it is attributed to the BSA charge contribution, since it has an overall negative charge at pH > 5.5 (PETERS, 1985). Figure 5.3C also present TEM images of uncoated NPs (images 1 and 3) and covalent conjugates (images 2 and 4), obtained by the negative staining technique. These images confirm the formation of a protein layer that surrounds the nanoparticles. This layer contains proteins that even after purification remained tightly bonded to the surface of the NPs by covalent bonds or other strong interactions. Very similar behavior was found in previous reports (KOKKINOPOULOU et al., 2017; SIMON et al., 2018).

Figure 5.3 - (A) FACS histograms for unconjugated NPs, non-covalent and covalent conjugates (B) FCS autocorrelation curves (symbols) and corresponding fit (straight lines): BSA-FITC (black diamonds ◊), BSA-FITC non-covalent conjugates (blue diamonds ◊) and BSA-FITC covalent conjugates (green diamonds ◊) (C) TEM image of uncoated NPs (1 and 3) and BSA covalent conjugates (2 and 4) and information about particle size and zeta potential of BSA covalent conjugates



C

BSA-NPs covalent conjugates	
Dp (nm)	152
PDI	0.065
ζ potential (mV)	-61.0



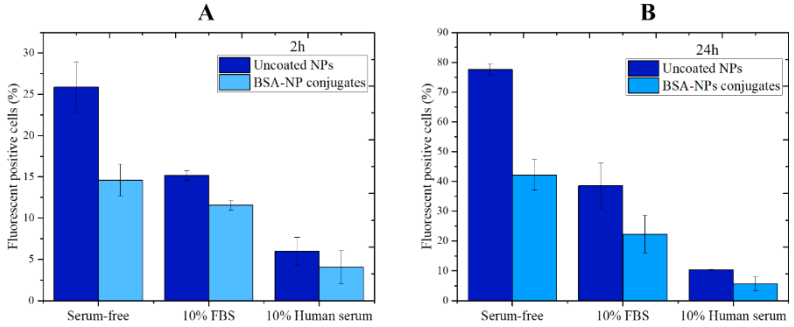
5.3.3. Cell uptake

The uptake of uncoated NPs and BSA-NPs covalent conjugates into macrophages (RAW264.7) was analyzed by flow cytometry (Figure 5.4). NPs and BSA-NPs conjugates were applied to cell culture medium without additional proteins or supplemented with fetal bovine serum (FBS) or human serum. This way, it was possible to observe how

conjugation with BSA affects NPs uptake by macrophages in different environments.

Flow cytometry analysis clearly indicate that, in all cases, the conjugation with BSA has reduced cell uptake by macrophages. Serum albumin is often reported as a dysopsonin protein (OGAWARA et al., 2004; THIELE et al., 2003), which means it inhibits cellular internalization by phagocytosis. This way, when conjugated to a NP, BSA hampers the recognition of BSA-NPs conjugates by macrophages, promoting the so-called “stealth effect” (CARACCIOLO et al., 2015; SCHÖTTLER et al., 2016). This effect avoids the clearance of the nanoparticles by the immune system and promotes a prolonged blood circulation time, enabling the nanoparticle to reach the desired target (KANG et al., 2015; SIMON et al., 2018).

Figure 5.4 - Cell uptake: Amount of fluorescent positive cells (%) for unconjugated NPs and BSA-NPs covalent conjugates during (A) 2h and (B) 24h of incubation.



Another important characteristic that can be observed through these results is that even when BSA-NPs conjugates are introduced in FBS or human serum, cellular uptake is still reduced. This means that the stealth properties obtained by conjugating NPs with BSA were preserved, reinforcing the idea that stable conjugates were formed.

The covalent modification of the NPs surface with BSA is an interesting strategy to produce stable nanocarriers, which are able to keep its stealth properties and to remain in the bloodstream for extended periods, improving the efficiency of treatments.

5.4. CONCLUSIONS

In the present work, PGlCL NPs were successfully conjugated with bovine serum albumin by thiol-ene reaction, producing BSA-NPs conjugates which are resistant to dissociation. FACS and FCS measurements revealed that the conjugates produced by BSA covalent attachment remained with a larger amount of proteins on its surface even after the purification processes, indicating a higher stability in comparison to the conjugates produced by non-covalent method. The conjugates visualization was possible through TEM imaging, and a protein layer attached to the surface of the NPs was clearly observed. Cell uptake assays revealed a reduced internalization of the NPs by macrophages after conjugation with BSA. These results confirm that covalent BSA-NPs conjugation is a feasible strategy to produce stable conjugates with stealth properties.

CHAPTER 6

6. CONCLUDING REMARKS

In this work, the synthesis and functionalization of the copolymer poly(globalide-co-ε-caprolactone) was performed by using clean high pressure technologies for future applications with biomedical purposes.

PGlCL e-ROP was performed using $scCO_2$ and $scCO_2$+DCM as solvents, under different globalide/ε-caprolactone feed ratios. The reactions were especially successful when conducted under the use of only $scCO_2$ as solvent. The copolymers synthesized with pure $scCO_2$ reached M_n values up to 25,000 Da, presented a well-defined melting point and a low formation of oligomeric products, and by this reason they were chosen to be further functionalized. The use of DCM as cosolvent increased the formation of cyclic oligomers, since it diluted the reaction media, favoring intramolecular backbiting. The presence of cyclic oligomers confers to PGlCL double melting point and wider molecular weight distribution.

PGlCL produced using pure $scCO_2$ in different Gl/CL ratios was successfully functionalized with N-acetylcysteine (NAC) through thiol-ene reaction. Higher globalide contents in PGlCL led to higher incorporation of NAC to the polymer chain, and the presence of NAC pendant groups disturbed the formation of crystalline domains, thus reducing the melting temperature of the copolymers. For copolymers with high contents of globalide, functionalization resulted in completely amorphous copolymers and a more hydrophilic character, desirable for biomedical applications where bioresorption is required. After functionalization with NAC, the polymer also presented antioxidant character, probably due to the presence of the the pendant thioether linkage formed after thiol-ene coupling. This property have an important role in applications where the material can combat cellular oxidative-stress.

After PGlCL functionalization with NAC, the work was expanded to the nanomaterials field, and then PGlCL nanoparticles were prepared and successfully conjugated with bovine serum albumin by thiol-ene reaction. As a result, it was produced stable and irreversible BSA-NPs conjugates. The occurrence of the covalent attachment was observed by FACS and FCS measurements, revealing that the BSA conjugates produced by covalent attachment remained with a larger amount of proteins in its surface in comparison to conjugates produced by non-

covalent method. The visualization of the BSA layer attached to the particles was possible through TEM imaging. Finally, cell uptake assays revealed that NPs presented stealth properties after conjugation with BSA, avoiding clearance by immune cells.

This work consisted of an unprecedent study, where it started to be built a platform of biomaterials based on PGlCL copolymers, synthesized using clean technologies. Here, it was possible to explore the process variables of PGlCL synthesis and understand their effect in the final properties of the material. Besides, it was possible to show different PGlCL modifications, which enabled the tuning of its properties for future applications in tissue engineering and in nanomedicine. There are still many challenges to be overcome in this area and also many modification strategies to be explored. This work should contribute to the progress of biomedical engineering and be a basis for future studies leading to improved biomaterials.

6.1. SUGGESTIONS FOR FUTURE STUDIES

- To produce fibers/scaffolds using PGlCL-NAC and test its degradation and cell adhesion;
- To functionalize PGlCL with other molecules/biomolecules containing thiol groups that can improve polymer features for biomedical applications, and produce fibers/scaffolds with the modified material;
- To encapsulate medicaments in PGlCL NPs and functionalize the surface of the the NPs with BSA or other proteins, aiming specific targeting;
- To produce magnetic PGlCL-NPs for diagnostic imaging or for tumor treatment, and functionalize it with BSA or other proteins for specific targeting.

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APPENDIX A - KINETIC STUDY OF PGlCL ENZYMATIC SYNTHESIS UNDER DIFFERENT KINDS OF STIRRING

This section presents a preliminary kinetic study of poly(globalide-co-ε-caprolactone) (PGlCL) using dichloromethane (DCM) as solvent, under magnetic and mechanical stirring. The samples obtained in the kinetic study were evaluated in terms of reaction yield, molecular weight and thermal properties to evaluate the effect of time and stirring on PGlCL e-ROP.

E-ROP reactions in dichloromethane (DCM) were carried out in two different reactors, with two different kinds of stirring, in order to observe the effect of non-homogeneities on the kinetics of globalide (Gl) and ε-caprolactone (CL) copolymerization. The first reactor employed a magnetic stirrer with a TeflonTM-coated stirring bar. The other reactor (Parr Instrument Company, model 4592, Moline, IL, USA) has a mechanical agitation using a straight blade impeller. Both reactors consist in jacketed stainless steel vessels with temperature and pressure control. Globalide and ε-caprolactone, both previously dried, were used in a mass ratio of 50/50 (globalide/ε-caprolactone). Dichloromethane (DCM) was used as solvent, in a mass ratio of 2:1 (solvent:monomers).

Novozym 435, also previously dried, was used in an amount of 5% in relation to the mass of monomers (total mass of globalide and ε-caprolactone). The enzyme, monomers, and the solvent were weighed on a precision scale balance (Shimadzu AUY220, Philippines with 0.0001 g accuracy) and fed to the reactor, which was immediately closed. The reaction temperature was kept in 65 °C, respecting the optimal enzyme activity temperature range (OLIVEIRA et al., 2004; RICHETTI et al., 2010; ROSA et al., 2008). The reactor pressure was maintained around 2.5 bar, which is the equilibrium pressure that the system reached at 65 ° C. At the end of the reaction time, more DCM was added to the reaction mixture to reduce the viscosity, helping to dissolve the product. After filtering off the enzyme, the filtrate was precipitated in cold ethanol. DCM and EtOH were used at the volumetric proportion of 1:6. The polymeric suspension was filtered and dried at room temperature in vacuum, up to constant mass.

Reactions were performed in different time intervals, ranging from 30 to 720 min, where each kinetic data point represented a different assay (destructive experiments). Yields were determined through the mass ratio between the mass of copolymer obtained and the mass of monomer fed.

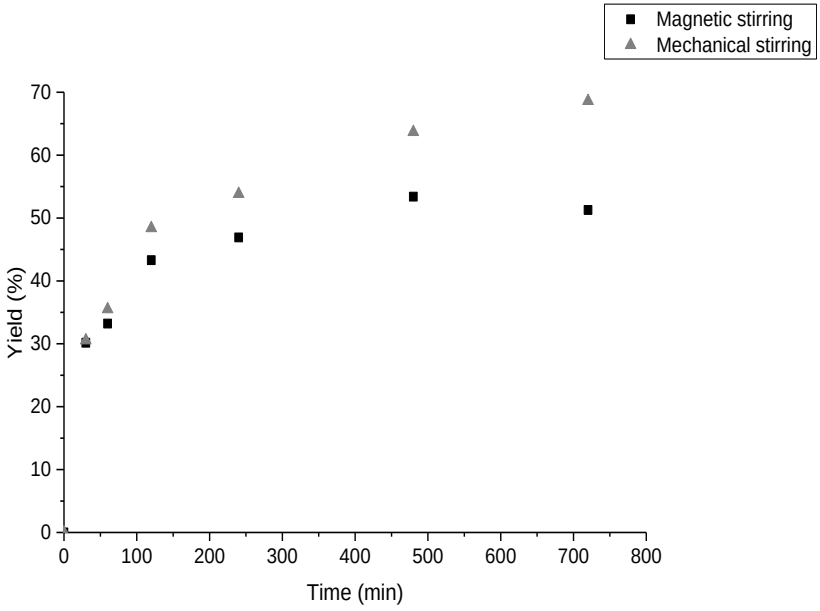
PGlCL samples obtained on kinetic study were characterized in terms of molecular weight (Gel Permeation Chromatography – GPC),

thermal properties and crystallinity (Differential Scanning Calorimetry – DSC). The assays were performed according item 3.2.3., on Chapter 3.

A1. YIELD AND MOLECULAR WEIGHT KINETIC CURVES

Figure A.1 presents the yield kinetic data for e-ROP of PGlCL 50/50 (Gl/CL feed mass ratio) synthesized on DCM, submitted to magnetic and mechanical stirring. For the reaction system stirred magnetically, it is possible to note that the yield reaction increases in a higher rate up to 120 minutes. After 120 min the yield increases in lower intensity, stabilizing its value in around 50%. During the polymerization reaction, the size of the copolymer chains increase, increasing the system viscosity. After 120 min this high viscosity affects the magnetic stirring, making it less intense and possibly even stopping the stirring completely. This way, after 120 min, mass transfer in the system happens basically by diffusion, which makes the polymerization reaction practically stop.

Figure A.1 - Yield kinetic behavior for PGlCL 50/50 (Gl/CL feed mass ratio) synthesis on DCM, under magnetic and mechanical stirring.

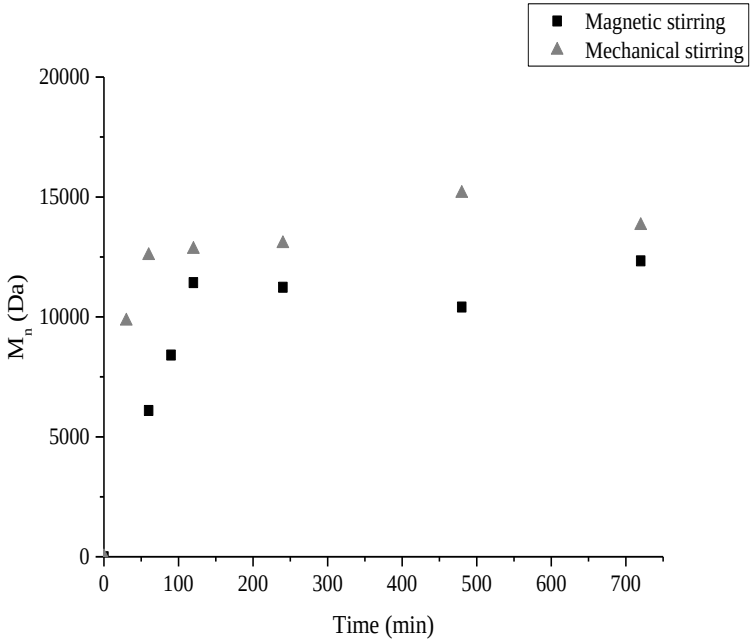


For the mechanically stirred system, the reaction yield also increases in a higher rate up to 120 min. However, after 120 min, the system maintained an increase on reaction yield, in a less intense way, reaching

almost 70% of conversion. The viscosity of the reaction media affects less the mechanical stirring than the magnetic stirring. Maintaining the stirring allows the monomer molecules to keep finding the active sites of the enzymes, leading to higher reaction yields.

Figure A.2 presents number average molecular weight (M_n) kinetic data of PGlCL 50/50 (Gl/CL feed mass ratio) synthesized on DCM, determined by GPC. For all kinetic data points, the inflection point of the broad polymer peak was used as the endpoint for integration procedures, as described in section 3.3.2, in Chapter 3. The same integration criteria was used in literature (KUNDU et al., 2011) and enables valid data comparisons maintaining the general trend in this data consistent with other studies (COMIM ROSSO et al., 2013; VAN DER MEULEN et al., 2008, 2011).

Figure A.2 - Number average molecular weight (Mn) kinetic behavior for PGlCL 50/50 (Gl/CL feed mass ratio) synthesis on DCM, under magnetic and mechanical stirring.



For magnetic stirring, M_n grows up to 120 min of reaction. Initially, after the monomer rings are opened, enzyme-activated polymer chains

(EAPC, polymer chains attached to the enzyme active site) reacts in a very intense way to oligomer/polymer chains in solution. This reaction regenerate the enzyme active site and generate growing polymer chains, increasing M_n values. The increase in M_n leads to lower mobility of the polymer chains, which causes a decrease in the polymerization rate. This way, the effect of polymer degradation reactions by the presence of water molecules becomes more prominent (COMIM ROSSO et al., 2013; GEUS, 2007; THURECHT et al., 2006). After 120 min, M_n keeps basically constant, which probably happens due to the establishment of an equilibrium between polymerization and degradation reactions which occurs simultaneously (KOBAYASHI; UYAMA; TAKAMOTO, 2000; KUNDU et al., 2011; SIVALINGAM; MADRAS, 2004).

For mechanical stirring, the same kinetic behavior of M_n can be observed, consisting of a growing chain step, and a step where an equilibrium of polymerization and degradation reactions is established, reaching after 90 min, values of M_n similar to those obtained under magnetic stirring. However, in comparison to the magnetic stirred system, the chain growth rate for mechanical stirred system is much higher. In 60 min of reaction, M_n reached values around 6,000 Da for magnetic stirred reaction, and around 13,000 Da for mechanical stirred reaction. The oligomeric chains formed during this interval of time are probably kept in contact to the enzyme pellets in a more efficient way with the use of a mechanical stirring, which allows the chains to grow faster.

A2. THERMAL PROPERTIES

Samples obtained in different reaction times, under mechanical stirring, were evaluated by DSC. These samples were chosen due to their more interesting kinetic results (yield and molecular weight). Results of crystallization temperature (T_c), melting temperature (T_m), melting enthalpy (ΔH_m) and degree of crystallinity are presented in Table A.1. The degree of crystallinity was calculated through the relation between ΔH_m of each sample, and the theoretical value of a 100% crystalline PCL sample, obtained from literature (CRESCENZI et al., 1972).

Table A.1 - Thermal properties of samples determined by DSC for samples obtained in different reaction times under mechanical stirring.

Time (min)	Stirring	T_c (°C)	T_m 1 (°C)	T_m 2 (°C)	ΔH_m (J/g)	X_c (%)
60	Mechanical	28	37	45	76.3	56
120		26	34	44	76.7	57
240		24	34	42	82.8	61
480		20	35	39	84.3	62

T_c: Crystallization temperature; T_m 1: First peak melting temperature; T_m 2: Second peak melting temperature; ΔH_m: Heat of fusion; X_c: Degree of crystallinity, calculated from the fusion enthalpy value of a PCL 100% crystalline sample (CRESCENZI et al., 1972).

All samples presented a double melting point, in the same way that the samples synthesized on $scCO_2$+DCM (section 3.3.3., in Chapter 3). This fact led us to further investigate the relation between the use of DCM and the presence of double melting points. As demonstrated by MALDI-TOF analysis, the use of DCM in PGlCL favors the formation of oligomeric cycles (intramolecular backbiting), probably generating small and imperfect crystals, responsible for the double melting peaks presence (melting-recrystallization mechanism).

Melting temperatures remained practically constant throughout the reaction time. ΔH_m, and consequently X_c experienced a subtle variation on their values as the reaction times increased, however, this variation is too small to be considered. Copolymers formed in all evaluated reaction times showed to be semi-crystalline.

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APPENDIX B – PGlCL DEGRADATION STUDY

This section presents a preliminary degradation study of poly(globalide-co-ε-caprolactone) (PGlCL). PGlCL degradation was evaluated in buffer and in the presence of a lipase from *Pseudomona cepacia.* Besides, a degradation assay was also performed in the presence of active sludge.

B1. Degradation of PGlCL and PGlCL-NAC films in buffer and lipase solutions

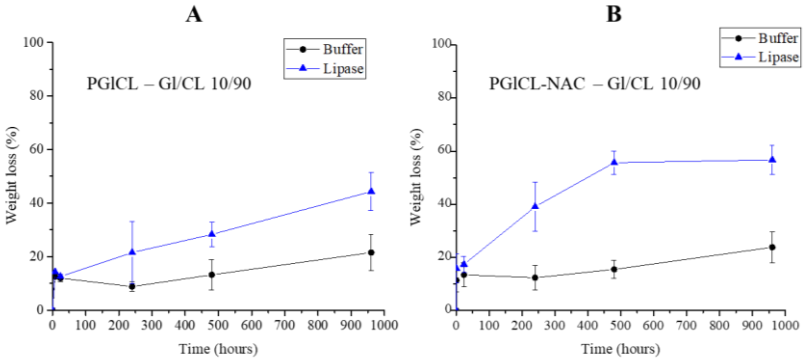
PGlCL and PGlCL-NAC films were prepared by casting 120 μL of a polymeric solution (120 mg/mL) onto circle Microscope Cover Slips with 25 mm diameter. PGlCL samples were diluted in chloroform, while PGlCL-NAC were diluted in a chloroform:ethanol mixture 9:1 (v/v). The samples were dried under vacuum at room temperature until a constant weight was achieved. The weights of the films were about 11-15 mg.

The degradation experiments were conducted at 37 °C by immersing each polymer film with the Cover Slips in 4 mL phosphate buffer solution (0.1 M, pH 7.4). In case of degradation by lipase, the films were immersed in phosphate buffer solution containing 0.05 mg *Pseudomonas cepacia*/mL phosphate buffer solution. The buffer-enzymatic solution was changed every 24 h to maintain the enzymatic activity. The Cover Slips were picked up over predetermined time intervals, washed with distilled water and dried under vacuum. The weights of the Microscope Cover Slips were measured before film casting and the weight of the Cover Slips with polymer was measured before and after degradation studies. The morphology of the films before and after degradation were characterized with a Gemini 1530 (Carl Zeiss AG, Oberkochem, Germany) scanning electron microscope operating at an accelerating voltage of 0.35 kV.

Figure B.1. Presents the degradation curves of PGlCL (A) and PGlCL-NAC (B) in a Gl/CL ratio of 10/90. As expected, degradation in the presence of a lipase have a significant effect on polymers degradation, in contrast to a slower hydrolytic degradation. For all cases, the highest weight loss were obtained during enzymatic degradation, since *Pseudomonas cepacia* lipase is able to catalyze the cleavage of ester bonds (QIN et al., 2016), resulting in the degradation and weight loss of the polymer. Comparing PGlCL and PGlCL-NAC, the functionalization with NAC was not enough to cause important effects in the degradation in buffer, both PGlCL and PGlCL-NAC reaching around 20% of weight

loss after 960 h. However, for degradation in enzymes, the functionalization with NAC resulted in a increase in degradation rate. After 480h of degradation polymer reached 55% of weight loss. For PGlCL non-functionalized, after 480h of degradation in the presence of enzymes, the weight loss was 28%, which means there was an increase of around 97% in the weight loss caused by enzymatic degradation, after functionalization with NAC.

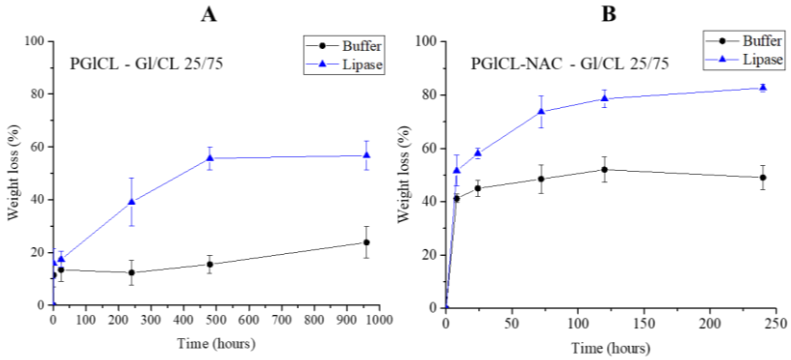
Figure B.1 - (A) Degradation curves of PGlCL (Gl/CL = 10/90) in buffer (circles) and in lipase solution (triangles); (B) Degradation curves of PGlCL-NAC (Gl/CL = 10/90) in buffer (circles) and in lipase solution (triangles).



On Figure B.2., for PGlCL with a Gl/CL ratio of 25/75, the effect of the functionalization with NAC in the weight loss caused by degradation is even more evident. This result is expected, since in this case, the degree of functionalization with NAC was higher (item 4.3.1.). In this case, it is possible even to observe an increase in the weight loss for degradation in buffer, that reached 50% after 240 h, while for non-functionalized PGlCL, only 24% was reached after 960 h.

As it was discussed in Chapter 4, the incorporation of NAC molecules covalently bonded to PGlCL increased the affinity of the polymeric material for water and reduced its degree of crystallinity, making it easier for the molecules of water to access the polymer chains and cleavage ester bonds, promoting degradation.

Figure B.2 - (A) Degradation curves of PGlCL (Gl/CL = 25/75) in buffer (circles) and in lipase solution (triangles); (B) Degradation curves of PGlCL-NAC (Gl/CL = 25/75) in buffer (circles) and in lipase solution (triangles).



Scanning electronic microscopy (SEM) images are shown in Figure B.3 and Figure B.4 and corroborate with the weight loss results. For both PGlCL and PGlCL-NAC it is possible to see clearly the difference in the film morphology after degradation in lipase, compared with time zero and with degradation in buffer, where the presence of the enzymes damaged more intensively the surface of the films. For PGlCL-NAC films, it is possible to observe that after 240 h in the presence of lipase, the film was almost completely degraded, being consistent with weight loss values.

It is also possible to notice that morphology of PGlCL film becomes completely different after functionalization with NAC (time zero images). PGlCL-NAC films present much more irregularities in the surface, and it is possible that this irregularities are caused by the folding of the polymer chains for the establishment of separate interactions between polar groups (coupled NAC molecules) and hydrophobic chains (PGlCL chains). The presence of this irregularities probably also facilitate the degradation of the functionalized material, since it caused an increase of the surface area accessible to the molecules of water and enzymes.

Figure B.3 - Scanning electronic microscopy images of PGlCL in a Gl/CL ratio of 25/75 before and after degradation in lipase and buffer

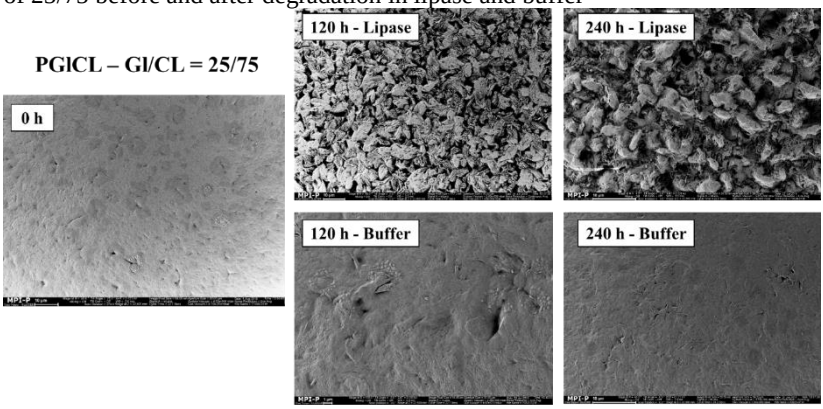
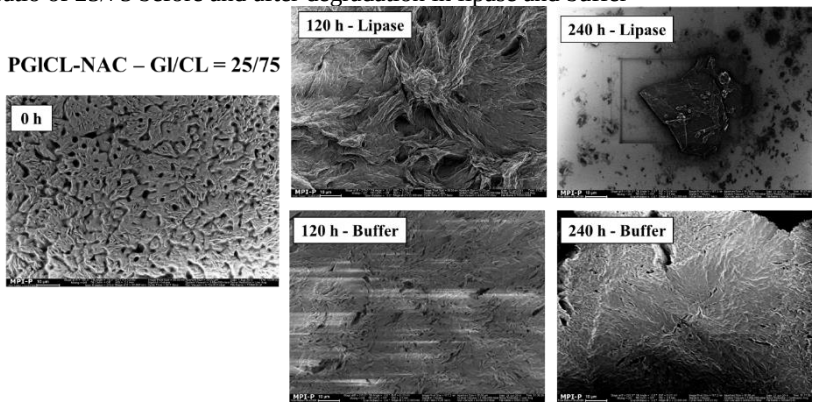


Figure B.4 - Scanning electronic microscopy images of PGlCL-NAC in a Gl/CL ratio of 25/75 before and after degradation in lipase and buffer



B2. BIODEGRADABILITY IN ACTIVATED SLUDGE

In this assay, it was tested PGlCL-NAC (Gl/CL = 25/75) biodegradability in activated sludge, using the method based on Organisation for Economic Cooperation and Development (OECD) guideline 301F (OECD, 1992). In summary, the biological oxygen demand (BOD) for each chemical was measured using the OxiTop control manometric closed system (WTW, Germany) over 28 days. The percentage of biodegradability was determined comparing the measured

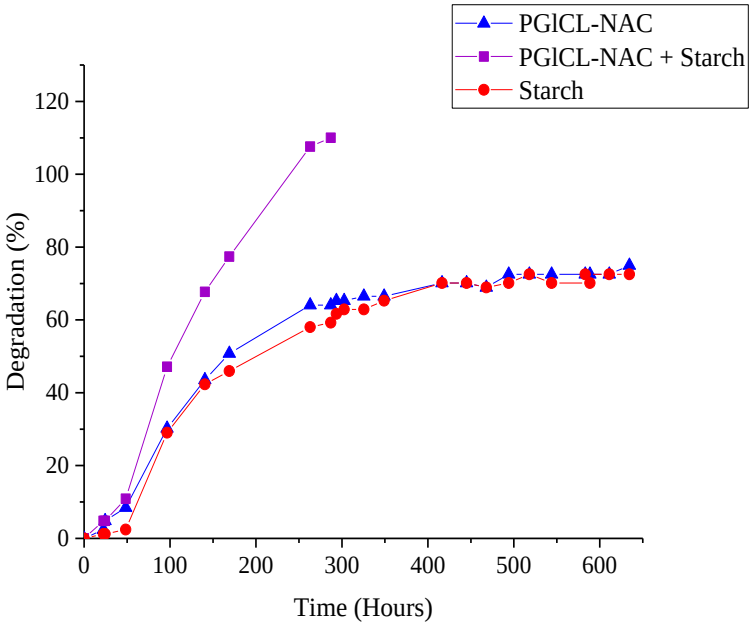
BOD and the calculated theoretical oxygen demand (ThOD) values. In a typical run, flasks containing the following compositions were used: (1) Two flasks for the inoculum "blanks", containing activated sludge and mineral medium with nutrients; (2) Two flasks for procedure control, containing the activated sludge, a reference compound readily biodegradable (in this case it was used starch) and mineral medium; (3) Two flasks for toxicity control, containing the inoculum, PGlCL-NAC, starch and mineral medium; (4) Two flasks for evaluating the biodegradability of the tested material, containing activated sludge, PGlCL-NAC and mineral medium.

The activated sludge sample used in all studies was from the same batch of collected at the waste treatment unit in the city of Mainz, Germany and it was aerated in the dark at 20 °C for 7 days before the start of the experiments. Activated sludge (2.8 mL) with a solid content of 3.9 g/L was mixed to 365 mL of mineral medium in amber bottles, giving a final concentration of approximately 30 mg/L, as described by the OECD procedure. It was used 24.16 mg of starch and 13 mg of PGlCL-NAC per bottle. Magnetic stirrer bars were also added. The screw-top measuring heads, containing sodium hydroxide pellets to adsorb produced carbon dioxide, were replaced. The flasks were stirred in an incubator cabinet in the dark at 20 °C. Oxygen consumption data via measurement of pressure loss were recorded over a 28 days period. At this time, the ThOD of each polymer was calculated as described in the OECD 301 guidelines.

Blank oxygen consumption values (BOD values representing background respiration in activated sludge) were deducted from the BOD of the test compound prior to determining the percentage biodegradability.

Figure B.5. present the degradation curves of PGlCL-NAC, PGlCL in the presence of starch as a toxicity control, and pure starch as a positive control. Starch is a readily biodegradable molecule, and the results obtained also showed this behavior, since in 28 days, it reached almost 75% of degradation. The mixture PGlCL-NAC + starch was used as a toxicity control, and the results clearly show that PGlCL-NAC did not present toxicity to the bacteria present in the activated sludge, since the mixture presented high degradation by the bacteria. PGlCL-NAC presented a degradation curve very similar to starch, also reaching almost 75% of degradation. This result shows that PGlCL-NAC is highly biodegradable and have a strong potential of application not only in the biomedical area, but also for the manufacturing of *green* biodegradable packages, for example.

Figure B.5 - Degradation curves obtained by the OECD biodegradability assays in activated sludge for PGlCL-NAC (triangles), PGlCL-NAC + Starch (squares) and Starch (circles).



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