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NOVEL BIODEGRADABLE MACROMONOMERS FOR BIOMEDICAL APPLICATIONS

Relatore: Prof. Davide MOSCATELLI

Co-relatore: Ing. Claudio COLOMBO

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Federica FERRARI Matr. 800786

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Sommario

L'efficacia delle formulazioni studiate per la somministrazione di farmaci tramite sistemi nanotecnologici (Drug Delivery Devices) si misura nella possibilità di distribuire in modo specifico dosi elevate di medicinali, riducendo così gli effetti collaterali delle terapie. Particolare attenzione viene posta nella sintesi di Nanoparticelle polimeriche, le quali presentano caratteristiche interessanti quali: biodegradabilità e biocompatibilità, rilascio controllato del farmaco, condizioni di sintesi non severe.
Queste sono in grado di risolvere i problemi di solubilità di farmaci idrofobici, grazie alle interazioni con la struttura polimerica di base. Posseggono inoltre vantaggi quali: elevata stabilità, efficace carico del farmaco, rilascio continuo degli agenti terapeutici.
Il progetto di Tesi presentato propone la sintesi di macromonomeri per nanoparticelle a base di poliesteri, adatti ad applicazioni biomedicali. Loro principale caratteristica è intesa essere la terminazione $–CH_3$, il cui scopo è l'aumento del carattere idrofobo del macromonomero. I gruppi metilici facilitano inoltre l'addizione di agenti di targeting rispetto a punti specifici della superficie delle particelle, migliorando così le proprietà di ritenzione nel corpo umano.
Macromonomeri con lunghezze di catena differenti sono stati polimerizzati secondo il metodo della polimerizzazione in emulsione, sia in condizioni batch che semibatch, con lo scopo di produrre particelle con caratteristiche dimensionali costanti. Le proprietà dei materiali sintetizzati sono state infine verificate con studi di Degrado e Stabilità.

Parole chiave: Drug Delivery, Nanoparticelle polimeriche, biocompatibilità, idrofobicità, macromonomeri a base di poliesteri, polimerizzazione in emulsione, studi di Degrado, studi di Stabilità.

Abstract

An efficient Drug Delivery Device can ideally provide for high dose of the therapeutic agent specifically to the diseased cells, thus minimizing side effects. Among the different classes polymeric nanoparticles (NPs) deserve special attention thanks to several attractive properties, including: biodegradability and biocompatibility, controlled release of payload, no severe synthesis conditions.
Polymeric NPs are able to dissolve hydrophobic drugs in polymeric matrix, solving in this way drug solubility problems as well as possess advantages such as: high stability, efficient drug load, sustained drug release.
The Work of Thesis presented aims at obtaining polyester-based macromonomers for NPs synthesis, suitable for biomedical applications.

Macromonomers main feature is intended to be a CH_3- termination, whose purpose lays in increasing their hydrophobicity. Methyl groups facilitate also the addition of targeting agents on specific places on NPs surfaces and enhance retention properties in human body. Macromonomers with different chain lengths were polymerized through free radical polymerization, in both batch and semibatch emulsion polymerization to produce nanoparticles (NPs) with narrow particle size distribution. As final step of the Thesis, material properties were verified with Degradation and Stability studies.

Keywords: Drug Delivery, Polymeric Nanoparticles, biocompatibility, hydrophobicity, polyester-based macromonomers, emulsion polymerization, Degradation studies, Stability studies.

1. Introduction

The Work of Thesis presented below aims at obtaining polyester-based macromonomers suitable for biomedical applications. Macromonomers main feature is intended to be a CH_3- termination, whose purpose lays in increasing their hydrophobicity. Methyl groups facilitate also the addition of targeting agents on specific places on NPs surfaces and enhance retention properties in human body. In the following Chapter the state of art and an introduction to the role of NPs as biomedical vehicles are given.

1.1 Nanomedicine

European Medicine Agency (EMA) defines Nanotechnology as "production and application of structures, devices and systems by controlling the shape and the size of materials at nanometer scale. Nanometer scale ranges from the atomic level, round 0.2 nm up to around 100 nm."

Nanomedicine is defined as application of nanotechnology in view of a medical diagnosis for treating or preventing diseases. It exploits the physical, chemical and biological properties of materials at nanometer scale. Current issues involve understanding the problems related to toxicity and environmental impact of the materials applied, **[1]** and **[2].** Functionalities can be added to nanomaterials by interfacing them with biological molecules or structures. The size of nanomaterials is similar to that of most biological molecules and structures; therefore, nanomaterials can be useful for both in vivo and in vitro biomedical research and applications. Up to now the integration of nanomaterials with biology has led to the development of diagnostic devices, contrast

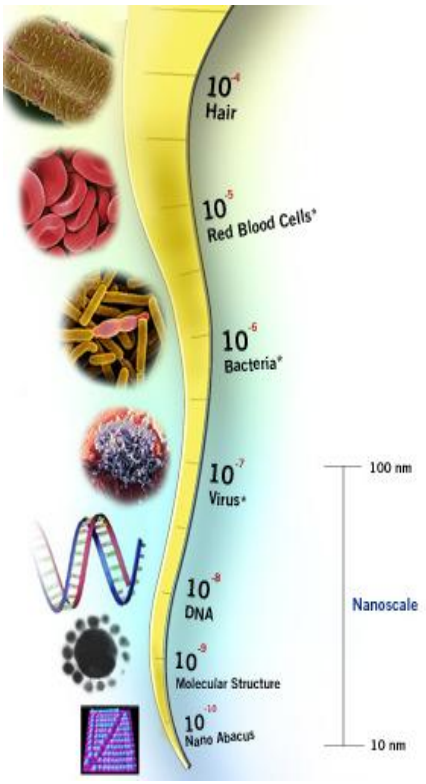


Figure 1.1- *Nanometer scale*

agents, analytical tools, physical therapy applications, and drug delivery vehicles.

Economically speaking Nanomedicine is a large industry, with nanomedicine sales reaching $6.8 billion in 2004, and with over 200 companies worldwide. Moreover a minimum of $3.8 billion in nanotechnology R&D is being invested every year. As this industry keep on growing, it is expected to have a significant impact on the economy. **[3]**

Why does nanomedicine represent such a huge promise for Health Care?

- Conventional medicine is reactive to tissue-level problems that are happening at the symptomatic level. Nanomedicine will diagnose and treat problems at the molecular level inside single cells, prior to traditional symptoms.
- Nanomedicine will be much more preventive than conventional medicine and comparatively inexpensive. Furthermore it could be more readily mass produced and distributed.

1.2 Drug delivery

A drug delivery system (DDS) is outlined as a formulation or a device that enables the introduction of a therapeutic substance in the body and improves its efficacy and safety by controlling the rate, time, and place of release of drugs in the body**. [3]**

This process includes the administration of the therapeutic product, the release of the active components and the subsequent transport of the active ingredients across the biological membranes to the site of action.

Drug delivery system represents an interface between the patient and the drug.[3]
Drugs may be introduced into the human body by various anatomical routes. They may be intended for systemic effects or specifically targeted. The choice of the route of administration depends on

- the disease
- the desired effect
- and availability of the product

A classification of various methods of systemic drug delivery by anatomical routes can be given as follows (see **Table 1.1**) :

Anatomical routes
Oral
Parenteral • Subcutaneous injection • Intramuscular injection • Intravenous injection • Intra-arterial injection
Transmucosal
Transnasal
Pulmonary (by inhalation)
Transdermal drug delivery
Intraosseous infusion

Table 1.1- *Anatomical routes for Drug delivery.*

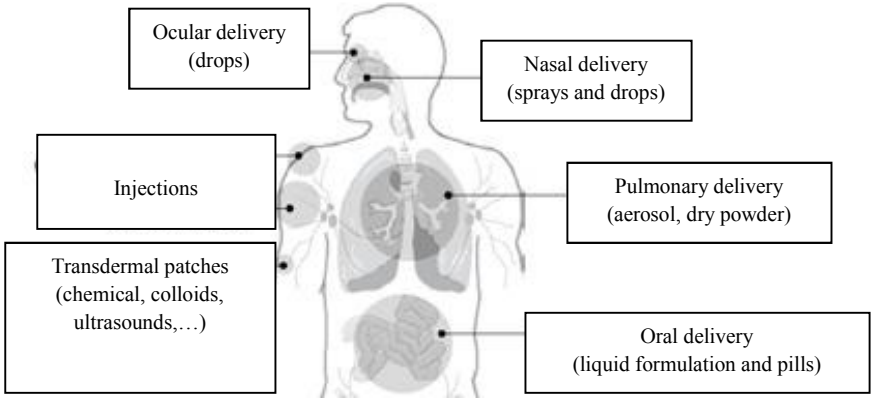


Figure 1.2- *Anatomical routes for Drug delivery.*

Nanotechnology has provided the possibility of delivering drugs to specific cells using Nanoparticles.
The overall drug consumption and side-effects may be lowered significantly by depositing the active agent in the desired region only and in no higher dose than needed. This highly selective approach would reduce costs and human suffering as well.

Targeted drug delivery is intended to reduce the side effects of drugs with concomitant decreases in consumption and treatment expenses.

Drug delivery systems, lipid- or polymer-based nanoparticles can be designed to improve the pharmacological and therapeutic properties of drugs. The strength of drug delivery systems lies in their ability to alter the pharmacokinetics and biodistribution of drugs. However, the pharmacokinetics and pharmacodynamics mechanisms are highly variable. Complex drug delivery mechanisms are being developed indeed, including the ability to get drugs through cell membranes and into cell cytoplasm. Efficiency is important because many diseases depend upon processes within the cell and can only be impeded by drugs that make their way into the cell.

1.2.1 Oral Drug Delivery

Oral route has been the one used the most both because of the ease of administration and the acceptance by patients. However this route shows some limits, as mentioned in **[3]**:

- Drugs taken orally have variable absorption rates for systemic effects and variable serum concentrations which may be unpredictable. This has

led to the development of sustained release and controlled-release systems.

- The high acid content and ubiquitous digestive enzymes of the digestive tract degrade some drugs well before they reach the site of absorption into the bloodstream. This is a particular problem for ingested proteins.
- Many macromolecules and polar compounds cannot effectively traverse the cells of the epithelial membrane in the small intestines to reach the bloodstream.
- Many drugs become insoluble at the low pH levels encountered in the digestive tract. Since only the soluble form of the drug can be absorbed into the bloodstream, the transition of the drug to the insoluble form can significantly reduce bioavailability.
- The drug may be inactivated in the liver on its way to the systemic circulation.
- Some drugs irritate the gastrointestinal tract and this is partially counteracted by coating.
- Oral route may not be suitable for drugs targeted to specific organs.

1.2.2 Parenteral Drug Delivery

Parenteral literally means "introduction of substances into the body by routes other than the gastrointestinal tract" but practically the term is applied to injection of substances by subcutaneous, intramuscular, intravenous, and intra-arterial routes.

Parenteral administration of the drugs is now an established part of medical practice and is the most commonly used invasive method of drug delivery.

Advantages of parenteral administration are as follows:

- Rapid onset of action.
- Predictable and almost complete bioavailability.
- Avoidance of the gastrointestinal tract with problems of oral drug administration.
- Provides a reliable route for drug administration in very ill and comatose patients, who are not able to ingest anything orally.

Major drawbacks of parenteral administration are as follows:

- Injection is not an ideal method of delivery because of pain involved and patient compliance becomes a major problem.
- Injections have limitations for the delivery of protein products, particularly those that require sustained levels.

Injections can be defined as:

1.2.2.1 Subcutaneous

This involves the introduction of the drug to a layer of subcutaneous fatty tissue by the use of a hypodermic needle. Large portions of the body are available for subcutaneous injection, which can be given by the patients themselves as in the case of insulin for diabetes. **Various factors that influence drug delivery by subcutaneous route are as follows:**

- Size of the molecules, as larger molecules have slower penetration rates than do smaller ones.
- Viscosity may impede the diffusion of drugs into body fluids.
- The anatomical characteristics of the site of injection, such as vascularity and amount of fatty tissue, influence the rate of absorption of the drug.

Subcutaneous injections usually have a lower rate of absorption and slower onset of action than intramuscular or intravenous injections.

Disadvantages of subcutaneous injection are as follows:

- The rate of absorption is difficult to control from the subcutaneous deposit.
- Local complications, which include irritation and pain at site of injection.
- Injection sites have to be changed frequently to avoid accumulation of the unabsorbed drug, which may cause tissue damage.

1.2.2.2 Intramuscular injections

These are given deep into skeletal muscles. **The onset of action after intramuscular injection is faster than with subcutaneous injection but slower than with intravenous injection.**

The absorption of the drug is diffusion controlled but it is faster because of high vascularity of the muscle tissue. Rate of absorption varies according to physicochemical properties of the solution injected and physiological variables such as blood circulation of the muscle and the state of muscular activity. Disadvantages of intramuscular route for drug delivery are as follows:

- Pain at the injection site.
- Limitation of the amount injected according to the mass of the muscle available.
- Complications include peripheral nerve injury and formation of hematoma and abscess at the site of injection.
- Inadvertent puncture of a blood vessel during injection may introduce the drug directly into the blood circulation.

Most injectable products can be given intramuscularly. Numerous dosage forms are available for this route: oil in water emulsions, colloidal suspensions, and reconstituted powders. The product form in which the drug is not fully dissolved generally results in slower, more gradual absorption and slower onset of action with longer lasting effects.

1.2.2.3 Intravenous administration

This involves injection in the aqueous form into a superficial vein or continuous infusion via a needle or a catheter placed in a superficial or deep vein. This is the only method of administration available for some drugs and is chosen in emergency situations because the onset of action is rapid following the injection. Theoretically, none of the drug is lost, and smaller doses are required than with other routes of administration.

The particles in the intravenous solution are distributed to various organs depending on the particle size. Particles larger than 7 μm are trapped in the lungs and those smaller than 0.1 μm accumulate in the bone marrow. Those with diameter between 0.1 and 7 μm are taken up by the liver and the spleen. This information is useful in targeting of a drug to various organs.

Disadvantages of the intravenous route are as follows:

- Immune reactions may occur following injections of proteins and peptides.

- Trauma to veins can lead to thrombophlebitis.
- Extravasation of the drug solution into the extravascular space may lead to irritation and tissue necrosis.
- Infections may occur at the site of catheter introduction.
- Air embolism may occur because of air sucked in via the intravenous line.

1.2.2.4 Intra-arterial

Direct injection into the arteries is not a usual route for therapeutic drug administration. Arterial puncture and injection of contrast material has been carried out for angiography. Most of the intra-arterial injections or arterial perfusions via catheters placed in arteries are for regional chemotherapy of some organs and limbs. Intra-arterial chemotherapy has been used for malignant tumors of the brain **[3]**.

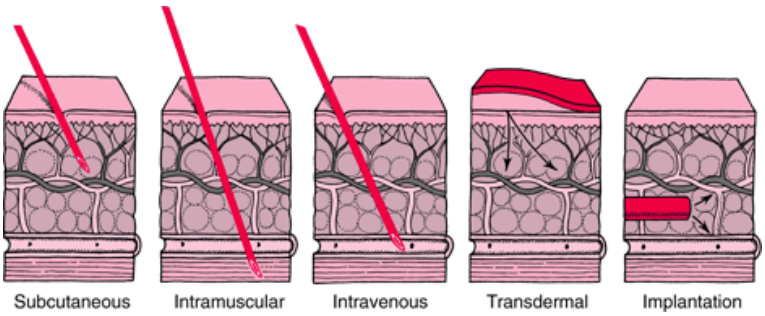


Figure 1.3- *Injections : Subcutaneous, Intramuscula, Intravenous, Transdermal, Implantation*

1.2.3 Transdermal Drug Delivery

Transdermal drug delivery is an approach used to deliver drugs through the skin for therapeutic use as an alternative to oral, intravascular, subcutaneous, and transmucosal routes. It includes the following categories of drug administration:

- Local application formulations, e.g., transdermal gel
- Transdermal patches
- Transdermal electrotransport
- Use of physical modalities to facilitate transdermal drug transport

- Minimally invasive methods of transdermal drug delivery, e.g., needle-free injections

1.2.4 Transmucosal Drug Delivery

Movement of drugs across the mucous membranes is by diffusion. At steady state, the amount of a substance crossing the tissue per unit of time is constant and the permeability coefficients are not influenced by the concentration of the solutions or the direction of nonelectrolyte transfer. As in the epidermis of the skin, the pathways of permeation through the epithelial barriers are intercellular rather than intracellular. The permeability can be enhanced by the use surfactants.

Delivery of biopharmaceuticals across mucosal surfaces may offer several advantages over injection techniques, which include the following:

- Avoidance of an injection
- Increase of therapeutic efficiency
- Possibility of administering peptides
- Rapid absorption when compared with oral administration
- Bypassing first pass metabolism by the liver
- Higher patient acceptance when compared with injectables
- Lower cost when compared with injectables

1.2.5 Nasal Drug Delivery

Drugs are administered nasally for topical and systemic effect. Topical administration includes agents for the treatment of nasal congestion, rhinitis, sinusitis, and related allergic and other chronic conditions. Various medications include corticosteroids, antihistaminics, anticholinergics, and vasoconstrictors. The focus in recent years has been on the use of nasal route for systemic drug delivery.

Advantages of nasal drug delivery:

- Highly vascularized subepithelial tissue
- Rapid absorption, usually within half an hour
- Avoidance of first pass effect that occurs after absorption of drugs from the gastrointestinal tract

- Avoidance of the effects of gastric stasis and vomiting, for example, in migraine patients
- Ease of administration by the patients, who are usually familiar with nasal drops and sprays
- Higher bioavailability of the drugs than in the case of gastrointestinal route or pulmonary route
- Most feasible route for the delivery of peptides

Disadvantages of nasal drug delivery:

- Diseases conditions of the nose may result in impaired absorption.
- Dose is limited because of relatively small area available for absorption.
- Time available for absorption is limited.

After a consideration of advantages as well as disadvantages, nasal drug delivery turns out to be a promising route of delivery and competes with pulmonary drug, which is also showing great potential. One of the important points is the almost complete bioavailability and precision of dosage.

1.2.6 Pulmonary Drug Delivery

Interest in this approach has been further stimulated by the demonstration of potential utility of lung as a portal for entry of peptides and the feasibility of gene therapy for cystic fibrosis. It is important to understand the mechanism of macromolecule absorption by the lungs for an effective use of this route.

1.2.6.1 Mechanisms of Macromolecule Absorption by the Lungs

The lung takes inhaled breaths of air and distributes them deep into the tissue to a very large surface, known as the alveolar epithelium, which is approximately 100 m2 in adults. This very large surface has approximately a half billion tiny air sacs known as alveoli, which are enveloped by an equally large capillary network.
The delivery of inhaled air to the alveoli is facilitated by the airways, which start with the single trachea and branch several times to reach the grape-like clusters of tiny alveoli. The alveolar volume is 4,000–6,000 ml when compared to the airway volume of 400 ml, thus providing a greater area for absorption for the inhaled substances. Large molecule drugs, such as peptides and proteins, do not easily pass through the airway surface because it is lined with a thick, ciliated mucus-covered cell layer making it nearly impermeable. The alveoli, on the

other hand, have a thin single cellular layer enabling absorption into the bloodstream.

Some barriers to the absorption of substances in the alveoli are as follows:

- Surfactant, a thin layer at the air/water interface, may trap the large molecules.
- A molecule must traverse the surface lining fluid which is a reservoir for the surfactant and contains many components of the plasma as well as mucous.
- The single layer of epithelial cells is the most significant barrier.
- The extracellular space inside the tissues and the basement membrane to which the epithelial cells are attached.
- The vascular endothelium, which is the final barrier to systemic absorption, is more permeable to macromolecules than is the pulmonary epithelium.

Although the mechanism of absorption of macromolecules by the lungs is still poorly understood, the following mechanisms are considered to play a part:

- Transcytosis (passage through the cells). This may occur and may be receptor mediated but it is not very significant for macromolecules > 40 kDa.
- Paracellular absorption. This is usually thought to occur through the junctional complex between two cells. The evidence for this route of absorption is not very convincing in case of the lungs. Molecules smaller than 40 kDa may enter via the junctional pores.

Once past the epithelial barrier, the entry of macromolecules into the blood is easier to predict. Direct absorption may also occur across the tight junctions of capillary endothelium.

A notable illustration of pulmonary drug delivery is given in **[4]** (see **Figure 1.4**):

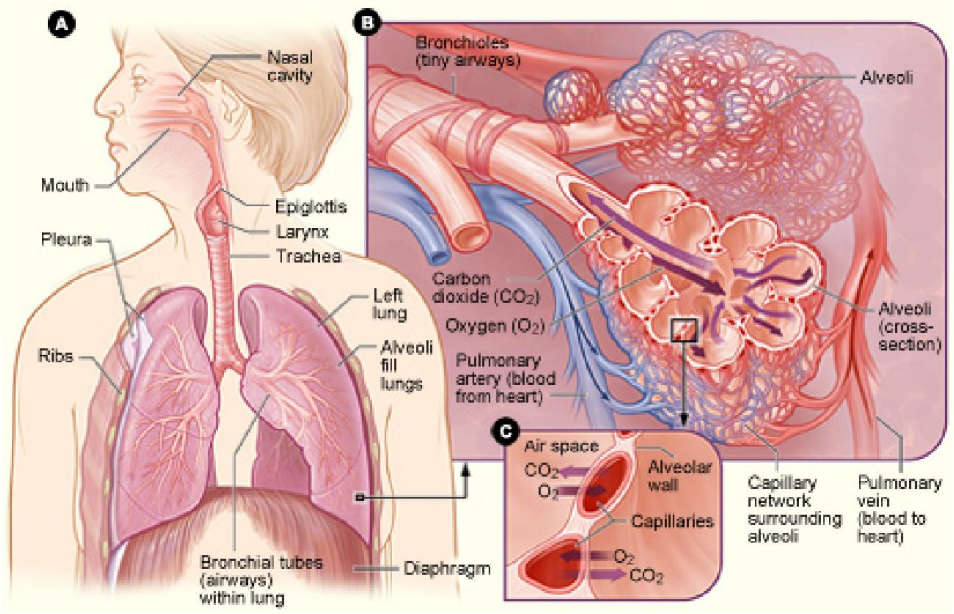


Figure 1.4- *Pulmonary Drug delivery system at different dimensional scales.*

Advantages of lungs for drug delivery are as follows:

- Large surface area available for absorption.
- Close proximity to blood flow.
- Avoidance of first pass hepatic metabolism.
- Smaller doses are required than by the oral route to achieve equivalent therapeutic effects.

Disadvantages of pulmonary drug delivery are listed as:

- The lungs have an efficient aerodynamic filter, which must be overcome for effective drug deposition to occur.
- The mucous lining the pulmonary airways clears the deposited particles toward the throat.
- Only 10–40% of the drug leaving an inhalation device is usually deposited in the lungs by using conventional devices

1.2.7 Cardiovascular Drug Delivery

Drug delivery to the cardiovascular system is different from delivery to other systems because of the anatomy and physiology of the vascular system; it supplies blood and nutrients to all organs of the body. Drugs can be introduced

into the vascular system for systemic effects or targeted to an organ via the regional blood supply.
Drug delivery to the cardiovascular system is not simply formulation of drugs into controlled release preparation but it includes delivery of innovative therapeutics to the heart. Details of cardiovascular drug delivery are described elsewhere **[5].**
Methods for local administration of drugs to the cardiovascular system include the following:

- Drug delivery into the myocardium: direct intramyocardial injection
- Drug delivery via coronary venous system
- Injection into coronary arteries via cardiac catheter
- Intrapericardial drug delivery
- Release of drugs into arterial lumen from drug-eluting stents

Examples of cardiac drug delivery have been represented in the **Figure 1.5 [6]**:

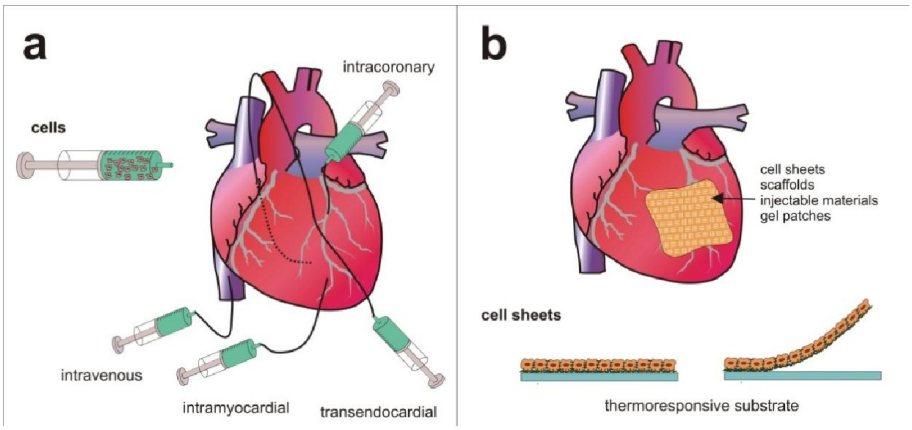


Figure 1.5- *Cardiac Drug delivery examples: a) Intracoronary; b) Interaction is promoted by cell sheets scaffolds*

1.2.8 Drug Delivery to the Central Nervous System

The delivery of drugs to the brain is a challenge in the treatment of central nervous system (CNS) disorders. The major obstruction to CNS drug delivery is the blood-brain barrier (BBB) **[3]** and **[7]**, which limits the access of drugs to the brain substance (**Figure 1.6**).
The blood-brain barrier (BBB) is a dynamic interface that separates the brain from the circulatory system and protects the central nervous system from

potentially harmful chemicals while regulating transport of essential molecules and maintaining a stable environment**. [8]**

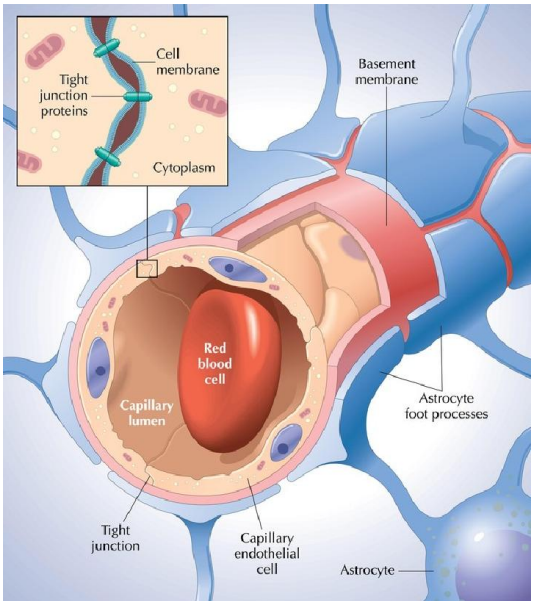


Figure 1.6- *Blood-brain barrier, limits the access of drugs to the brain substance*

Most CNS-disorder research is directed toward the discovery of drugs and formulations for controlled release; little attention has been paid to the method of delivery of these drugs to the brain. Various methods of delivering drugs to the CNS are shown in **Table 1.2**:

Various methods of drug delivery to the central nervous system (CNS)
Systemic administration of therapeutic substances for CNS action
Intravenous injection for targeted action in the CNS
Direct administration of therapeutic substances to the CNS
Introduction into cerebrospinal fluid pathways: intraventricular, subarachnoid pathways
Introduction into the cerebral arterial circulation
Introduction into the brain substance
Direct positive pressure infusion
Drug delivery by manipulation of the blood-brain barrier
Drug delivery using novel formulations
Gels
Liposomes
Microspheres
Nanoparticles
Chemical delivery systems
Drug delivery devices
Pumps
Catheters
Implants releasing drugs
Use of microorganisms for drug delivery to the brain
Bacteriophages for brain penetration
Bacterial vectors
Cell therapy
CNS implants of live cells secreting therapeutic substances
CNS implants of encapsulated genetically engineered cells producing therapeutic substances
Cells for facilitating crossing of the blood-brain barrier
Gene transfer
Direct injection into the brain substance
Intranasal instillation for introduction into the brain along the olfactory tract
Targeting of CNS by retrograde axonal transport

Table 1.2- *Drug Delivery to the CNS*

1.2.9 Drug Formulations

There is constant evolution of the methods of delivery, which involves modifications of conventional methods and discovery of new devices. A classification of technologies that affect the release and availability of drugs is shown in the following **Table 1.3**:

Issue	Oral	Intravenous	Intramuscular/ subcutaneous	Transnasal	Transdermal	Pulmonary
Delivery to blood circulation	Indirect through GI tract	Direct	Indirect absorption from tissues	Indirect	Indirect	Indirect
Onset of action	Slow	Rapid	Moderate to rapid	Rapid	Moderate to rapid	Rapid
Bioavailability	Low to high	High	High	Moderate	Low	Moderate to high
Dose control	Moderate	Good	Moderate	Moderate to good	Poor	Moderate to good
Administration	Self	Health professional	Self or health professional	Self	Self	Self
Patient convenience	High	Low	Low	High	Moderate	High
Adverse effects	GI upset	Acute reactions	Acute reactions	Insignificant	Skin irritation	Insignificant

GI = gastrointestinal

Table 1.3- *Release and availability of drugs*

1.2.10 Sustained Release

Sustained release (SR) preparations are not new but several new modifications are being introduced. They are also referred to as "long acting" or "delayed release" when compared to "rapid" or "conventional" release preparations. The term sometimes overlaps with "controlled release," which implies more sophisticated control of release and not just confined to the time dimension. Controlled release implies consistency, but release of drug in SR preparations may not be consistent. The following are the rationale of developing SR:

- To extend the duration of action of the drug
- To reduce the frequency of dosing
- To minimize the fluctuations in plasma level
- Improved drug utilization
- Less adverse effects

- Limitations of SR products are as follows:
- Increase of drug cost.
- Variation in the drug level profile with food intake and from one subject to another.
- The optimal release form is not always defined, and multiplicity of SR forms may confuse the physician as well as the patient.
- SR is achieved by either chemical modification of the drug or modifying the delivery system, e.g., use of a special coating to delay diffusion of the drug from the system. Chemical modification of drugs may alter such properties as distribution, pharmacokinetics, solubility, or antigenicity. One example of this is attachment of polymers to the drugs to lengthen their lifetime by preventing cells and enzymes from attacking the drug.

1.2.11 Controlled Release

Controlled release implies regulation of the delivery of a drug usually by a device. The control is aimed at delivering the drug at a specific rate for a definite period of time independent of the local environments. The periods of delivery are usually much longer than in case of SR and vary from days to years. Controlled release may also incorporate methods to promote localization of drug at an active site. Site-specific and targeted delivery systems are the descriptive terms used to denote this type of control.

1.2.12 Programming the Release at a Defined Time

Approaches used for achieving programmed or pulsatile release may be physical mechanisms such as swelling with bursting or chemical actions such as enzymatic degradation. Capsules have been designed that burst after a predetermined exposure to an aqueous environment. Physical factors that can be controlled are the radius of the sphere, osmotic pressure of the contents, and wall thickness as well as elasticity.

1.2.13 Ideal Properties of Material for Drug Delivery

Properties of an ideal macromolecular drug delivery or biomedical vector are as:

- Structural control over size and shape of drug or imaging-agent cargo-space.
- Biocompatible, nontoxic polymer/pendant functionality.
- Precise, nanoscale-container and/or scaffolding properties with high drug or imaging-agent capacity features.

- Well-defined scaffolding and/or surface modifiable functionality for cell-specific targeting moieties.
- Lack of immunogenicity.
- Appropriate cellular adhesion, endocytosis, and intracellular trafficking to allow therapeutic delivery or imaging in the cytoplasm or nucleus.
- Acceptable bioelimination or biodegradation.
- Controlled or triggerable drug release.
- Molecular level isolation and protection of the drug against inactivation during transit to target cells.
- Minimal nonspecific cellular and blood-protein binding properties.
- Ease of consistent, reproducible, clinical-grade synthesis.

1.3 Nanoparticles (NPs)

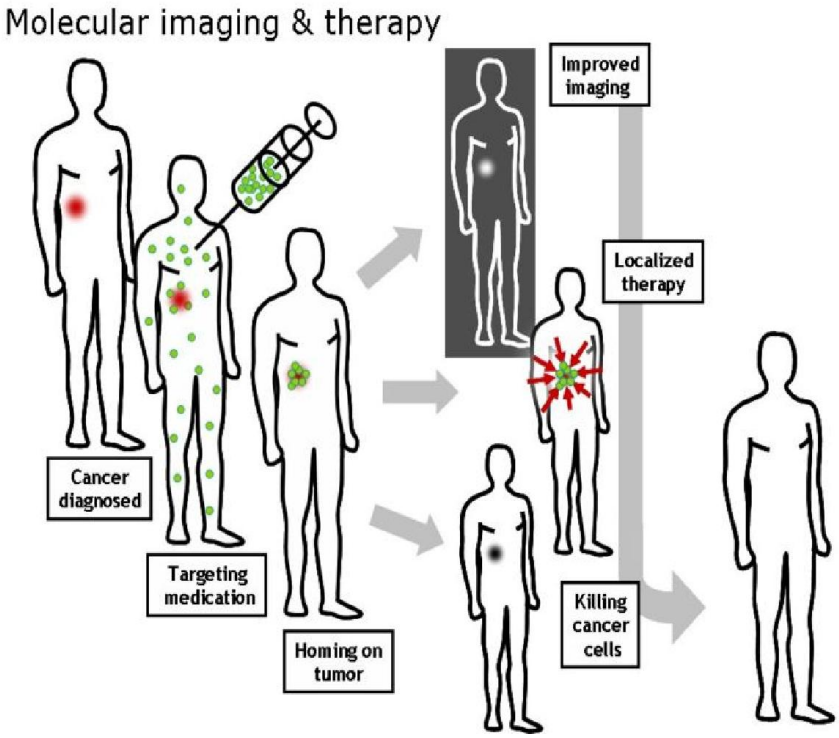


Figure 1.7- *Nanoparticles in prevention, diagnosis and treatment of diseases*

Nanoparticles for medical applications are defined as particles with a size between 1 and 1000 nm **[9],** thereby offering the optimal interaction with cellular entities. Efficacy of NPs as delivery vehicles is highly size-and shape-dependent: they affect the movement in and out the vasculature (**Figure 1.8**) **[10]**.

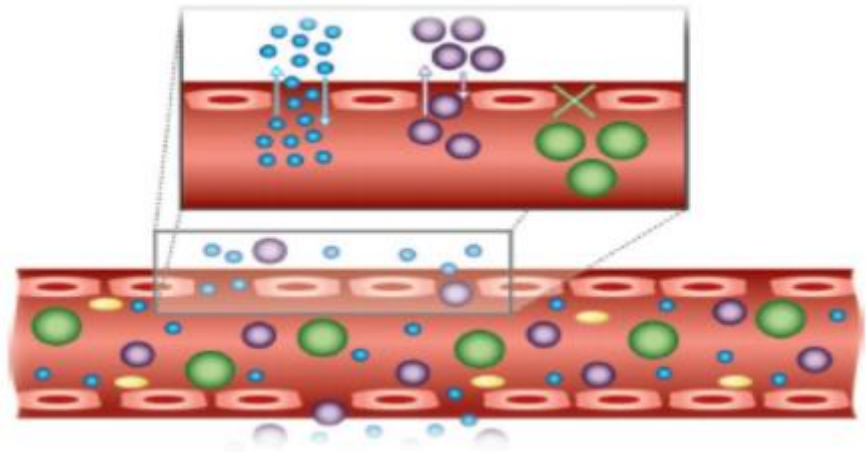


Figure 1.8- Efficacy of NPs as delivery vehicles is highly size- and shape- dependent

These biomimetic features, together with high surface to mass ratio and ease of introducing new properties or modifying already existing ones give NPs the potential to make the difference in prevention, diagnosis and treatment of deseases (**Figure 1.7**).

Nanoparticles are also used to avoid multidrug resistance (MDR) mechanisms. Mechanisms of MDR include decreased uptake of drugs, reduced intracellular drug concentration by activation of the efflux transporters, modifications in cellular pathways by altering cell cycle checkpoints, increased metabolism of drugs, induced emergency response genes to impair apoptotic pathways and altered DNA repair mechanisms **[11].**

- Imaging
- Drug delivery
- Biosensor

Are the principle fields of application in medicine. Examples of nano devices investigated for biomedical purposes are presented in **Figure 1.9 [12]**.

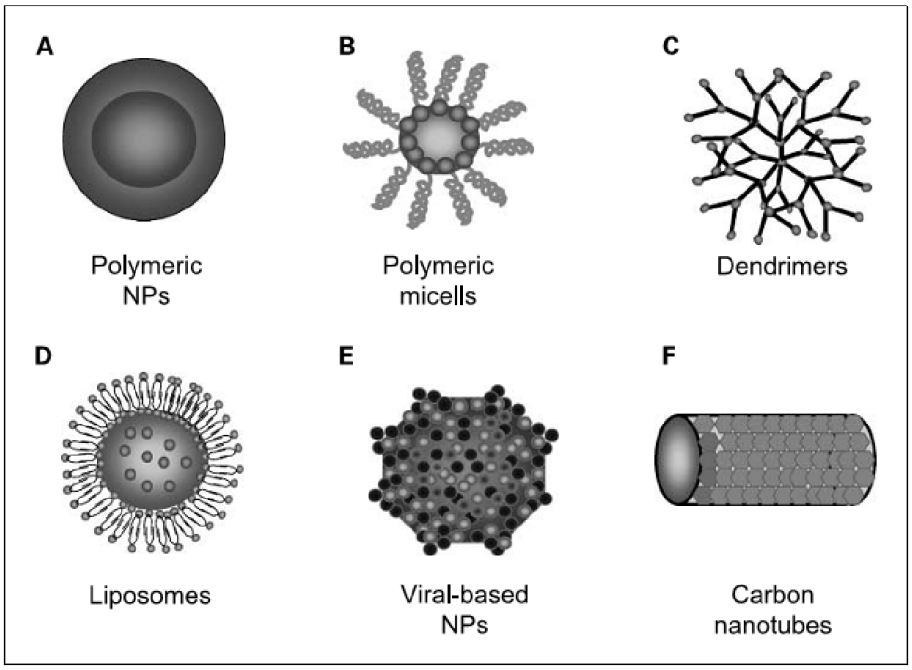


Figure 1.9- *Devices applied for biomedical purposes: A) polymeric NPs, B) polymeric micells, C) Dendrimers, D) Liposomes, E) Viral-based NPs, F) Carbon nanotubes*

Achievable advantages with nanoparticle drug delivery systems can be summerize as:

- Provide targeted delivery
- Improve bioavailability
- Decrease toxic side effects
- Protect the drug from degradation
- Increase the aqueous solubility of the drug
- Reduce total body dose of drugs
- Simultaneously deliver multiple drugs
- Improve crossing of biological barriers
- Produce a prolonged release of drugs
- Offer appropriate form for all routes of administration

1.3.1 Nanoparticle systems as contrast agents in imaging

Nps unique features are being exploited also as contrast agent in biomedical imaging. Contrast agents function to enhance image contrast and improve the quality of the analysis, by making visible what otherwise would't be detected. Ultrasound, magnetic resonance imaging (MRI), X-rays are examples of non-invasive imaging tecniques that can take advantage from the divelopement of NPs as contrast agents.

In this perspective a significant improvement could be linked to the developent of diagnostic procedures to image patogenic processes on a molecular level, **[9]** and **[3]**. Other fields that could benefit are guided surgery, imaging of gene expression, monitoring drug efficacty.

A NP contrast agent exploits several mechanisms, according to which imaging modality they are addressed for. Compared to conventional contrast agents,NP systems offer the possibility of targeting precise sites . **Table 1.4** represents some possible imaging application for nano-scale devices.

Nano-device	Application
Gold nanoparticles	X-ray contrast agent
Magnetic NPs	MRI contrast agent
Quantum dots	Optical labels in fluorescence imaging

Table 1.4- *Possible imaging application for nano-scale devices.*

1.3.2 Multifunctional NPs

On the wave of what has been discovered until now, multifunctional NPs have been proposed: they endow different functionalities such as targeting, monitoring and treating the site of desease. A potential multifunctional NP structure in illustrated in **Figure 1.10**.

However this is strictly connected to additional costs and further synthesys steps , which means also advanced chemical tecniques. Beside in vivo effects increase significantly. The trade-off between additional funcionality and complexity is still on a debate.

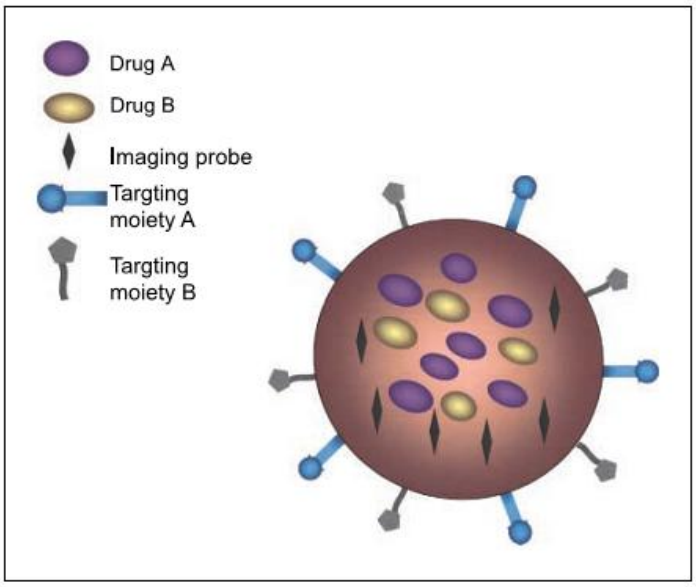


Figure 1.10- *Example of multifunctional NP.*

It is possible to summerize different properties which can be incorporated in a multifuncional NP system (**Table 1.5**) **[12]**:

Property	Function
Therapeutic agent	Released to treat diseased site
Targeting lingand	Recognizes target cells to increase efficiency and reduce toxicity
Imaging agent	Report real-time NP distribution and monitor drug transport so that therapeutic efficacy can be evaluated
Cell-penetrating agent	Facilitate entry of NP systems in cells.

Table 1.5- *Properties of multifunctional NP systems*

1.3.3 PEGylated NPs

Independently from the final function the particles have been studied for, they need to be able to maintain in the body long enough to reach their site of action. An undeniable obstacle to the long term circulation is clearance by the phagocyte system (MPS). Therefore NPs are engineered to avoid such elimination.

PEG is a coiled, hydrophilic polyether compound that can be covalently attached, adsorbed or grafted to the surface of a NP. PEG coating reduces MPS uptake and

increases circulation time when compared to uncoated particles. **[13]** This is manly because PEG has the lowest level of protein or cellular adsorption of any known polymer **[14]** , thus making a NP coated with PEG unrecognizable for the plasma proteins in blood stream.

It has been shown that polyethylene glycol (PEG) chains can reduce the opsonization process, thus preventing recognition by monocytes and macrophages, and allowing a longer circulation time for the NPs **[15]** .
NPs have large payloads

- stability,
- signal enhancement
- capacity for multiple, simultaneous applications owing to their unique size and high surface area: volume ratio **[16].**

While they are bigger than molecules and many proteins, yet smaller than cells, they behave differently to other therapies and imaging agents, affecting their in vivo applications.

For example, as said in **[16]** in cancer tissue, NPs not only extravasate from the leaky tumor vasculature to a higher degree than healthy tissue, but also remain in the area by the enhanced permeability and retention (EPR) effect .

The most acknowledged theory to explain why PEG improves stealth propertis is based on interactions between proteins and the PEGylated surfaces. When blood proteins approach the particles they compress the surface layer of PEG chains. Upon compression, the PEG layer is forced into a higher energy conformation, which will create an opposing force. This force is completely able to balance the attaractive force between the plasma proteins and the particle, thus resulting in less or no attachment of proteins to the surface **[17].**

Peg layer can take two different conformations. If the surface coverage is low, the PEG chains will usually take on a “mushroom” formation, while if high surface is covered, we will have a “brush” configuration. A high surface density will make sure that no gaps in the PEG layer are present, but it will also lead to restriceted mobility, thus reductin steric properties of the PEG layer. For this reason we can assume that the optimal coverage lies somewhere in between the two possible ones. (**Figure 1.11**)

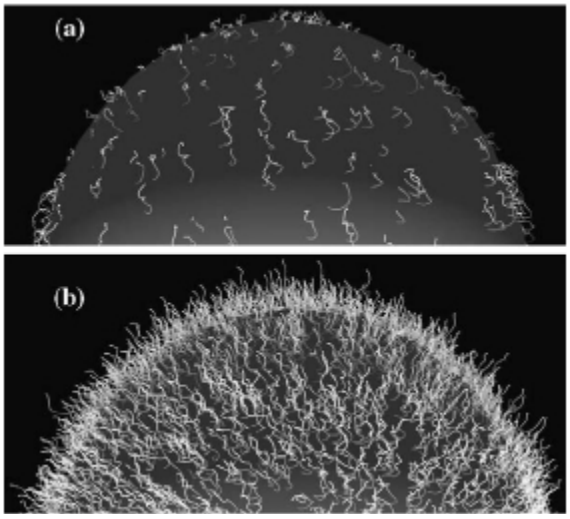


Figure 1.11 – *a) Low PEG density, "Mushroom" conformation b) High PEG density leads to "Brush" conformation.*

Despite these advantages, some fundamental challenges hamper NP deployment to the clinic. These include uptake by the reticuloendothelial system (RES), in which NPs are rapidly shuttled out of circulation to the liver, spleen or bone marrow, and nonspecific binding of NPs to nontargeted or nondiseased areas.
The addition of PEG to the NP surface (PEGylation) can reduce many of these challenges. In both drug-delivery and imaging applications, the addition of PEG to NPs reduces RES uptake and increases circulation time versus uncoated counterparts **[17]** .

All NPs contain at least two fundamental spatial components: the core and the corona that interact with the environment or solvent. (Figure 1.12)

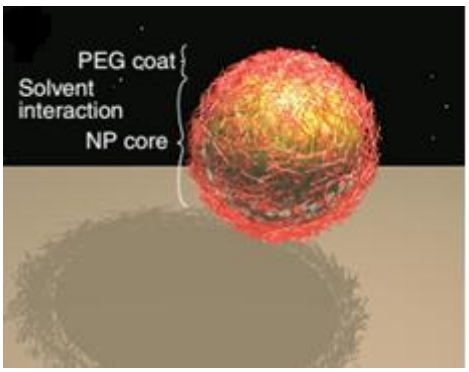


Figure 1.12 – *Structure of a PEGylated NP*

It has to be highlighted the area in which NP interfaces with the external environment. PEG chains modify this interface layer and increase circulation time.
Circulation half-time ($t_{½}$) describes blood pool residence and is the period over which the concentration of circulating NPs remains above 50% of the injected dose, analogous to a drug's half-life .NP efficacy requires sufficient t ½ to not only reach the target, but also remain in the affected area (at concentrations sufficiently above background tissue) long enough for image capture or drug delivery.

The RES system prevents site-specific accumulation because it removes the NPs from circulation, acting as a competitor to the intended target site **[15].** Moreover, the NPs must clear from the non-targeted area to produce imaging contrast or dosing efficiency.
The ideal $t_{½}$ is dependent on the application considered.

1.4 Nanotoxicology

Nanotoxicology is a branch of bio-nano-science, which deals with the substances that migrate out of biomaterials. For instance, as to polymers, many low-molecular-weight leachable components exhibit some level of physiologic activity and cell toxicity. It is reasonable to say that a biomaterial should not give off anything from its mass unless it is specifically designed to. Toxicology also deals with methods to evaluate how well this design criterion is met when a new biomaterial is under development **[18].**

In other words, Nanotoxicological studies are used to determine whether and how biometerials properties may represent a threat to human health. Nanoparticles , inter alia, play a remarkable role in toxicity.

Their size is an important factor in the occurrence of disease. Some studies on the different sizes of carbon and titanium oxide showed that reduction in nanoparticle size increases its toxicity in the lungs. Some of the particle features such as

- Size
- surface chemistry
- crystallinity
- coating
- longevity of particles

represent important parameters.

There is an additional risk of toxicity associated with polymeric NPs not widely studied yet. This toxicity could include (but not limited to) hepatotoxicity, nephrotoxicity etc. and can have long term impacts not easily evaluated in short term in-vivo clinical trials either in animals or humans. Since the degradation of polymers to either their monomers or other degradation products in the body cannot be accurately predicted (unclear metabolic pathway), this is a real risk, especially in medicines intended for long term patient use. Even if the toxicity is ignored, there is additional hepatic load of metabolism, again an area of concern for long term medication.

However polymers still represent a landmark for biomaterials. The major advantages of polymers is

- stability,
- lower cost
- predictable characteristics.

1.5 Focus : Cancer therapy

The small size of nanoparticles endows them with properties that can be very useful in oncology.
In order to substantially improve effective cancer therapy, we must improve our knowledge of cancer
pathophysiology, discover new anticancer drugs, and develop novel biomedical technologies. Consequently,
cancer therapy has become a multidisciplinary challenge requiring close collaboration among clinicians, biological and material scientists, and biomedical engineers **[11]** .

Another notable property of nano-sized systems consists in high surface area, which allows many functional groups to be attached to a nanoparticle, which can seek out and bind to certain tumor cells **[19]** .

Additionally, the small size of nanoparticles (10 to 100 nanometers), allows them to preferentially accumulate at tumor sites (because tumors lack an effective lymphatic drainage system). Research into multifunctional nanoparticles that would detect, image, and then proceed to treat a tumor is under way.

1.5.1 Current issues in cancer chemotherapy

Chemotherapy is the major therapeutic approach for the treatment of cancer and other serious diseases, such as cardiovascular restenosis and acquired immune deficiency syndrome. In general, the clinical application of conventional anticancer drugs involves high patient risks because the drugs are not specific to cancer cells **[20]** (**Figure 1.13**). Most patients must tolerate severe side effects, decreased quality of life and repeated treatments. The inefficiency and side effects of chemotherapy have been primarily associated with the formulation and biodistribution of the drug, toxicity to normal cells, and the acquisition of drug resistance by the cancer cells. Thus, researchers are continuously seeking to overcome these issues.

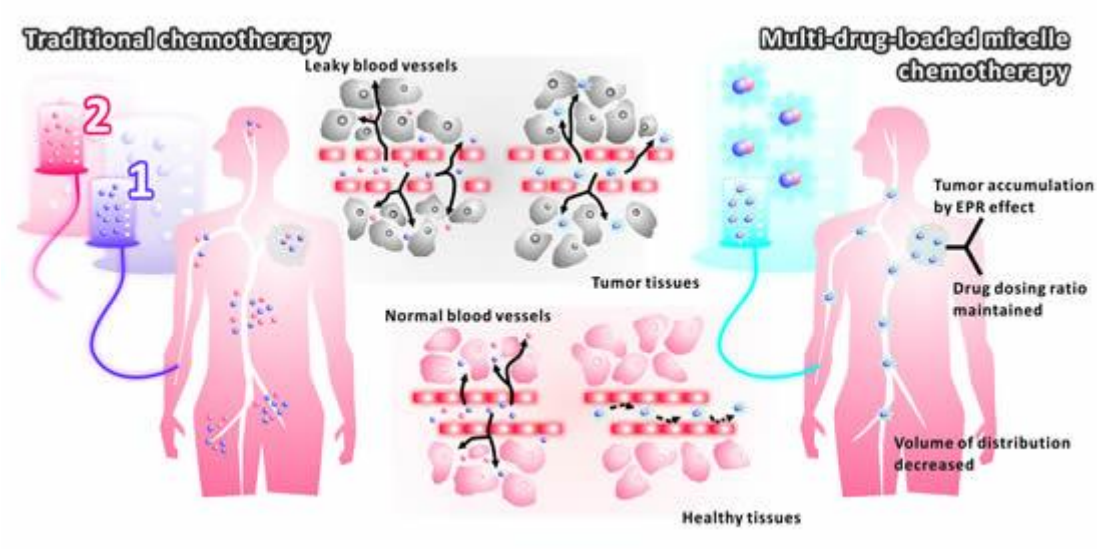


Figure 1.13 – *Traditional combination drug delivery versus polymeric micelle-mediated combination* drug delivery.

Another serious problem in chemotherapy is the acquisition of drug resistance by cancer cells. Unlike most normal tissues, the interstitium of a tumor tissue is characterized by high hydrostatic pressure, leading to an outward convective interstitial flow that can flush the drug away from the tumor.
Furthermore, even if the drug is successfully delivered to the tumoral interstitium, its efficacy

Active targeting is thought to be able to contribute in solving this problem **[21]** and **[22]**.
Cancer cells often display increased cell surface expression of proteins that may be found at low levels on normal cells (tumor-associated antigens), as well as proteins that are found exclusively on cancer cell surfaces (tumor-specific antigens) (**Figure 1.14**). Active drug targeting is usually achieved by chemical attachment to a targeting component that strongly interacts with antigens (or receptors) displayed on the target tissue, leading to preferential accumulation of the drug in the targeted organ, tissue, or cells. The use of a targeting moiety not only decreases adverse side effects by allowing the drug to be delivered to the specific site of action, but also facilitates cellular uptake of the drug by receptor mediated endocytosis, which is an active process requiring a significantly lower concentration gradient across the plasma membrane than simple endocytosis **[11]** .

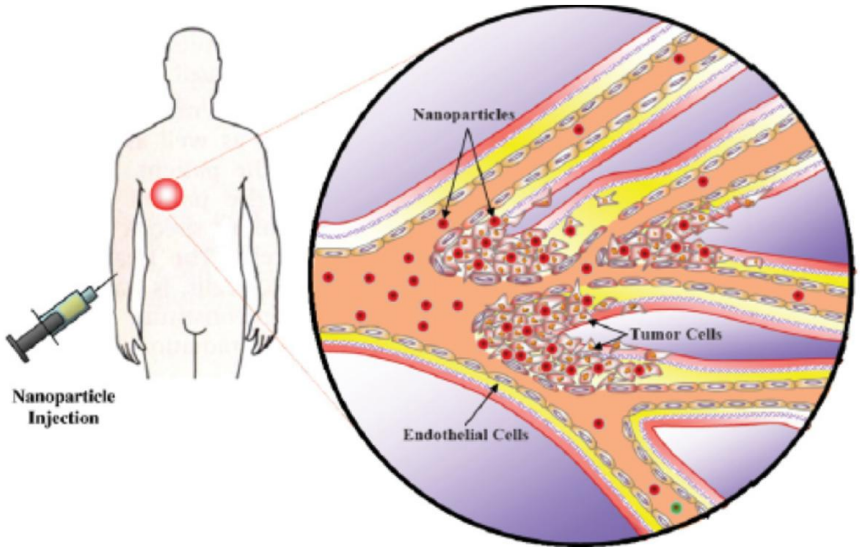


Figure 1.14 – *Active targeting.*

1.6 Biomaterials

IUPAC definition

Material exploited in contact with living tissues, organisms, or microorganisms.

Note 1: The notion of exploitation includes utility for applications and for fundamental research to understand reciprocal perturbations as well.

Note 2: This general term should not be confused with the terms *biopolymer* or *biomacromolecule*. The use of "polymeric biomaterial" is recommended when one deals with polymer or polymer device of therapeutic or biological interest **[23]**

A **biomaterial** is any matter or surface that interacts with biological systems. Biomaterials science includes elements of

- medicine
- biology
- chemistry
- tissue engineering
- materials science

A biomaterial should not be **toxic**, unless it is specifically engineered for. Since the non-toxic requirement is the norm, toxicology for biomaterials has evolved into a sophisticated science (see **section 1.4**).
The understanding and measurement of biocompatibility is unique to biomaterials science. Unluckily Biocompatibility has not a universal meaning **[18]**; No immune response is necessary in order to let the system work in the human body, wherever it is placed. For this reason, Biocompatibility is often defined in terms of performance or success at a specific task.

Biomaterials incorporated into medical devices are implanted into tissues and organs. Hence, the key principles governing cells structure, tissues and organs, and the fundamental mechanisms of disease processes are critical aspects to workers in the field.

Consideration of the anatomical site of an implant is essential.
An intraocular lens may go into the lens capsule or the anterior chamber of the eye. A hip joint will be implanted in-bone across an articulating joint space.

Each of these sites challenges the biomedical device designer with special requirements for geometry, size, mechanical properties, and bioresponses.
In particular, in this work of Thesis polymers for biomedical application will be deepened. As stated in **[24]** polymers represent the largest and most versatile class of biomaterials, being extensively applied in multitude of biomedical applications. This versatility is due to the relative ease with which polymers can be designed and prepared with a wide variety of structures and appropriate physical, chemical, surface and biomimetic properties.
Biopolymers offer indeed an alternative to traditional biocompatible materials (metallic and ceramic) and non-biodegradable polymers for a large number of applications, **[25]** and **[26]** .

In this case two polymers have been considered for drug delivery applications:

- PLA
- PLGA

These polymers are degraded by simple hydrolysis of the ester bonds, which does not require the presence of enzymes. The hydrolytic products from such degradation process are then transformed into non-toxic byproducts that are removed through normal cellular activity.

1.6.1 Lactic acid

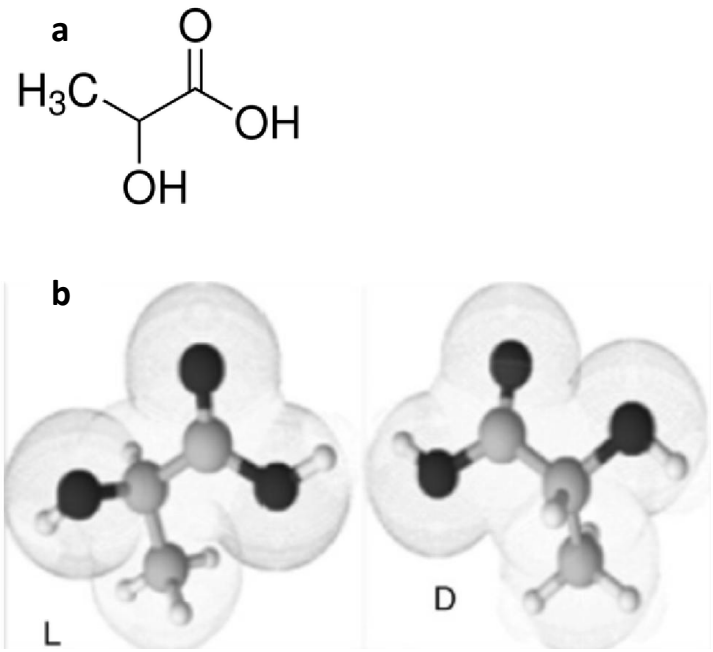


Figure 1.15 – *a) Lactic acid; b) Enantiomers L- and D-*

Lactic acid (2-hydroxypropionic acid) is a simple chiral molecule. It presents two enantiomeric structures: L- and D-lactic acid , which differ in their effect on polarized light. The optically inactive D, L or meso form is an equimolar (racemic) mixture of D(−) and L(+) isomers .
Lactic acid is considered the most potential monomer for chemical applications because it contains a carboxylic and a hydroxyl group **[25]**.
Lactic acid production has a great worldwide attributed to its versatile applications in food, pharmaceutical, textile, leather, and chemical industries and as monomer in the production of biodegradable polymers (PLA).
It is able to influence the metabolic function of cells in a variety of ways indeed, as it can serve as an energy substrate and given its uncharged character and small size, it can permeate through the lipid membrane. Also, lactate is capable of entering cells via the monocarboxylate transporter (MCT) protein shuttle system **[27].** Once inside the cell, lactate is converted to glucose, serving as an energy source.
In addition to its role as an energy substrate for cells, lactic acid has been shown to have antioxidant properties that may serve to protect cells from damage due to free radicals that are naturally produced throughout the cell life cycle **[25].**

Lactic acid can be produced by **fermentative or chemical** synthesis.

1.6.1.1 Chemical synthesis

The chemical synthesis is mainly based on the hydrolysis of lactonitrile by a strong acid, where a racemic mixture of the two forms (D(−) and L(+)) lactic acid is produced.

1.6.1.2 Fermentative synthesis

The biotechnological production of lactic acid has received significant attention, since it is an attractive process in terms of its environmental impact and its combination of low production cost from sugarcane fermentation, decreased fossil-based feedstock dependency, reduced CO_2 emission, biocatalyst use, and high product specificity. Also production of optically pure L- or D-lactic acid can be performed, depending on the strain selected.

Approximately 90% of the total lactic acid produced worldwide is made by bacterial fermentation and the remaining portion is produced synthetically by the hydrolysis of lactonitrile.
The fermentation processes to obtain lactic acid can be classified according to the type of bacteria used **[25].**

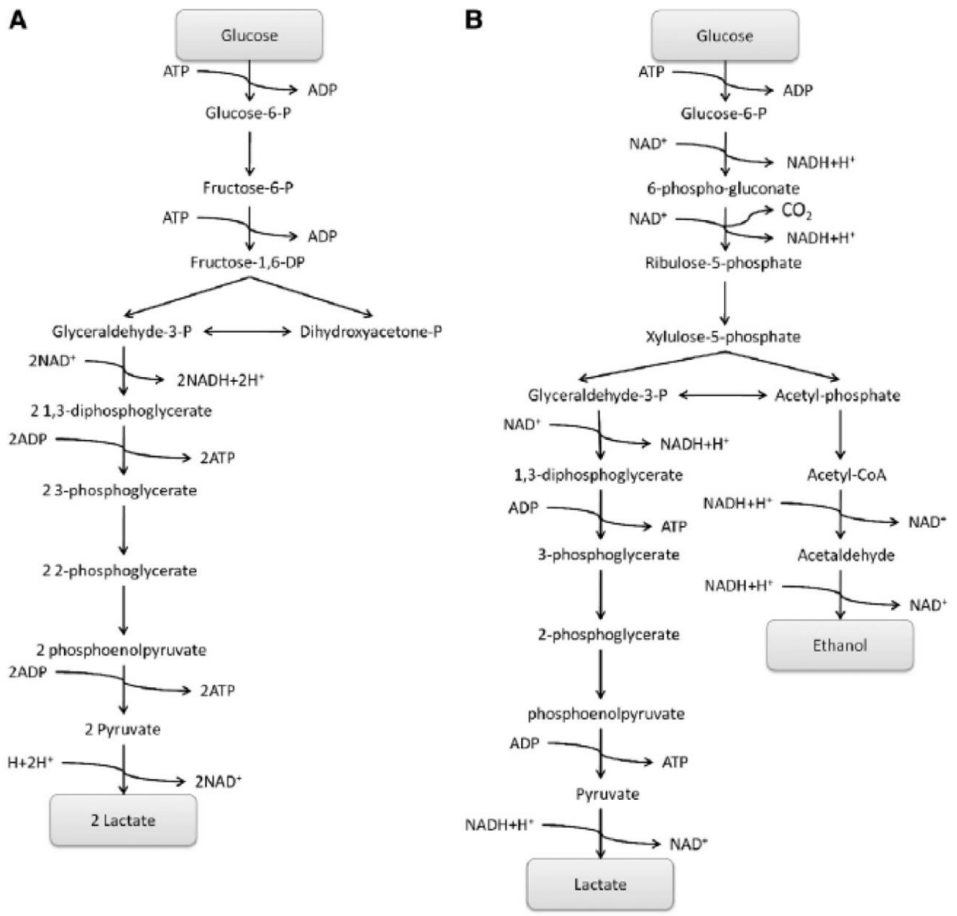


Figure 1.16 - *Metabolic pathways for lactic acid production: A)Embden-Meyerhof-Parnas B) 6-phosphogluconate/phosphoketolase*

1.6.2 Poly-Lactic-Acid (PLA)

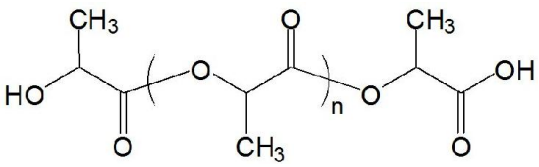


Figure 1.17 - *PLA*

Regarding PLA, it has an extensive mechanical property profile and it is thermoplastic with high biocompatibility and biodegradability properties.

PLA is obtained from lactic acid and converted back to the latter one when hydrolytically degraded.

Although there are multiple ways to produce PLA, none of them is simple or easy to perform: PLA synthesis requires rigorous control of conditions (temperature, pressure and pH), the use of catalysts and long polymerization times, which implies high energy consumption. In order to understand reaction behavior, kinetic studies of PLA synthesis have been conducted by means of modern simulators, which offer a powerful tool to determine the optimal conditions for obtaining the desired PLA polymer for a specific application.

PLA is a highly versatile biodegradable polymer, which can be tailor-made into different resin grades for processing into a wide spectrum of products. Since lactic acid is a chiral molecule existing in L and D isomers, the term "poly-lactic acid" refers to a family of polymers: pure poly-L-lactic acid (PLLA), pure poly-D-lactic acid (PDLA), and poly-D,L-lactic acid (PDLLA) .
The L-isomer is a biological metabolite and constitutes the main fraction of PLA.
Depending on the composition both the optically active L- and D, L-enantiomers, PLA can crystallize in three forms (α, β, and γ) **[28]**.
PLLA has gained great attention because of its excellent biocompatibility and mechanical properties. However, its long degradation times coupled with the high crystallinity of its fragments can cause inflammatory reactions in the body. In order to overcome this, PLLA can be used as a material combination of L-lactic and D, L-lactic acid monomers, being the latter rapidly degraded **[25]**.

1.6.2.1 Properties

Stereochemistry and Thermal history have direct influence on PLA crystallinity, and consequently, on its properties in general. PLA with PLLA content higher than 90% tends to be crystalline, while the lower optically pure is amorphous. The melting temperature (T_m), and the glass transition temperature (T_g) of PLA decrease with decreasing amounts of PLLA **[25]**.
Physical characteristics such as density, heat capacity, mechanical and rheological properties of PLA are dependent on its transition temperatures.

PLA also can be tailored by formulation involving co-polymerizing of the lactide with other lactones-type monomers, a hydrophilic macromonomers (polyethylene glycol (PEG)), or other monomers with
functional groups (such as amino and carboxylic groups, etc.), and blending PLA with other materials .

Blending can radically alter the resultant properties, which depend sensitively on the mechanical properties of the components as well as the blend microstructure and the interface between the phases prepared a series of blends of the biodegradable polymers poly(D,L-lactic acid) and poly(ε-caprolactone) by varying mass fraction across the range of compositions. Polymers made from ε-caprolactone are excellent drug permeation products. However, mechanical and physical properties need to be enhanced by copolymerization or blending **[25]**.

PLA degrades primarily by hydrolysis, after several months exposure to moisture. Degradation occurs in two stages.

- First, random chain scission of the ester groups leads to a reduction in molecular weight.
- In the second stage, the molecular weight is reduced until the lactic acid and low molecular weight oligomers are naturally metabolized by microorganisms to yield carbon dioxide and water .

Degradation rate is mainly determined by polymer reactivity with water and catalysts. Any factor which affects the reactivity and the accessibility, such as particle size and shape, temperature, moisture, crystallinity, % isomer, residual lactic acid concentration, molecular weight, water diffusion and metal impurities from the catalyst, will affect the polymer degradation rate **[25]**.
In vivo and in vitro degradation have been evaluated for polylactide surgical implants.

Lactic acid polymers	Glass transition temperature Tg (°C)	Melting temperature Tm (°C)	Density (g/cm3)	Good solubility in solvents
PLLA	55-80	173-178	1.290	Chloroform,furan,dioxane dioxilane
PDLLA	43-53	120-170	1.25	PLLA solvents and acetone ethyl lactate
PDLA	40-50	120-150	1.248	tetrahydrofuran,ehtylacetate, dimethylsulfoxide

Table 1.6 - *Lactic acid polymers properties*

1.6.2.2 Poly-lactid acid synthesis

PLA can be prepared by different polymerization process from lactic acid including:

- polycondensation
- ring opening polymerization
- direct methods like azeotopic dehydration and enzymatic polymerization .

Currently, direct polymerization and ring opening polymerization are the most used production techniques **[25]**.

Polycondensation includes solution polycondensation and melts polycondensation, and is the least expensive route. However, it is very difficult to obtain a solvent-free high molecular weight poly-lactic acid for these routes. In direct polycondensation, solvents and/or catalysts are used under high vacuum and temperatures to remove water produced in the condensation.
The resultant polymer is a low to intermediate molecular weight material.

[29] reports the synthesis of PLA by direct polymerization without catalysts, solvents and initiators by varying the temperature from 150 to 250 °C and the pressure from atmosphere pressure to
vacuum for 96 h. The Mitsui Toatsu Chemical Company polymerized poly-DL-lactic acid (PDLLA) using direct solution polycondensation, in which lactic acid, catalysts, and organic solvent with high boiling point
were mixed in a reactor.
Polycondensation method produces oligomers with average molecular weights several tens of thousands and other side reactions also can occur, such transesterification, resulting in the formation of ring structures as lactide. These

side reactions have a negative influence on properties of the final polymer. Those byproducts are not negligible, but can be controlled by the use of different catalysts and functionalization agents, as well as by varying the polymerization conditions **[25]** .

Lactid acid direct condensation is carried out in three stages:

- removal of the free water
- oligomer polycondensation
- melt condensation of high molecular weight PLA.

In first and third stages the removal of water is the rate-determining step. For the second one, the rate determining step is the chemical reaction, which depends on the catalyst used. The direct polycondensation of lactic acid in bulk is not applied on a large scale, because of the competitive reaction of lactide formation and the simultaneously occurring degradation process.
In the sequential melt/solid-state polycondensation, besides the three mentioned steps is utilized an additional fourth stage. In the fourth stage, the melt-polycondensated PLA is cooled below its melting temperature, followed by particle formation, which then is subjected to a crystallization process.

Ring-opening polymerization (ROP) is the most commonly route to achieve high molecular weight. This process occurs by ring opening of the lactic acid cyclic dimer (lactide) in the presence of catalyst. The process consists of three steps:

- polycondensation
- depolymerization
- ring opening polymerization

This route requires additional steps of purification which is relatively complicated and expensive. Catalytic ring-opening polymerization of the lactide intermediate results in PLA with controlled molecular weight.
By controlling residence time and temperatures in combination with catalyst type and concentration, it is possible to control the ratio and sequence of D- and L-lactic acid units in the final
polymer .

On the other hand, **ring-opening polymerization** of lactide can be carried out in melt, bulk, or in solution and by cationic, anionic, and coordination-insertion mechanisms depending on the catalyst. Various types of initiators have been successfully tested, but among them, **stannous octoate is usually preferred**

because it provides high reaction rate, high conversion rate, and high molecular weights, even under rather mild polymerization conditions .

Azeotropic dehydration is a direct method for synthesis of high molecular weight PLA. In this route, the removal of water formed from the reaction medium becomes relatively easier and a higher molecular weight of the PLA is achievable .

Enzymatic polymerization emerges as one of the most viable alternatives. It can be carried out under mild conditions. This methodology can provide adequate control of the polymerization process, but the literature about enzymatic polymerization is relatively poor **[25]** .

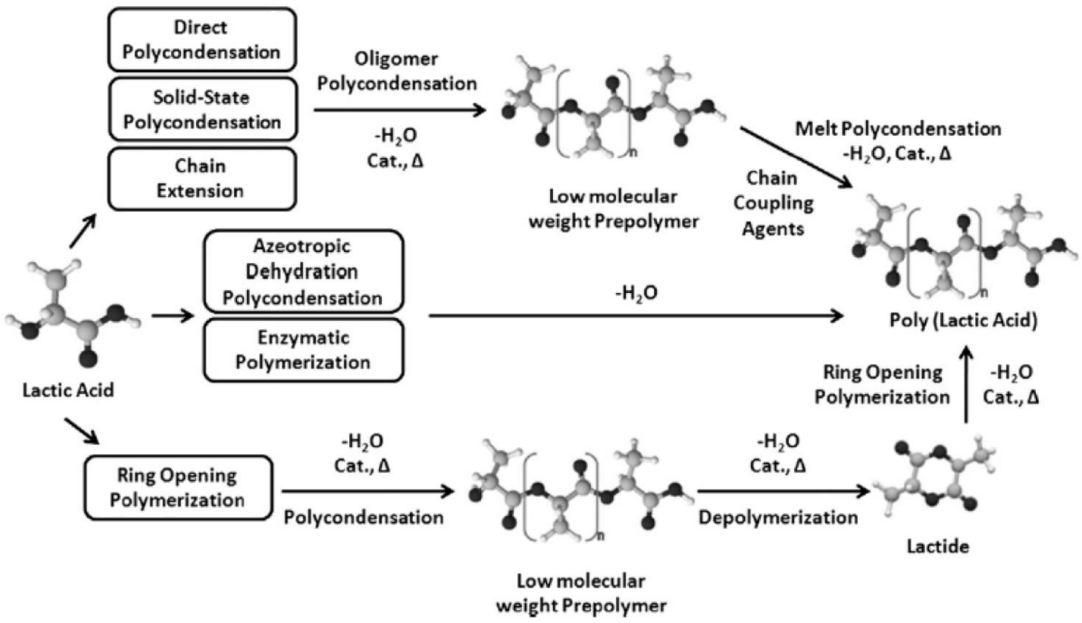


Figure 1.18 - Synthesis methods for Poly(Lactic Acid)

1.6.3 Poly-Lactic-Co-Glycolic- Acid (PLGA)

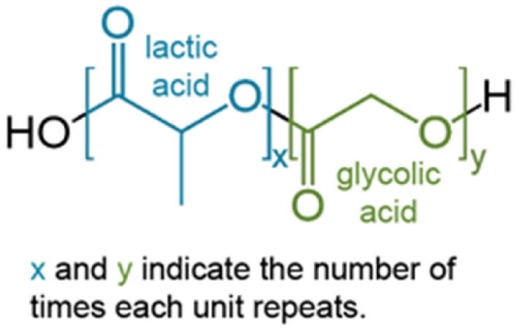


Figure 1.19 - PLGA

PLGA are a family of FDA-approved biodegradable polymers that are physically strong and highly biocompatible and have been extensively studied as delivery vehicles for drugs, proteins and various other macromolecules such as DNA, RNA and peptides.

Also, it is possible to tune the overall physical properties of the polymer-drug matrix by controlling the relevant parameters such as polymer molecular weight, ratio of lactide to glycolide and drug concentration to achieve a desired dosage and release interval depending upon the drug type. However the potential toxicity from dose dumping, inconsistent release and drug-polymer interactions require detailed evaluation **[30]** .

1.6.3.1 Properties

The physicochemical properties of optically active PDLA and PLLA are nearly the same. In general, the polymer PLA can be made in highly crystalline form (PLLA) or completely amorphous (PDLA) due to disordered polymer chains. PGA is void of any methyl side groups and shows highly crystalline structure in contrast to PLA. PLGA can be processed into almost any shape and size, and can encapsulate molecules of virtually any size. It is soluble in wide range of common solvents including chlorinated solvents, tetrahydrofuran, acetone or ethyl acetate.

In water, PLGA biodegrades by hydrolysis of its ester linkages.

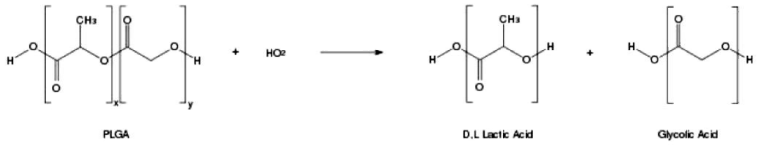


Figure 1.20- *Hydrolysis of poly lactic-co-glycolic acid*

Presence of methyl side groups in PLA makes it more hydrophobic than PGA and hence lactide rich
PLGA copolymers are less hydrophilic, absorb less water and subsequently degrade more slowly.

Due to the hydrolysis of PLGA, parameters that are typically considered invariant descriptions of a solid formulation can change with time, such as the glass transition temperature (T_g), moisture content and molecular weight **[30]** . The effect of these polymer properties on the rate of drug release from biodegradable polymeric matrices has been widely studied.

The change in PLGA properties during polymer biodegradation influences the release and degradation rates of incorporated drug molecules.
PLGA physical properties themselves have been shown to depend on multiple factors, including the initial molecular weight, the ratio of lactide to glycolide, the size of the device, exposure to water (surface shape) and storage temperature. Mechanical strength of the PLGA is affected by physical properties such as molecular weight and polydispersity index. These properties also affect the ability to be formulated as a drug delivery device and may control the device degradation rate and hydrolysis.

Recent studies have found, however, that the type of drug also plays a role in setting the release rate **[30]**.
Mechanical strength, swelling behavior, capacity to undergo hydrolysis and subsequently biodegradation rate of the polymer are directly influenced by the degree of crystallinity of the PLGA, which is further dependent on the type and molar ratio of the individual monomer components in the copolymer chain.
Crystalline PGA, when co-polymerized with PLA, reduces the degree of crystallinity of PLGA and as a result increase the rate of hydration and hydrolysis.

As a rule, higher content of PGA leads to quicker rates of degradation with an exception of 50:50 ratio of PLA/PGA, which exhibits the fastest degradation, with higher PGA content leading to increased degradation interval below 50% **[30] (see also Chapter 3)**.

Degree of crystallinity and melting point of the polymers are directly related to the molecular weight of the polymer.

1.6.3.1 Pharmacokinectic and Biodistribution Profile

As a drug delivery specific vehicle, PLGA must be able to deliver its payload with appropriate duration, biodistribution and concentration for the intended therapeutic effect. Thus, design essentials, including material, geometry and location must incorporate mechanisms of degradation and clearance of the vehicle as well as active pharmaceutical ingredients (API).
Biodistribution and pharmacokinetics of PLGA follows a non-linear and dose-dependent profile **[30]**. Moreover **[30]** suggests that both blood clearance and uptake by the mononuclear phagocyte system (MPS) may depend on dose and composition of PLGA carrier systems. Additionally whole-body autoradiography and quantitative distribution experiments indicate that some formulations of PLGA, such as nanoparticles, accumulate rapidly in liver, bone marrow, lymph nodes, spleen and peritoneal macrophages.
The degradation of the PLGA carriers is quick on the initial stage (around 30%) and slows eventually.

1.7 Biodegradable macromonomers for NPs synthesis through Free Radical Polymerization

Polyesters and copolyesters such as poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), and poly(ε-caprolactone)(PCL) have gained importance in Material Science during the last years because of their biocompatible features and low cost. Their potentiality and all the characteristics that make them suitable for biomedical applications, such as biocompatibility, no-citotoxic interactions, sustainable degradation are well known in Literature, as mentioned in **[25], [30]** and **[31].** These polyesters find indeed many applications in tissue engineering field, as sutures and as adhesion barriers. They also can be regarded as well-established polymers for drug delivery applications, in particular for NPs synthesis, **[26].** Nowadays, PLA- and PLGA- based NPs are prepared mainly by nanoprecipitation, solvent evaporation, salting-out and emulsification-diffusion methods, being ease, mild, and low energy input processes for the preparation of polymeric nanoparticles.
However NPs produced through these procedures have critical drawbacks related to **[32]**

- Requirement of an organic solvent for high molecular weight polyester dissolution, which must be completely removed from the final solution for all the biological and medical purposes;
- Large particle size distribution (PSD) whereas a narrow particle size distribution is preferable for drug delivery applications;
- Reproducibility of the final NPs features is unlikely obtained;

[32] gave a specific hint, and on its trace has been developed the preliminary study presented in this Thesis.The authors observed the features presented by PCL macromonomers, which were synthetised through a two-step procedure: the synthesis of macromonomers, based on ε-caprolactone functionalized with a vinyl end group (**Figure 1.21**) and their subsequent polymerization through free radical polymerization (FRP) in order to obtain NPs.

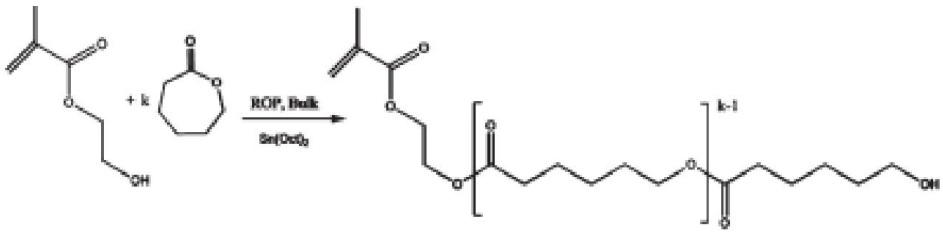


Figure 1.21 - *PCL macromonomers*

Macromonomers were synthesized through Ring opening polymerization (ROP), applying FDA approved catalyst and co-catalyst . In order to avoid transesterification, which causes deviations from the controllable behavior of ROP, the reaction was stopped before complete conversion was reached. ROP technique allows indeed the synthesis of polymers with high and controlled molecular weight, starting from specific cyclic esters.

Emulsion FRP guarantees the final biocompatibility of the NPs suspensions; in fact, the reaction was carried out in water without using any solvent and adopting a FDA accepted emulsifier such as Tween80. Also, small and monodisperse NPs production is allowed.

Starting from this work, the same process found application in the synthesis of PLA- and PLGA- based NPs aimed at biological purposes, here introduced.

1.7.1 Ethylate monomers

In this work of Thesis attention has been focused on PLA- and PLGA-based NPs.
The aim is to obtain polyester-based macromonomers suitable for an emulsion polymerization process. The macromonomers should also terminate with a CH_3- in order to increase their hydrophobicity.

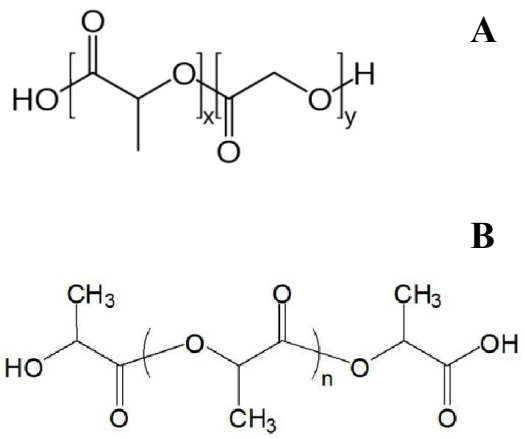


Figure 1.22- A*) PLGA; B) PLA*

For this reason a two-step procedure is proposed, consisting in the synthesis of macromonomers through ROP and successive coupling reaction with HEMA succinate.
Coupling reaction reveales necessary, in order to avoid the hydrophilic characteristics otherwise presented by macromonomers, promoted by the –OH termination.

Besides, since the –OH is one of the most commonly used groups for the addition of targeting agents, methyl terminations could allow the binding of targeting agents on specific place on the NPs surface. Thus functionalization selectivity and reproducibility could be enhanced.

For instance, **Figure 1.23** represents the ideal structure of a PLA-based hydrophobic macromonomer.

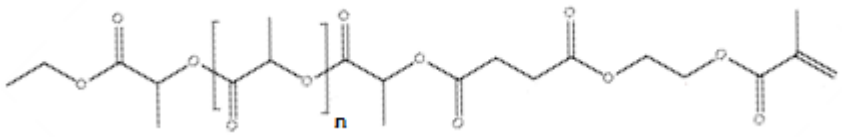


Figure 1.23 - *Ideal structure of a PLA-based macromonomer.*

Macromonomers with different chain lengths were then polymerized through free radical polymerization, in both batch and semibatch emulsion polymerization to produce nanoparticles (NPs) with narrow particle size distribution.
As final step of theThesis, material properties were verified with Degradation and Stability studies.

2. Materials e Methods

In this Chapter the methodology used to produce macromonomers is reported. As explained in **Chapter 1**, the aim is to obtain polyester-based macromonomers suitable for an emulsion polymerization process. These macromonomers should also terminate with a CH3- in order to increase their hydrophobicity. Also, since –OH is one of the most commonly used group for the addition of targeting agents, macromonomers terminating with a methyl will allow the binding of targeting agents on specific place on the NPs surface.

To achieve this result Ring Opening Polymerization (ROP) reactions were performed at first, by using Stannous (II)2 ethyllexanoate as catalyst, Lactide and Glycolide as starting monomers and Ethyl Lactate as co-catalyst. In order to remove the –OH group, Coupling Reactions with HEMA succinate were promoted.
The last phase of this work of Thesis focused on Nanoparticles (NPs) synthesis, referring to Sodium dodecyl sulfate (SDS) and TWEEN 80 as surfactants.

Degradation and stability studies were executed with the purpose of testing and verifying the properties of the synthetized materials.

In the following Chapter all the steps developed are reported in detail.

2.1 ROP

IUPAC definition:

A polymerization in which a cyclic monomer yields a monomeric unit which is acyclic or contains fewer cycles than the monomer.

Note:

If the monomer is polycyclic, the opening of a single ring is sufficient to classify the reaction as ring-opening polymerization. **[33]**

Ring-opening polymerization (ROP) encompasses polymerization of cyclic compounds (monomers) with at least one heteroatom or a double bond in the

molecule. It is a form of chain-growth polymerization, in which the terminal end of a polymer chain acts as a reactive center, where further cyclic monomers can react by opening their ring and form a longer polymer chain. **Propagating center can be**

- **radical**
- **anionic**
- **cationic**

ROP represents the most versatile synthesis method of major groups of biopolymers, particularly when Molecular Weight control is required.

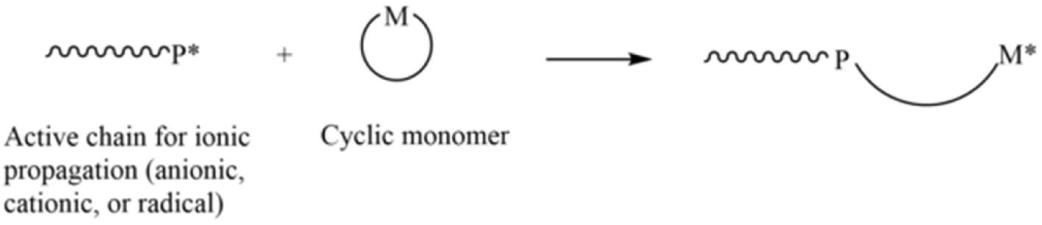


Figure 2.1 – *ROP scheme*

The driving force for the ring-opening of cyclic monomers is via the relief of bond-angle strain or steric repulsions between atoms at the center of a ring, as quoted in **[34]** .

Cyclic monomers that are polymerized using ROP include a variety of structures, such as **[35]:**

- alkanes, alkenes,
- compounds containing heteroatoms in the ring:
    - oxygen: ethers, acetals, esters (lactones, lactides, and carbonates), and anhydrides,
    - sulfur: polysulfur, sulfides and polysulfides,
    - nitrogen: amines, amides (lactames), imides
    - phosphorus: phosphates, phosphonates, phosphites
    - silicon: siloxanes, carbosilanes and silanes.

The development of macromolecules with strictly defined structures and properties, engineered for biomedical applications, leads to complex and advanced architecture and diversification of the hydrolysable polymers as well **[36].**

2.1.1 Theory

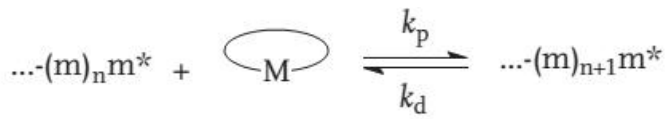
2.1.1.1 Thermodynamics and Kinetics of ROP

The ability of a cyclic monomer to polymerize according to the ring - opening mechanism is determined by two equally important factors: the conversion of monomer molecules into macromolecules must be allowed both thermodynamically and kinetically. In other words this means that:

- monomer - macromolecule equilibrium must be shifted to the macromolecule side;
- the corresponding polymerization mechanism could enable conversion of the monomer molecules into the polymer repeating units. (**Equation 2.1**) monomer - macromolecule equilibrium must be shifted to the macromolecule side;
- the corresponding polymerization mechanism could enable conversion of the monomer molecules into the polymer repeating units. (**Equation 2.1**)

(2.1)

where M denotes the monomer molecule, and m is the macromolecule repeating unit derived from the M monomer; whereas an elementary reaction of the macromolecular chain growth can be written as **[37]:**



(2.2)

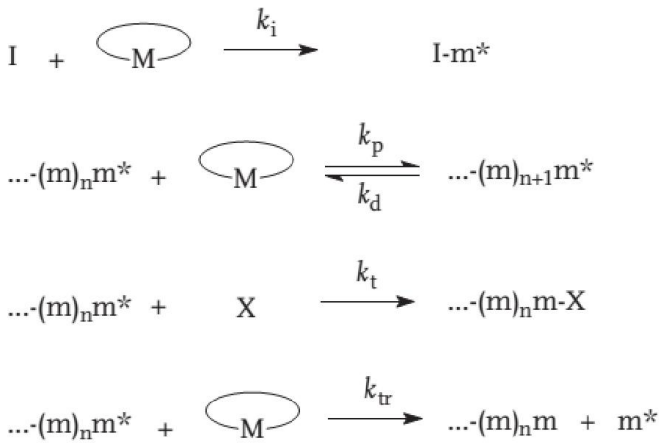
where m^* denotes the active species, k_p and k_d are the rate constants of propagation and depropagation, respectively.

Depending on the monomer and catalytic/initiating system and the nature of the resulting active species, several mechanisms can operate in the ring-opening polymerization.
The mechanisms most often employed include coordination, covalent, ionic (anionic or cationic), metathetic, radical and enzymatic.

In contrast to the polymerization of a large majority of unsaturated monomers, the ROP of cyclic monomers is often accompanied by the presence of a relatively high concentration of the unreacted monomer when the process comes to equilibrium. This feature is related to a pronounced reversibility of the

propagation step (i.e. relatively high k_d in comparison to k_p; **Equation 2.2**). Thus, a value of the equilibrium monomer concentration ($[M]_{eq}$) is usually taken as a measure of the monomer thermodynamic polymerizability.

The reaction of a monomer with initiating agents (**Equation 2.3a**) should lead to active species capable of adding new monomer molecules (**Equation 2.3b**); moreover, they should be added faster than they undergo any side reactions, such as termination (**Equation 2.3c**) or transfer to monomer (**Equation 2.3d**) **[37]** .



(2.3 a)

(2.3 b)

(2.3 c)

(2.3 d)

where I denotes the initiator molecule, m^* is the active species, X is the terminating agent, and k_p, k_d, k_t, k_{tr} are the rate constants of propagation, depropagation, termination and transfer, respectively. For an idealized, living polymerization: $k_t = 0$ and $k_{tr} = 0$.

The formal **thermodynamic criterion** of a given monomer polymerizability is related to a sign of the free Gibbs energy of polymerization (**Equation 2.1**):

$$\Delta G_p(xy) = \Delta H_p(xy) - T\Delta S_p(xy) \quad \textbf{(2.4)}$$

where x and y denote monomer and polymer states, respectively [i.e.: x and/or y = l (liquid), g (gaseous), c (solid amorphous), c ′(solid crystalline), s (solution)], $\Delta H_p(xy)$ and $\Delta S_p(xy)$ are the corresponding enthalpy and entropy of polymerization, and T is the absolute temperature.

In agreement with the general rules of the thermodynamics of chemical processes, only when $\Delta G_p(xy)$ <0 polymerization is possible.

ΔG_p may be expressed as a sum of standard enthalpy of polymerization ($\Delta G°_p$) and a term related to instantaneous monomer molecules and growing macromolecules concentrations:

$$\Delta G_p = \Delta G_P^O + RTln(\frac{[\ldots-(m)_{i+1}m*]}{[M]*[\ldots-(m)_i m*]}) \quad \textbf{(2.5)}$$

where R denotes the gas constant.

Following **Flory ' s assumption** that the reactivity of an active center, located at a macromolecule of a sufficiently long macromolecular chain, does not depend on its polymerization degree (Dp_i) and taking into account that $\Delta G_P^O = \Delta H_p{}^o$ - T $\Delta S_p{}^o$
(where $\Delta H_p{}^o$ and $\Delta S_p{}^o$ denote a standard polymerization enthalpy and entropy, respectively), we obtain:

$$\Delta G_p = \Delta H°_p - T(\Delta S°_p + Rln([M]) \quad \textbf{(2.6)}$$

When equilibrium is reached ΔG_p = 0, that is, when polymerization is complete the monomer concentration $[M]_{eq}$ assumes a value determined by standard polymerization parameters ($\Delta H_p{}^o$ and $\Delta S_p{}^o$) and polymerization temperature **[37]** (**Equation 2.7a and 2.7b**)

$$\ln[M]_{eq} = \frac{\Delta H°_p}{RT} - \frac{\Delta S°_p}{R} \quad \textbf{(2.7 a)}$$

$$[M]_{eq} = \exp(\frac{\Delta H°_p}{RT} - \frac{\Delta S°_p}{R}) \quad \textbf{(2.7 b)}$$

2.1.1.2 Ring-opening polymerization of cyclic esters

Polylactones and polylactides can be prepared by two different approaches

- by polycondensation of hydroxy-carboxylic acids
- by ring-opening polymerization (ROP) of cyclic esters.

The polycondensation technique is less expensive than ROP, but it is difficult to obtain high molecular weight polymers, to achieve specific endgroups, and to prepare well-defined copolyesters **[36]**.

The ROP of lactones and lactides has been thoroughly investigated during the last 40 years, due to its versatility to produce a variety of biomedical polymers in a controlled manner.

There are several reasons for studying the polymerization of cyclic esters: for example the possibility to exploit the potential of synthetic polymer chemistry to prepare a variety of polymers with control of the major variables affecting polymer properties. Experimental conditions have to be optimized in order to find the best polymerization system for technological and industrial processes. Factors such as economy, toxicology and technical apparatus development are not negligible.

Polylactones and polylactides of high molecular weight are exclusively produced by the ring-opening polymerization of the corresponding cyclic monomers.

For instance, a polyester is formed when cyclic esters react with a catalyst or initiator. **Figure 2.2** presents the reaction pathway for the ring-opening polymerization of cyclic esters.

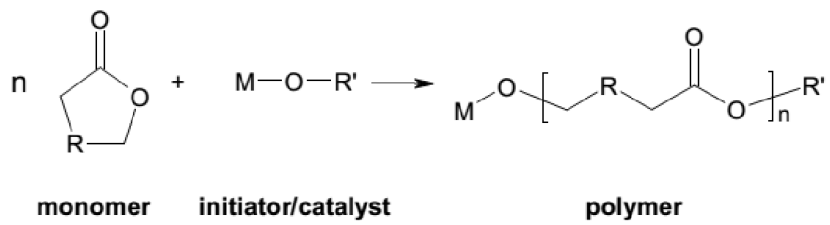


Figure 2.2 – *Schematic representation of the ring opening polymerization of a cyclic ester. R= $(CH2)_{0-3}$ and/or (CHR'')*

Each macromolecule formed will generally contain one chain end terminated with a functional group originating from the termination reaction and one terminus end-capped with a functional group originating from the initiator.

By altering the catalyst or initiator and the termination reaction, the nature of the functional groups can be varied to fit the specific application.

The types of initiators and end-groups play an important role in both the thermal stability and hydrolytic stability of the resulting polyester **[36] .**

The ring-opening reaction can be performed either as a bulk polymerization, or in solution, emulsion or dispersion.

A catalyst or initiator is necessary to start the polymerization. Under rather mild conditions, high-molecular weight aliphatic polyesters of low polydispersity can be prepared in short periods of time.

2.1.1.3 Organometallic compounds as initiators for the ROP of lactones and lactides

The synthesis of novel initiators and the ROP of existing or new monomers and macromonomers substituted with functional groups provide a very interesting and promising strategy to produce structurally advanced macromolecules. A large variety of organometallic compounds, i.e. metal alkoxides and metal carboxylates, have been studied in order to achieve effective polymer synthesis.

Many reactions catalyzed by metal complexes are highly specific, and, by careful selection, of metal and ligands, reactions can be generated to form a desired polymer structure **[36]**.

Figure 2.3 summarizes some of the most frequently used initiators and catalysts.

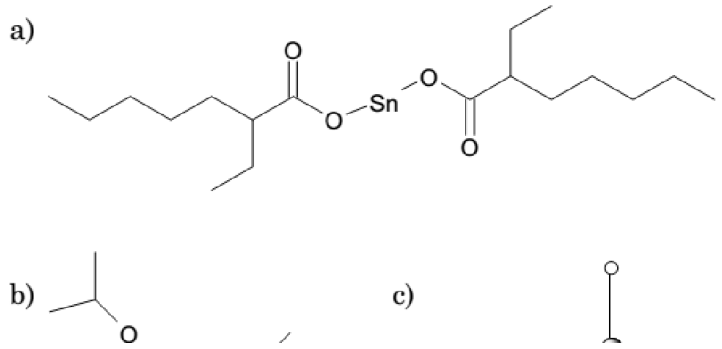


Figure 2.3 – *Chemical structure of initiators used in ROP of lactones and lactides. A)stannous octoate b)aluminium isopropoxide c)lanthanide isopropoxide.*

2.1.1.4 Transesterification reactions

It is well known from the ring-opening polymerization of lactones and lactides that the catalyst or initiator causes transesterification reactions at high temperatures **[36]** or at long reaction times. **Intermolecular transesterification** reactions modify the sequences of copolylactones and prevent the formation of block copolymers. **Intramolecular transesterification** reactions, i.e. back-biting, cause degradation of the polymer chain and the formation of cyclic oligomers. Both

types of transesterification reactions broaden the molecular weight distribution.

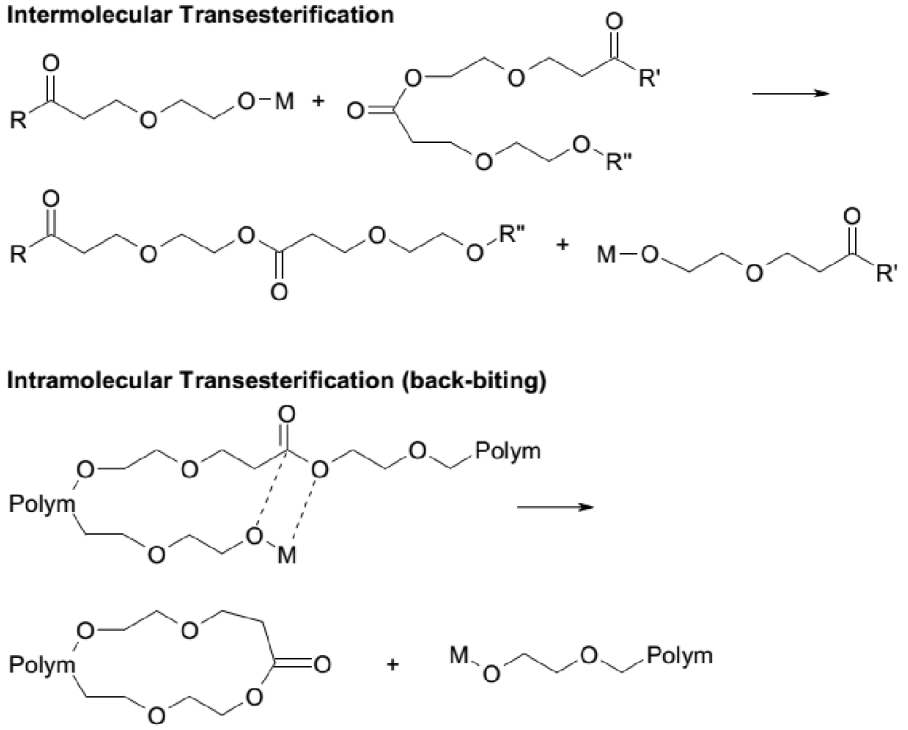


Figure 2.4 – *Reaction scheme for intermolecular and intramolecular transesterification*

As displayed in **Figure 2.4**, each intramolecular transesterification randomly breaks the polymer chain. In this way, an attack on the polymer chain leads to a free residual polymer and a new randomized, modified polymer.

Parameters that will influence the number of transesterifications are

- Temperature
- reaction time
- type and concentration of catalyst or initiator.

The lactide configuration influences the degree of transesterification reactions taking place during polymerization. The contribution of transesterification processes in the case of D,L-lactide was found to be considerably higher than that observed when L,L-lactide was polymerized. The difference in number of side-reactions was attributed partly to the polymer chain stiffness. The

poly(D,L-lactide) is more flexible than the poly(L,L-lactide) due to the atactic lactide blocks **[36]**.

2.1.2 Materials

2.1.2.1 Stannous(II) 2-ethylhexanoate

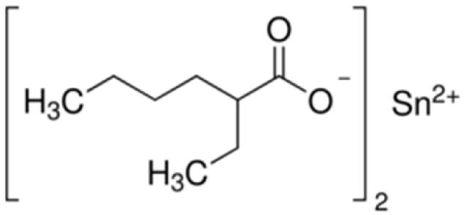


Figure 2.5 – *Stannous(II) 2-ethylhexanoate*

Compound	State	Purity	MW
Stannous(II) 2-ethylhexanoate	Liquid	>99%	405.12 g/mol

Stannous(II) 2-ethylhexanoate, commonly referred to as stannous octoate [$Sn(Oct)_2$], is a frequently used catalyst in the ROP of lactones and lactides.

It has been used in the following procedure as catalyst, being approved by the FDA.

Stannous octoate has been researched thoroughly in the past and is generally used for bulk polymerization of lactides because of its solubility in lactide, catalytic activity and racemization of the polymer. This catalyst promises good reaction rate, conversion of greater than 90%, low levels of racemization, that is less than 1% and providing higher molecular weight polymer, as stated in **[37]** and **[38]** .

$Sn(Oct)_2$ is not thought to be the actual initiator since the molecular weight does not depend on the monomer-to-SnOct2 molar ratio. The most promising mechanism is a coordination insertion mechanism where a hydroxyl functional group is thought to coordinate to $Sn(Oct)_2$, forming the initiating tin-complex .

Investigations of the coordination-insertion mechanism have resulted in two slightly different reaction pathways **[38]**:

- Kricheldorf and coworkers have proposed a mechanism where the coinitiating alcohol functionality and the monomer are both coordinated to the $Sn(Oct)_2$-complex during propagation.
- Penczek and coworkers have presented a mechanism where the $Sn(Oct)_2$-complex is converted into a tin-alkoxide before complexing and ring-opening of the monomer.

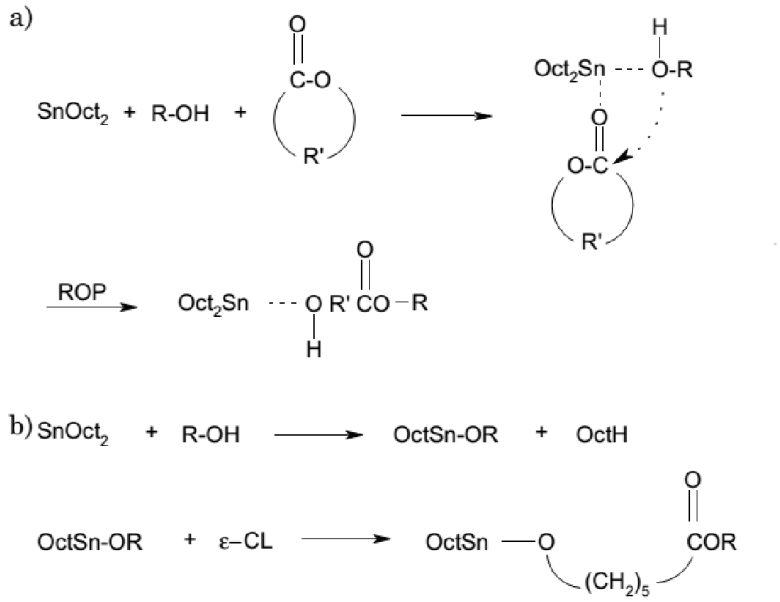


Figure 2.6 – *The main ROP mechanism proposals with stannous(II)2-ethylhexanoate as catalyst: a) complexation of a monomer and alcohol prior to ROP b) formation of a tin-alkoxide before ROP of ε-caprolactone.*

If water coexists with the alcohol/stannous octoate initiating system, it converts tin(II) alkoxide into quiescent hydroxyl end capped chains and stannous hydroxides which are less reactive than stannous alkoxide. This decreases concentration of active propagating species and thus resulting in slower polymerization **[38]** .

$$Sn(Oct)_2 + ROH \leftrightarrow Oct - Sn - OR + Oct - H$$
$$Oct - Sn - OR + ROH \leftrightarrow Sn(OR)_2 + Oct - H$$

In this work of Thesis in particular, Stannous(II) 2-ethylhexanoate was used to catalyze lactide ROP. The specific mechanism is reported in **Figure 2.7**:

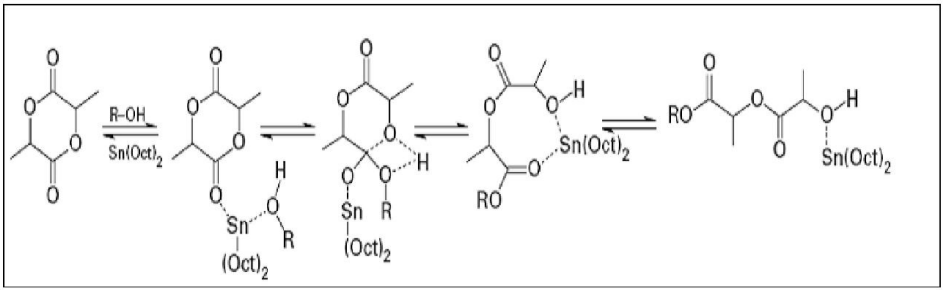


Figure 2.7 – *Generalized coordination-insertion mechanism of lactide to PLA;*

2.1.2.2 Lactide

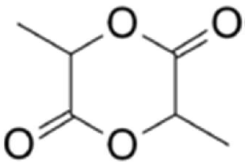


Figure 2.8 – *Lactide*

Compound	State	Purity	Melting point	MW
Lactide	Solid (cristal)	98%	92-94 °C	144 g/mol

Lactide is the cyclic di-ester of lactic acid, i.e., 2-hydroxypropionic acid. Lactic acid cannot form a lactone as other hydroxy acids do because the hydroxyl group is too close to the carboxylic group. Instead, lactic acid first forms a dimer.

The dimer contains a hydroxyl group at a convenient distance from the carboxylic group for the formation of a lactone. Indeed, the dimer readily forms a six-membered cyclic diester known as lactide..

Lactic acid is chiral; two enantiomeric forms, (R)-lactic acid and (S)-lactic acid, exist. Thus, lactide formed from two equivalents of lactic acid consists of two stereocenters. Three different stereoisomers of lactide are known **[39].**

For the experimental work described in this Thesis L,L-lactide has been used.

2.1.2.3 Glycolide

Glycolide was used as starting monomer, together with Lactide

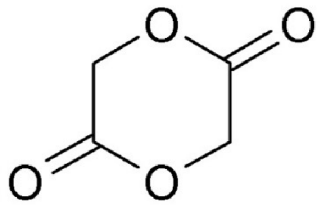


Figure 2.9– *Glycolide*

Compound	State	Purity	Melting point	MW
Glycolide	Solid (powder)	>99%	82-86 °C	116 g/mol

2.1.2.4 Ethyl-Lactate

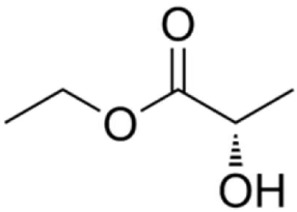


Figure 2.10 – *Ethyl Lactate*

Compound	State	Purity	Melting point	MW
Etil-Lactate	Liquid	>99%	-26 °C	118.13 g/mol

Ethyl lactate was used as co-catalyst.

It is also known as lactic acid ethyl ester, an ester formed by lactic acid and ethanol, commonly used as a solvent **[40]**. Due to its relatively low toxicity, ethyl lactate is used commonly in pharmaceutical preparations, food additives and fragrances. Drug delivery properties and biocompatibility have been investigated in Literature in several occasions (i.e. **[41]**).

2.1.3 Experimental Section

In the section below a detailed description of experimental procedures is given, referring to the ROP reaction of lactide and glycolide.

2.1.3.1 ROP of Lactide

The ROP reaction adopted to synthesize macromonomers was performed using the following procedure:

A mixture of $Sn(Oct)_2$ and Etyl Lactate at a given molar ratio ($\frac{1}{200}$) was prepared and left under continuous magnetic stirring at room temperature for 5 min.

Meanwhile the desired amount of Lactide has been placed in a 100 ml glass reactor under magnetic stirring and heated in an oil bath at 125-130 °C.

$Sn(Oct)_2$ + Etyl Lactate solution was added to the melted monomer to initiate the reaction.

The reaction was stopped after 4 hours, thus reaching >90% conversion. Monomers obtained have been transferred in glass vials and refrigerated to T_{amb}, while waiting for further use.

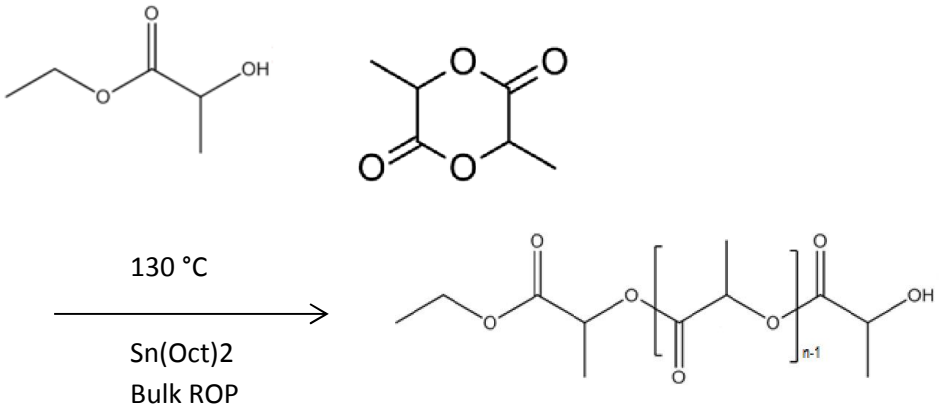


Figure 2.11 – *Lactide ROP*

The procedure refers to the synthesis of macromonomers, which differ only in the chain length:

Macromonomers
ETL-LA_4
ETL-LA_6
ETL-LA_8

Table 2.1 – *Macromonomers synthetized*

2.1.3.2 ROP of Lactide and Glycolide

The procedure described in the previous section was followed in this case as well.
Lactide and glycolide mixture contained 50% glycolide and 50% lactide on molar base. According to available data in Literature **[30]** and **[42]** up to fifty percent GA in weight, are adopted to avoid the crystallinity characteristics of poly(lactic acid) (PLA).

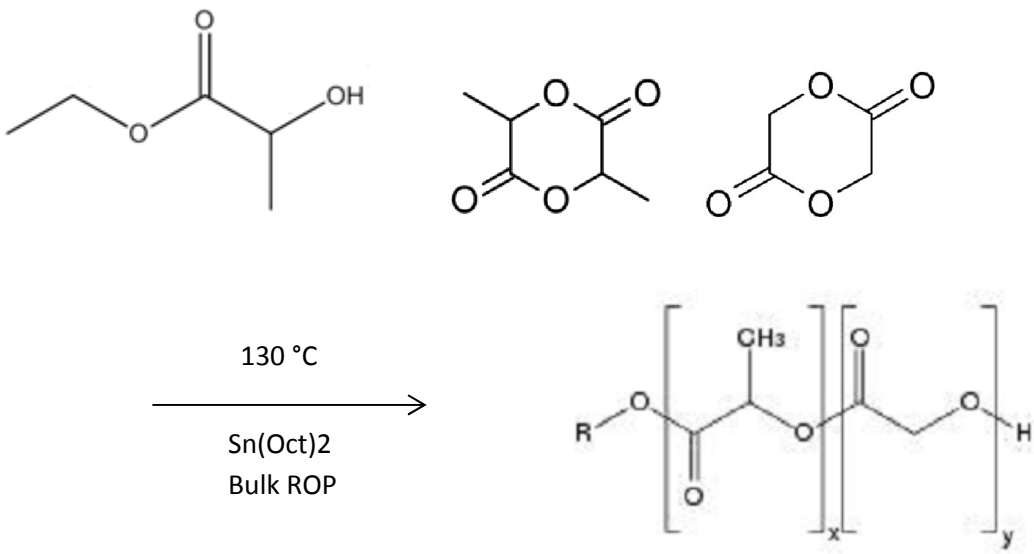


Figure 2.12 – *Lactide and Glycolide ROP*

The procedure refers to the synthesis of monomers which differ only in the chain length:

Macromonomers
ETL-PLGA$_4$
ETL-PLGA$_6$
ETL-PLGA$_8$

Table 2.2 – *Macromonomers synthetized*

2.2 Coupling reaction

Coupling reaction revealed necessary to the purpose reported in **Chapter 1**, i.e. the synthesis of macromonomers with hydrophobic terminations, aimed in avoiding degradation or interactions with biological systems as long as possible.

2.2.1 Materials

2.2.1.1 HEMA succinate

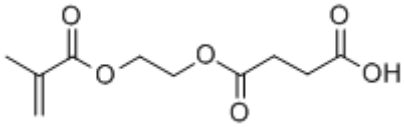


Figure 2.13 – *HEMA succinate*

Compound	State	Purity	MW
HEMA COOH	Liquid	>99%	230.216 g/mol

HEMA succinate is a carboxylic acid-containing methacrylate, obtained by promoting the reaction between Hema and succinic anhydride. It combines the bonding characteristics of succinic acid with the reactivity of HEMA **[43]** .

2.2.1.2 4-Dimethylaminopyridine (DMAP)

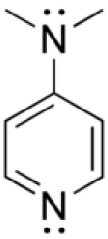


Figure 2.14 – *DMAP*

Compound	State	Purity	Melting point	MW
DMAP	Solid	>99%	110-113 °C	122.17 g/mol

4-Dimethylaminopyridine (DMAP) is a derivative of pyridine. This colourless solid is a useful nucleophilic catalyst for a variety of reactions **[44].** DMAP has however a relatively high toxicity and is particularly dangerous because of its ability to be absorbed through the skin. It is also corrosive.

2.2.1.3 N,N'-Dicyclohexylcarbodiimide (DCC)

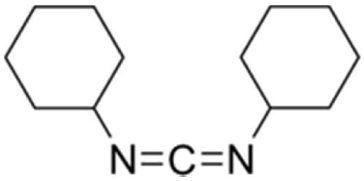


Figure 2.15 – *DCC*

Compound	State	Purity	Melting point	MW
DCC	Solid	>99%	34 °C	206.33 g/mol

***N,N'*-Dicyclohexylcarbodiimide** is an organic compound whose primary use is as coupling agent. Under standard conditions it exists in the form of white crystals with a heavy, sweet odor **[45]**. The low melting point of this material allows it to be melted for easy handling. It is highly soluble in dichloromethane, tetrahydrofuran, acetonitrile and dimethylformamide, but insoluble in water. The compound is often abbreviated DCC.

However DCC is a potent allergen and a sensitizer, often causing skin rashes.

2.2.1.4 Dichloromethane (DCM)

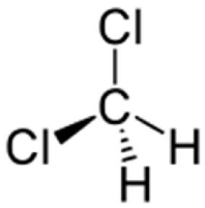


Figure 2.16 – *DCM*

Compound	State	Purity	Melting point	MW
DCM	Liquid	>99%	-96.7 °C	84.93 g/mol

Dichloromethane (**DCM**, or **methylene chloride**) is an organic solvent. It is colorless and volatile. Although it is not miscible with water, it is miscible with many organic solvents **[46]**.

DCM's volatility and ability to dissolve a wide range of organic compounds makes it a useful solvent for many chemical processes.

DCM is the least toxic of the simple chlorohydrocarbons, but it is not without health risks, as its high volatility makes it an acute inhalation hazard. DCM is also metabolized by the body to carbon monoxide potentially leading to carbon monoxide poisoning. Acute exposure by inhalation has resulted in optic neuropathy and hepatitis. Prolonged skin contact can result in DCM dissolving some of the fatty tissues in skin, resulting in skin irritation or chemical burns. It may be carcinogenic, as it has been linked to cancer of the lungs, liver, and pancreas in laboratory animal.

The reason why DCM has been chosen as solvent for biological applications is due to its volatility. Promoting evaporation of this solvent is indeed relatively easy (Boiling point : 39 °C at 1 atm), so that removing completely DCM can be achieved under no severe conditions.

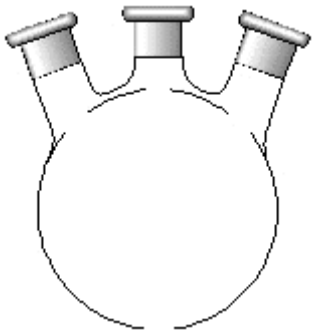
2.2.2 Experimental Section

2.2.2.1 Coupling Reaction

Molar ratios on which the reaction was based:

- 1 M eq **HemaCOOH**
- 1.05 M eq **Macromonomer**
- 0.10 M eq **DMAP**
- 1.10 M **DCC**
- **DCM** used as solvent. Initial concentration: $0.25\ \frac{mol\ HemaCOOH}{l}$

As we can see Hema succinate was used as limiting agent.



- HEMA-COOH
- DMAP
- MACROMONOMER
- DCM

were placed in a three necked flask, under inert nitrogen atmosphere and magnetic stirring. Meanwhile the reactor was cooled by an ice and water bath, to keep T=0°C.

- DCC
- DCM (about 8 ml)

Have been placed in a 10 ml syringe and fed with a syringe pump, whose rate had been set to 10 ml/h.

The reaction was stopped after 24 hours. In particular temperature control resulted crucial during the first hour reaction, that coincided with the feeding time.

Considering for example the ETL-LA_4 coupling reaction:

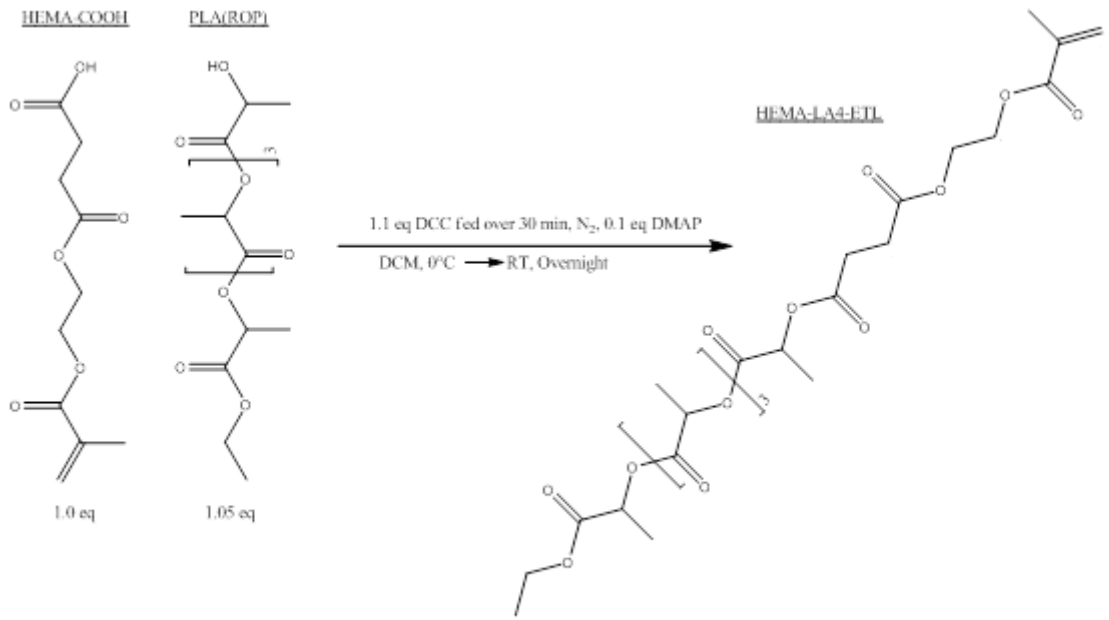


Figure 2.17 – *Example of Coupling reaction*

As we can see the reaction products will not have hydrophilic termination, which suggest us that the terms of degradation will be longer. Any supposition will be verified by further experimental data. As byproduct DCU is formed. It consists in a white powder, removed by vacuum filtration.

All the macromonomers synthetized with the ROP technique were subjected to coupling reactions. Macromonomers samples after coupling reactions are listed below, in **Table 2.3**:

LA_X Macromonomers
HEMA ETL LA_0 *
HEMA ETL LA_4
HEMA ETL LA_6
HEMA ETL LA_8

* HEMA ETL LA_0 macromonomer refers to Ethyl Lactate coupling reaction with Hema succinate

$PLGA_X$ Macromonomers
HEMA ETL $PLGA_4$
HEMA ETL $PLGA_6$
HEMA ETL $PLGA_8$

Table 2.3 – LA_X and $PLGA_X$ *Macromonomers after coupling reaction*

2.2.2.2 Coupling Reaction Work up

After the 24-hour reaction a procedure was suggested, in order to remove undesired compounds. The technique which revealed most efficient is described below:

- **Vacuum filtration:** the mixture of solid (DCU) and liquid was poured through a filter paper in a Buchner funnel. The solid was trapped by the filter and the liquid was drawn through the funnel into the flask below, by means of a vacuum.

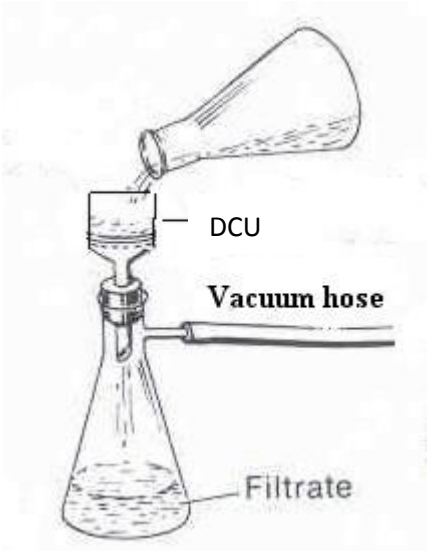


Figure 2.18 – *Vacuum Filtration*

- After the first filtration the liquid phase was placed in freezer overnight, T=-20°C, in order to promote insolubility of the residual DCU. At T_{amb} DCU is not completely removed because it is soluble in DCM, so that in the first filtration not all the byproduct formed during the reaction is separated.

- A **second vacuum filtration** was necessary, to complete the DCU separation.

- **Acid wash**: it was performed in a separating funnel. HCl aq 1M was used, to remove non reacted DMAP, by promoting the reaction between H^+ and Nytrogen. A glass stick has been used for stirring. Acid wash needs to be repeated twice: it represents a tradeoff indeed, since every passage improves the separation. However part of the desired product is lost every time. Optimizing the operating conditions, two acid washes revealed sufficient.

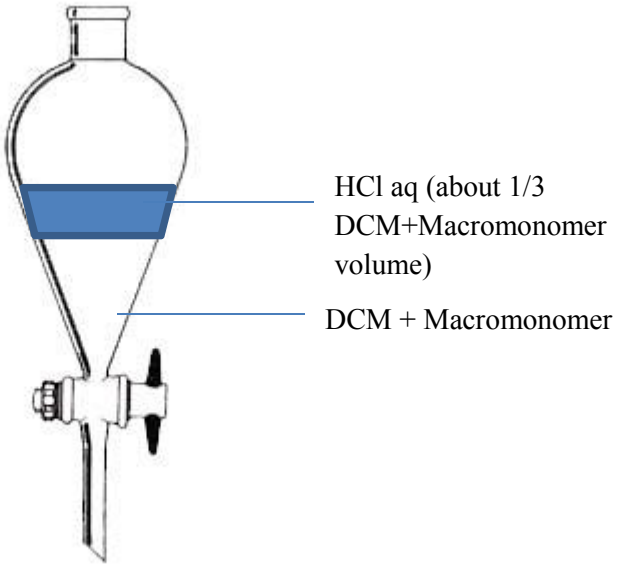


Figure 2.19 – *Acid wash*

- **NaCl wash**: residual water was saturated with NaCl aq, so that in the lower phase we will reasonably separate only organic compounds (DCM and the desired macromonomer).

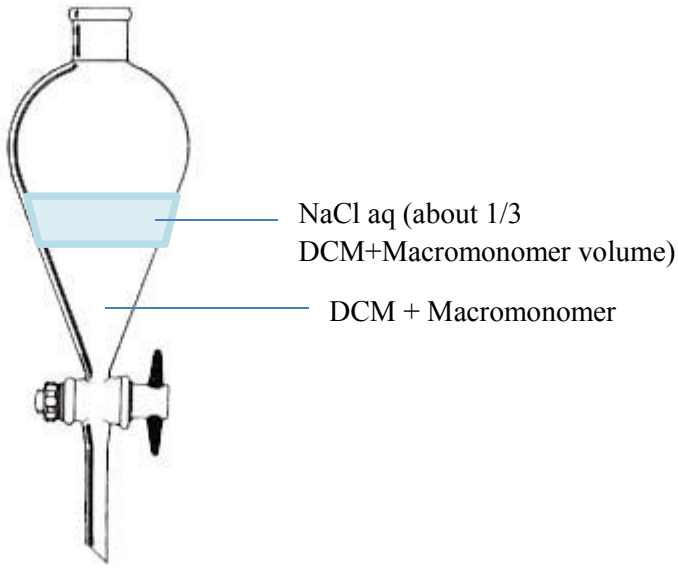


Figure 2.20 – *NaCl wash*

- **DCM evaporation:** it was promoted by exploiting a rotary evaporator, in order to remove efficiently and gently the solvent.

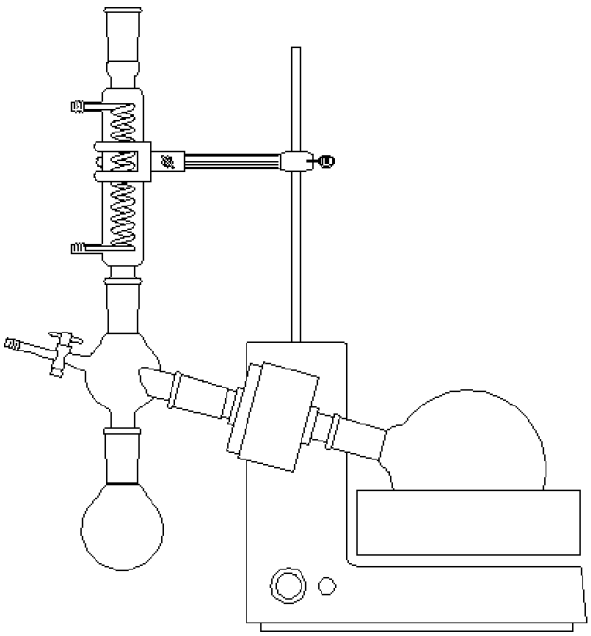


Figure 2.21 – *Rotovapor*

2.3 Emulsion Polymerization

NPs for biomedical use were synthetized through emulsion free radical polymerization
PLA and PLGA based macromonomers found application in the production of NPs, whose features were tested afterward with degradation studies.

Emulsion FRP guarantees the final biocompatibility of the NPs suspensions and allows small and monodisperse NPs production. Emulsion polymerization was performed as batch and semibatch reaction.

- Batch emulsion polymerization was applied in order to synthetized nanoparticles, which revealed valuable to characterize the properties of the materials proposed;
- Semibatch emulsion polymerization was used to copolymerize the macromonomers prepared with PEG, whose primary role in the interaction with living tissues is well known, **[15]**. Macromonomers were introduced gradually in the reactor, by fixing a rate which allowed both to maximize reactants conversions and to reduce aggregates formation.

The procedure is fully explained in the following sections.

2.3.1 Theory

IUPAC definition
Emulsion polymerization: Polymerization whereby monomer(s), initiator, dispersion medium, and possibly colloid stabilizer constitute initially an inhomogeneous system resulting in particles of colloidal dimensions containing the formed polymer.
Batch emulsion polymerization: *Emulsion polymerization* in which all the ingredients are placed in a reactor prior to reaction **[23]** .

Colloids possess specific properties of great practical interest. In these systems, although one component is micro-dispersed into another component, the size of the dispersed species is much larger than that of a molecular mixture (a conventional chemical solution where the solute and solvent molecules are of comparable size). On the other hand, these dispersed species are not large

enough to rapidly sediment because of gravity; they show a sedimentation rate in the order of 10^{-4} cm/s or even slower, being subject to an irregular, random movement (**Brownian motion**). Colloids are therefore constituted by a finely dispersed (discontinuous) phase, homogeneously distributed in a dispersion medium (continuous phase). Both the continuous and the discontinuous phase may be solid, liquid or gaseous **[47]**.

Phase of Colloid	Dispersing (solventlike) Substance	Dispersed (solutelike) Substance	Colloid Type	Example
Gas	Gas	Gas	—	None (all are solutions)
Gas	Gas	Liquid	Aerosol	Fog
Gas	Gas	Solid	Aerosol	Smoke
Liquid	Liquid	Gas	Foam	Whipped cream
Liquid	Liquid	Liquid	Emulsion	Milk
Liquid	Liquid	Solid	Sol	Paint
Solid	Solid	Gas	Solid foam	Marshmallow
Solid	Solid	Liquid	Solid emulsion	Butter
Solid	Solid	Solid	Solid sol	Ruby glass

Table 2.4 – *Colloids*

The properties of colloidal character appear when the dimensions of the dispersed phase are between 1 and 1000 nm, but no strict limit is imposed. In some cases, for example in emulsions, the dispersed units may be larger. When the dimensions of the dispersed phase are smaller than 1 nm the colloidal behavior coincides with that of molecular solutions. On the contrary, dispersions of particles generally bigger than 1 μm are called suspensions and may sediment relatively fast if their density and size are big enough.

Emulsions are defined as micro-heterogeneous systems, constituted by at least one non-miscible liquid dispersed in another one in form of droplets, whose diameter is usually between 50 and 5000 nm.

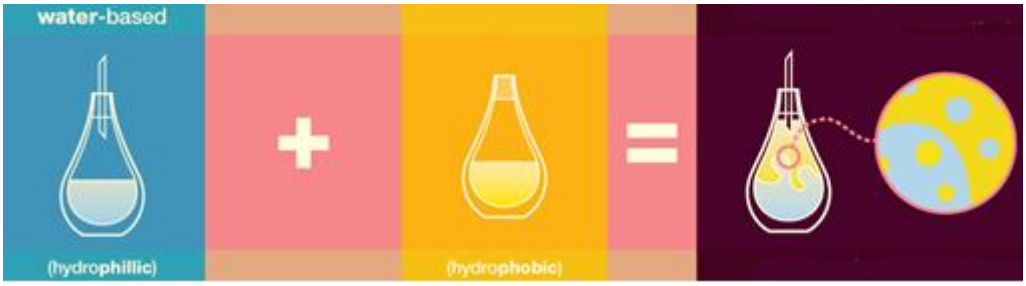


Figure 2.22 – *Emulsion*

Those systems feature a minimum stability, that can be enhanced by adding suitable substances, like surfactants, finely ground solids etc.

Emulsion polymerization is a very interesting technique, whose main features can be listed as:

- Particle size in-between 5 nm and 5 μm.
- Mainly water soluble initiators are used.
- Emulsifiers play a critical role in the polymerization reaction, by keeping the droplets and the particles under continuous, steady suspension.
- The final product is a stable latex.

The basic components of an emulsion polymerization system are **[47]** :

- the monomer (or the monomers in case of a copolymerization)
- the dispersing medium (most commonly water),
- the emulsifier
- the water soluble initiator.

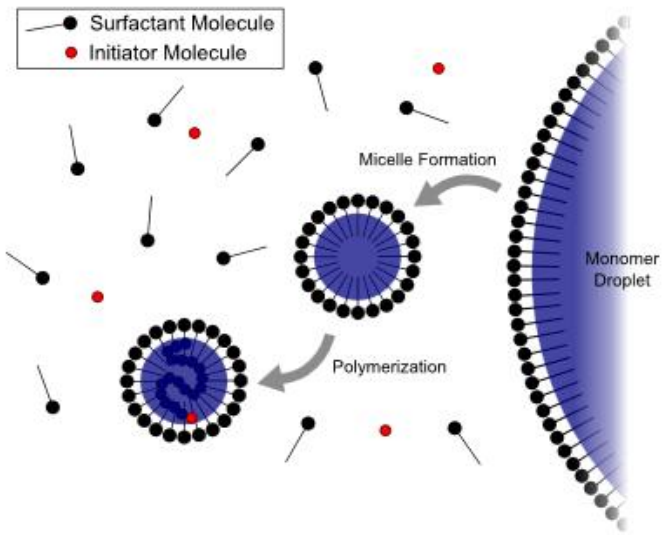


Figure 2.23 – *Emulsion Polymerization*

Both the nature and the amount of the surfactant play an important role in the reaction evolution. If **ionic surfactants** are considered (steric surfactants can also be used),the surfactant molecule carries a net charge on the polymer particles, thus making them repel each other and prevent coagulation. These emulsifiers display the following properties:

•They are water soluble due to their polar head, if their tail is not too long.

•They lower the surface energy between an aqueous and an oil phase, arranging themselves at the interphase in such a way that their polar head is in the aqueous phase, while their hydrocarbon tail in the oil phase.

•At low concentrations these surfactant molecules on the interphase are in equilibrium with the surfactant molecules in the bulk. Above a characteristic concentration, referred to as the **Critical Micelle Concentration** (CMC), aggregates of surfactant molecules are formed, referred to as micelles, which are in equilibrium with free surfactant molecules in bulk. Micelles are constituted by 20-100 surfactant molecules, while their typical diameter is 5-10 nm with their number in the solution being a function of the surfactant concentration.

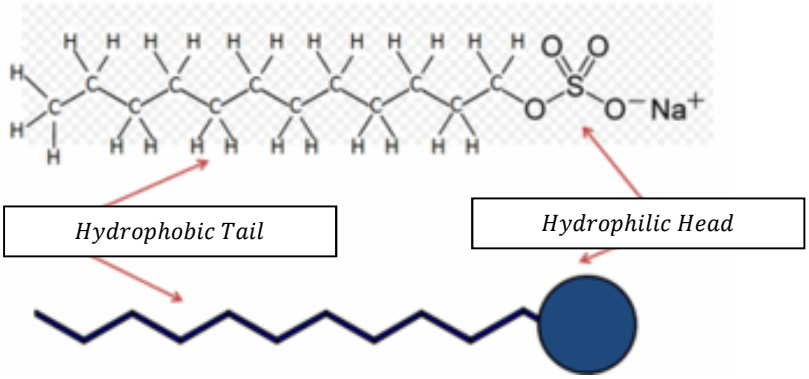


Figure 2.24 -$NaC_{12}H_{25}SO_4$, *Sodium dodecyl sulfate represents an example of ionic surfactant*

The physical picture of emulsion polymerization is based on the original qualitative picture of Harkins (**Figure 2.25**) **[48]**: when in the water-surfactant system a non- or slightly- water soluble monomer is added, the latter is distributed as follows: a small portion stays in the bulk as independent molecules, a greater, yet relatively small, portion enters inside the micelles, as proved by the fact that the micelles grow bigger in size while the monomer is being added. The greatest monomer portion however lies dispersed in form of droplets, whose actual size depends upon the shear rate and is typically between 1-10 μm.

The initiator lies in the aqueous phase, where the free radicals for the initiation of the polymerization appear. The rate of these radicals production is typically of the order of 10^{13}radicals per ml per second.

Monomer droplets are not polymerization sites, given the fact that the initiators used are usually non-monomer soluble.

Therefore the actual polymerization reaction takes place almost exclusively inside the micelles, where the water soluble initiator and the organic, non-water soluble monomer meet. The micelles are actually likely to be the polymerization locus, both due to their high concentration and their much greater surface to volume ratio as opposed to the monomer droplets. Despite the fact that the initiator is water soluble, the added hydrophobic monomers seek an organic, hydrophobic environment, that means the inner part of the micelles. During the

polymerization, the micelles swell. Thus, three types of particles exist in the system:

- monomer droplets
- non active micelles where no polymerization is taking place
- active micelles, where polymerization is taking place.

The system undergoes a major change when a small percentage of the monomer is converted to polymer. Just a small percentage, ca. 0.1%, of the initial micelles is actually activated. While these active micelles grow bigger in size, containing both monomer and polymer, they adsorb increasingly more emulsifier molecules from the bulk. Soon enough the emulsifier concentration in the bulk becomes lower than CMC, thus some of the inactive micelles are destabilized and destroyed to independent molecules **[48].**

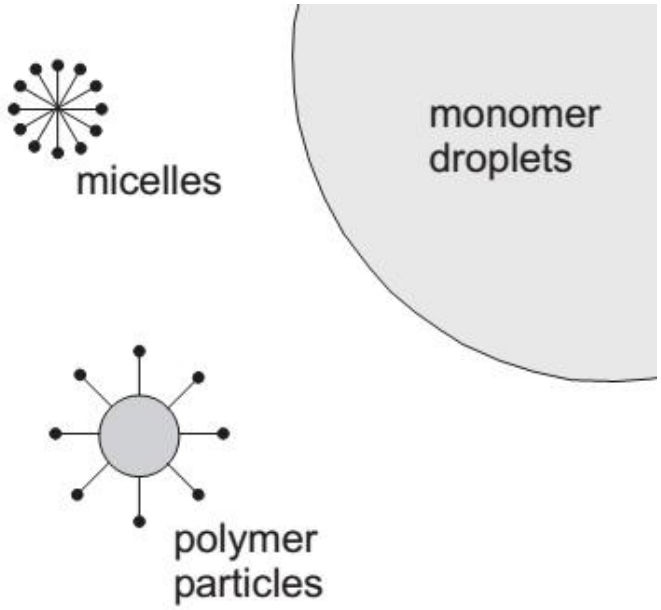


Figure 2.25 – *Picture of Harkins*

At a conversion around 2-15%, depending on each system's nature, the active micelles have grown too much in comparison to the initial ones, therefore they are no longer considered to be micelles, but polymer particles instead. At this stage all inactive micelles have vanished and actually all the emulsifier content has been adsorbed on the polymer particles.

Consequently, the monomer droplets are no longer stable and if the shear would cease, they would aggregate. The polymerization is continued in the polymer particles at the same rate, as the monomer concentration in the latter is kept constant via monomer diffusion from the initial monomer droplets. During this critical stage of the emulsion polymerization, the number of the polymer particles remains constant, while the monomer droplets shrink as the polymer particles grow bigger. This stage is characterized by steady-state conditions.

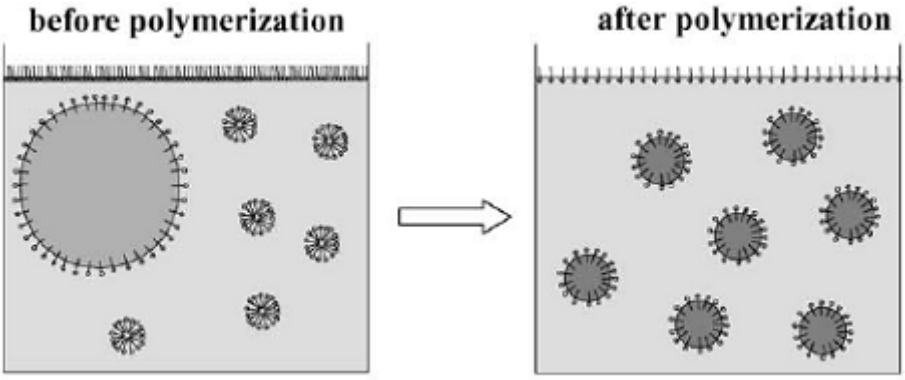


Figure 2.26 – *Growth of polymeric NPs*

Finally, at a conversion of 50-80% the monomer droplets have totally vanished and all the non- reacted monomer is actually contained in the polymer particles. At this stage, the polymerization proceeds with an ever decreasing rate, while the monomer concentration in the polymer particles is continuously decreasing as well. Conversions of up to 100% are fairly common. The final latex diameter is typically 50-200 nm, being a size in-between this of the initial micelles and the initial monomer droplets.

2.3.2 Materials

2.3.2.1 Sodium dodecyl sulfate (SDS)

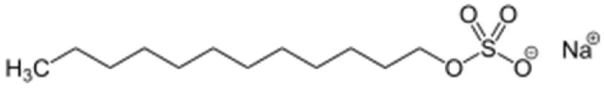


Figure 2.27 - *SDS*

Compound	State	Purity	Melting point	MW
SDS	Solid (powder)	>99%	-206 °C	288.372 g/mol

Sodium dodecyl sulfate (SDS or NaDS), sodium laurilsulfate or sodium lauryl sulfate (SLS) is an organic compound. It is an anionic surfactant used in many cleaning and hygiene products **[50]**.

The salt is of an organosulfate consisting of a 12-carbon tail attached to a sulfate group, giving the material the amphiphilic properties required of a detergent. Being derived from inexpensive coconut and palm oils, it is a common component of many domestic cleaning products.

2.3.2.2 Potassium persulfate (KPS)

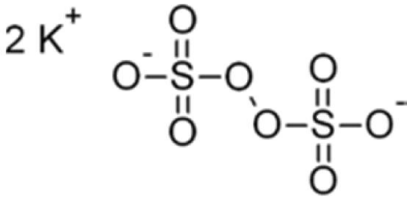


Figure 2.28 - *KPS*

Compound	State	Purity	Melting point	MW
KPS	Solid (powder)	>99%	< 100 °C	270.322 g/mol

Potassium persulfate is an inorganic compound. Also known as potassium peroxydisulfate or KPS, it is a white solid that is highly soluble in water. This salt is a powerful oxidant, commonly used to initiate polymerizations **[51]** .

2.3.2.3 Polyethylene glycol (PEG)

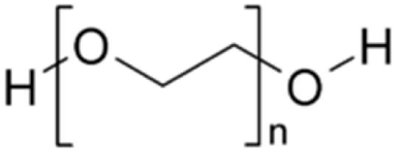


Figure 2.29 - *PEG*

Compound	State	Purity
PEG	Liquid	>99%

Polyethylene glycol (PEG) is a polyether compound with many applications, from industrial manufacturing to medicine **[52]** .

PEGylation is the act of covalently coupling a PEG structure to another larger molecule, for example, for therapeutic necessity. PEG is soluble in water, methanol, ethanol, acetonitrile, benzene, and dichloromethane, and is insoluble in diethyl ether and hexane. It is coupled to hydrophobic molecules to produce non-ionic surfactants.

When attached to various protein medications, polyethylene glycol allows a slowed clearance of the carried protein from the blood. This makes for a longer-acting medicinal effect and reduces toxicity, and allows longer dosing intervals.

2.3.2.4 TWEEN 80

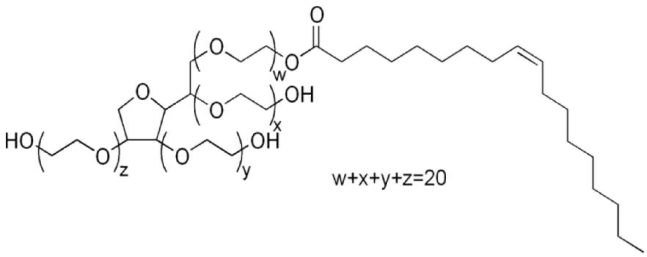


Figure 2.30- *TWEEN 80*

Compound	State	Purity	MW

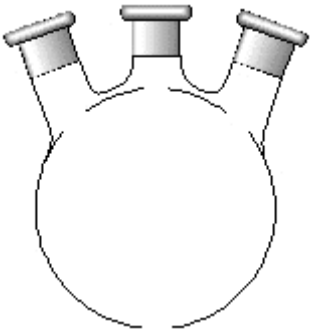
$C_{64}H_{124}O_{26}$	Liquid	>99%	1310 g/mol

Polysorbate 80 (common commercial brand names include Alkest TW 80 and Tween 80) is a polysorbate surfactant whose stability and relative non-toxicity allows it to be used as a detergent and emulsifier in a number of domestic, scientific, and pharmacological applications **[53]**.
Polysorbate 80 is applied in pharmaceutical applications to stabilize emulsions and suspensions.
In this work of thesis it has been used as stabilizing agent.

2.3.3 Experimental Section

2.3.3.1 Batch emulsion polymerization

In order to avoid the formation of undesired aggregates, NPs with 2.5% concentration latex were synthetized.
Relative ratios are based on the macromonomers.



- 10% SDS
- 45 ml H_2O

Have been placed in a three necked flask, under inert nitrogen atmosphere and magnetic stirring. Meanwhile the reactor was heated up to T= 80 °C in an oil bath.

- MACROMONOMER
- 3 ml Solvent (DCM or Ethanol)

Using a non-water soluble solvent (i.e. DCM) allows to promote emulsion polymerization for long-chain macromonomers .

In order to avoid the formation of aggregates, the mixture should be injected slowly.

- 50 mg KPS
- 5 ml H_2O

The reaction time was set to 2.5 hours, since almost complete conversion is reached **[42]** .

The following table summarizes the NPs synthetized and the solvent applied for each reaction.

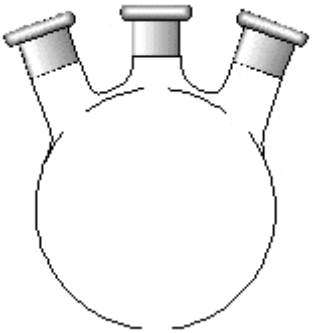
PLA NPs	
Macromonomer	**Solvent**
HEMA ETL LA_0	Ethanol
HEMA ETL LA_4	Ethanol
HEMA ETL LA_6	DCM
HEMA ETL LA_8	DCM

PLGA NPs	
Macromonomer	**Solvent**
HEMA ETL $PLGA_4$	Ethanol
HEMA ETL $PLGA_6$	DCM
HEMA ETL $PLGA_8$	DCM

Table 2.5–Hema ETL LA_X and $PLGA_X$ NPs

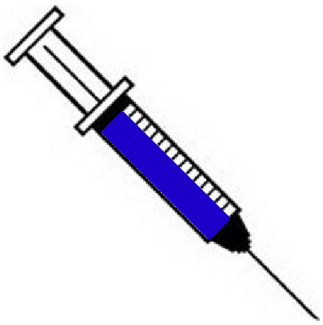
2.3.3.2 Semibatch emulsion polymerization

PEGylated particles represent a further step in the characterization of the PLA-PLGA macromonomers and of the study of their possible application as biomedical vehicles.The procedure through which PEGylation was achieved is reported:



- TWEEN
- PEG 2000
- 40 ml H2O

Have been placed in a three necked flask, under inert nitrogen atmosphere and magnetic stirring. Meanwhile the reactor was heated up to T= 80 °C in an oil bath.



- MACROMONOMER
- 2 ml Solvent (DCM)

The solvent allows to handle a homogeneous system, where reactants can fully interact.

Macronomomer and solvent need to be fed with a syringe pump, whose rate was set to 3 ml/h.

- 50 mg KPS
- 2.5 ml H_2O

The reaction was stopped after 2.5 h.

2.4 NPs for biomedical applications

Biodegradable nanoparticles are frequently used to improve the therapeutic value of various water soluble/insoluble medicinal drugs and bioactive molecules by improving bioavailability, solubility and retention time **[49]**. These nanoparticle–drug formulation reduces the patient expenses and risks of toxicity. Biodegradable polymeric nanoparticles are highly preferred because they provide controlled/sustained release property, subcellular size and biocompatibility with tissue and cells. Apart from this, these nanomedicines are stable in blood, non-toxic, nonthrombogenic, nonimmunogenic, noninflammatory, do not activate neutrophils, biodegradable, avoid reticuloendothelial system and applicable to various molecules such as drugs, proteins, peptides, or nucleic acids. The general synthesis and encapsulation of biodegradable nanomedicines are represented in **Figure 2.27** and better reported in **Chapter 1** . Drug molecules are either bound to surface as nanosphere or encapsulated inside as nanocapsules **[49].**

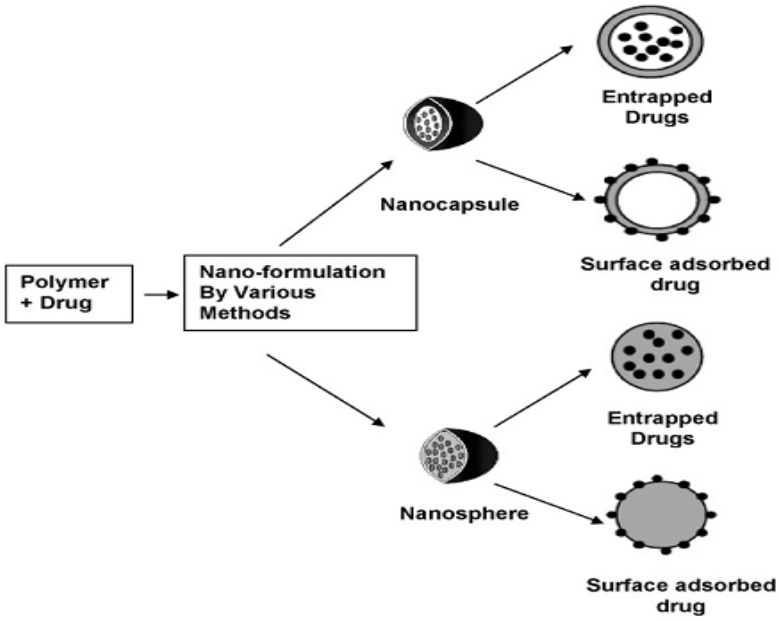


Figure 2.31 – *Type of biodegradable nanoparticles. According to the structural organization biodegradable particles are classified as nanoparticles and nanospheres. The drug molecules are either entrapped inside or adsorbed on the surface.*

The most widely used polymers for nanoparticles are poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and their co-polymers, poly(lactide-co-glycolide) (PLGA). These polymers are known for both their biocompatibility and resorbability through natural pathways. Additionally, the degradation rate and accordingly the drug release rate can be influenced, as stated in **[49]** and **[32]** .

Macromonomers used to synthetized NPs:	
Hema-ETL LA_0	**-**
Hema-ETL LA_4	Hema-$PLGA_4$
Hema-ETL LA_6	Hema-$PLGA_6$
Hema-ETL LA_8	Hema-$PLGA_8$

Table 2.6 – *Final Hema ETL LA_X and $PLGA_X$ macromonomers*

2.5 Degradation study

As final part of this work, a preliminary degradation study of these materials was performed. In order to verify macromonomers properties, degradation in Water was performed at first.

PBS Stability Study and Cell Medium degradation were realized to demonstrate PEGylated NPs stability and degradation features.

Degradation study was carried out as:

- Degradation in H_2O;
- Degradation in PBS (stability study);
- Degradation in Cell medium.

2.5.1 Degradation Study in H_2O

Degradation study was first lead in H_2O. It revealed a valuable way to better understand NPs properties. NPs produced have been placed in cuvettes and heated up to 50 $\pm$ 1°C. Degradation grade was characterized at different times. Particles size measurements were performed through dynamic light scattering (Malvern, Zetanano ZS) **[42]** over one month.

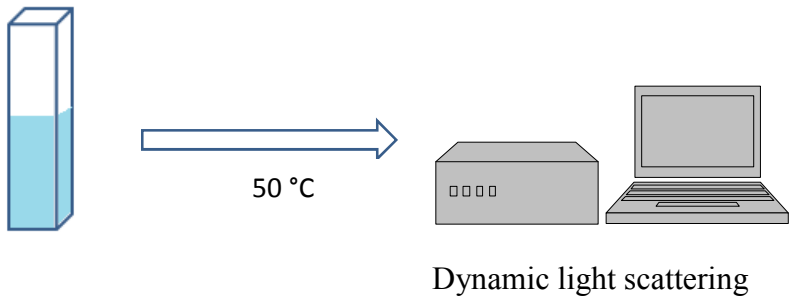


Figure 2.32 – *Schematic representation of Dynamic Light scattering used during the degradation study*

2.5.1.1 Experimental section- Degradation in H_2O

Degradation Study has been performed with samples diluted to1%:

- 1.2 ml H_2O;
- 0.8 ml macromonomers;

have been used. Measures were realized each day.

2.5.2 Stability Study in Phosphate buffered saline (PBS)

PEGylated NPs were subjected to PBS Stability study.We are expecting results comparable to what has been obtained with the degradation study in H_2O, that means NPs resistance at least one month long.
Degradation grade has been characterized at different times. Particles size measurements were performed through dynamic light scattering (Malvern, Zetanano ZS).

2.5.2.1 Phosphate buffered saline (PBS)

Phosphate buffered saline (abbreviated PBS) is a buffer solution commonly used in biological research **[54]** . It is a water-based salt solution containing sodium phosphate, sodium chloride and, in some formulations, potassium chloride and potassium phosphate. The osmolarity and ion concentrations of the solutions match those of the human body (isotonic).
PBS has many uses because it is isotonic and non-toxic to most cells. These applications include substance dilution and cell container rinsing.

2.5.2.2 Experimental section

Stability study was performed with the following operative conditions:

In a cuvette:

- 1ml PBS
- 1ml NPs

During the first day, 2 measurements where performed. In the following weeks about 2 or 3 measurements for week were realized. DLS measurements were carried out at 37°C using 137° backscatter angle. After the first measure, values of attenuator and measure position have been kept constant. This permitted to perform the analysis with standardized operating condition, thus optimizing particles characterization:

NPs	Attenuator	Measure position
PEG Hema ETL LA_0	6	4,65
PEG Hema ETL LA_4	6	4,65
PEG Hema ETL LA_6	7	4,65
PEG Hema ETL LA_8	7	4,65
PEG Hema ETL $PLGA_4$	5	4,65
PEG Hema ETL $PLGA_6$	7	4,65
PEG Hema ETL $PLGA_8$	7	1,25

Table 2.7–*Attenuator and Measure position, PBS stability study*

2.5.3 Degradation Study in Cell Medium

PEGylated NPs degradation rate was also tested in Cell medium. It represents a degradation study. We are expecting a quicker degradation compared to H_2O degradation study.

2.5.3.1 Cell medium

Is intended to simulate the interaction between NPs and blood stream. Usually cell culture media contains phenol red, a pH indicator that will turn yellow at low pH and purple at high pH.

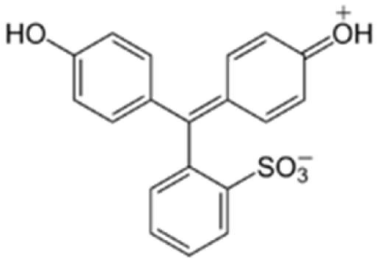


Figure 2.33- *Phenol red as pH indicator*

Living tissues grow usually at a near-neutral pH; that is, a pH close to 7. The pH of blood ranges from 7.35 to 7.45, for instance. When cells are grown in tissue culture, the medium in which they grow is held close to this physiological pH. A small amount of phenol red added to this growth medium will have a pink-red color under normal conditions.

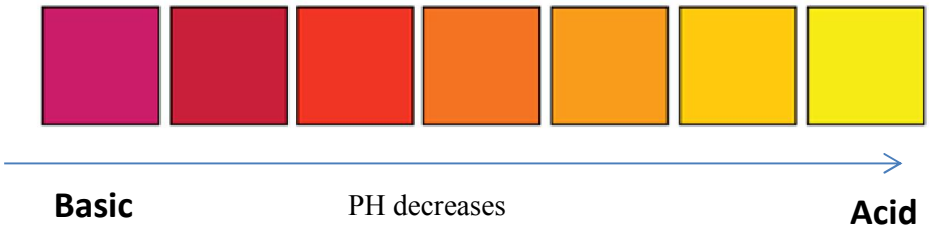


Figure 2.34- *PH and colours correspondences*

2.5.3.2 Experimental section

Degradation was performed with the following operative conditions:

- 1ml Medium
- 1mlNPs

Degradation grade has been characterized at different times: particles size measurements were performed through dynamic light scattering (Malvern, Zetanano ZS) 3 times a day.
DLS measurements were carried out at 37°C using 137° backscatter angle. After the first measure, values of attenuator and measure position have been kept constant. This permitted to perform the analysis with standardized operating condition, thus optimizing particles characterization:

NPs	Attenuator	Measure position
PEG Hema ETL LA_0	6	4,65
PEG Hema ETL LA_4	6	1,25
PEG Hema ETL LA_6	6	4,65
PEG Hema ETL LA_8	7	4,65
PEG Hema ETL $PLGA_4$	7	1,25
PEG Hema ETL $PLGA_6$	7	4,65
PEG Hema ETL $PLGA_8$	6	4,65

Table 2.8–*Attenuator and Measure position, Cell medium degradation*

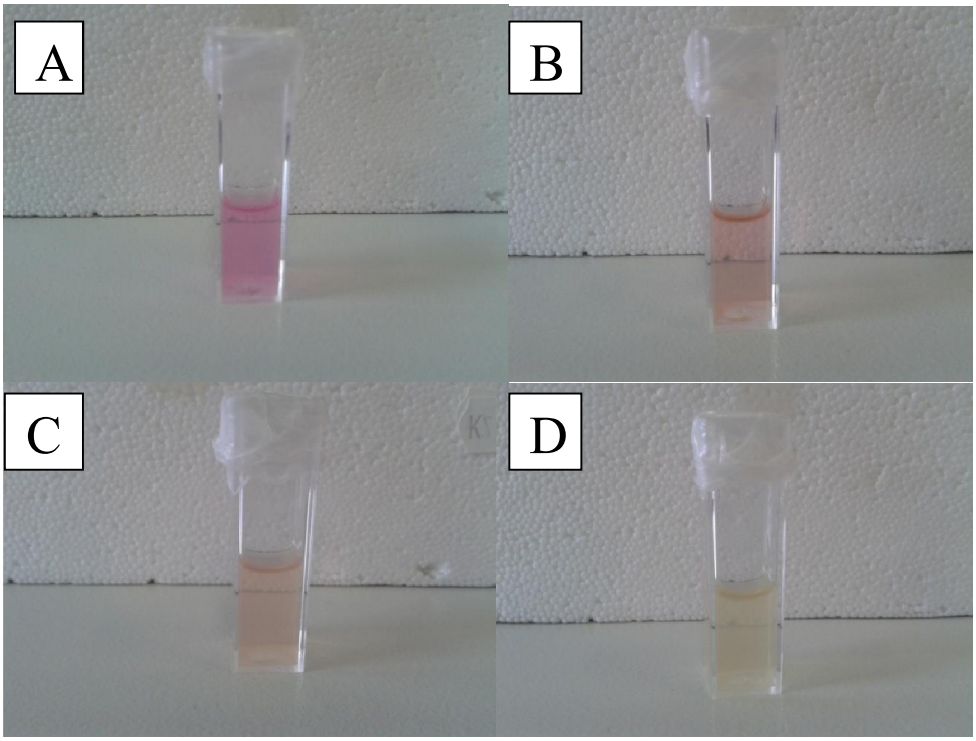


Figure 2.35- *The figure represents the phases which scan the degradation steps: A) initial phase; B) and C) point out two sequential stages of degradation; D) final phase, when NPs have already deteriorated. In this case we expect that light scattering does relieve no more particles, but micelles. Micelles formation denote NPs disruption indeed.*

2.6 Characterization techniques

In order to have complete picture of the situation, macromonomers and NPs have been investigated with:

- H –NMR
- Dynamic Light Scattering

2.6.1 HNMR

Proton NMR (also Hydrogen-1 NMR, or 1H NMR) is the application of nuclear magnetic resonance in NMR spectroscopy with respect to hydrogen-1 nuclei within the molecules of a substance, in order to determine the structure of its molecules **[55]** .In samples where natural hydrogen (H) is used, practically all the hydrogen consists of the isotope 1H (hydrogen-1; i.e. having a proton for a nucleus). A full 1H atom is called protium.
Nuclear magnetic resonance (NMR) is a physical phenomenon in which nuclei in a magnetic field absorb and re-emit electromagnetic radiation.

- A spinning charge generates a magnetic field. The resulting spin-magnet has a magnetic moment (μ) proportional to the spin.

- In the presence of an external magnetic field (**B0**), two spin states exist, **+1/2** and **-1/2**.

- The difference in energy between the two spin states is dependent on the external magnetic field strength, and is always very small. **Figure 2.39** illustrates that the two spin states have the same energy when the external field is zero, but diverge as the field increases.

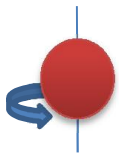


Figure 2.36- Magnetic moment

Strong magnetic fields are necessary for NMR spectroscopy. The international unit for magnetic flux is the tesla (**T**).

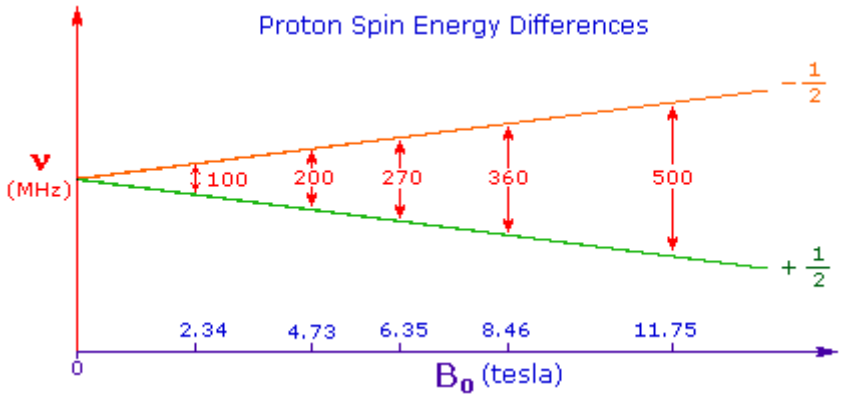


Figure 2.37 - *Two spin states have the same energy when the external field is zero, but diverge as the field increases*

The NMR spectrometer must be tuned to a specific nucleus. The procedures used to obtain the spectrum vary, but the simplest is known as the continuous wave (CW) method. A solution of the sample in a uniform 5 mm glass tube is oriented between the poles of a powerful magnet, and is spun to average any magnetic field variations.

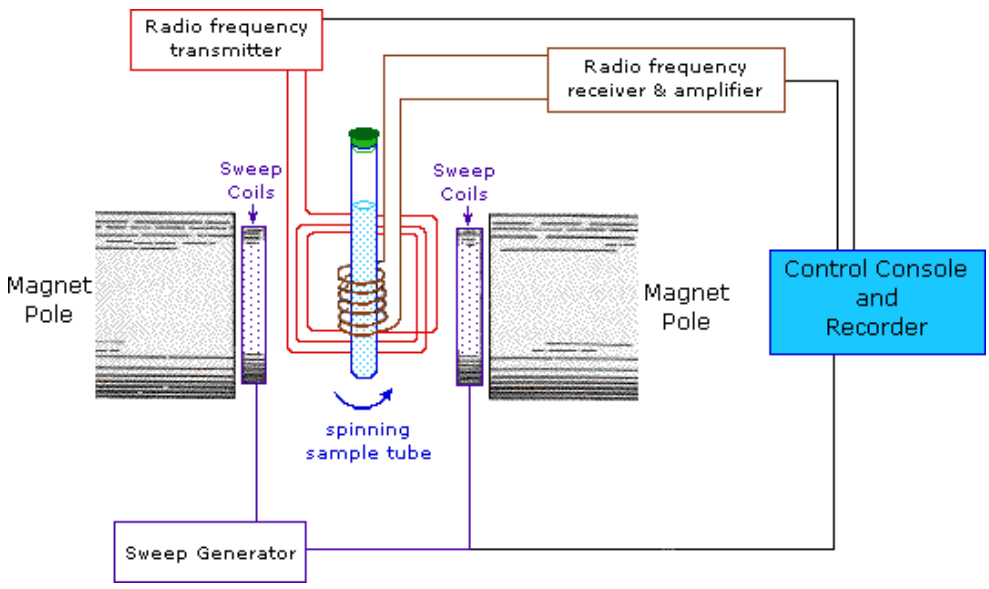


Figure 2.38 – *HNMR schematic inner structure*

Since electrons are charged particles, they move in response to the external magnetic field (Bo) so as to generate a secondary field that opposes the much stronger applied one. This secondary field shields the nucleus from the applied field, so Bo must be increased in order to achieve resonance. This is the reason why the proton nuclei in different compounds behave differently in the NMR experiments **[56]** .

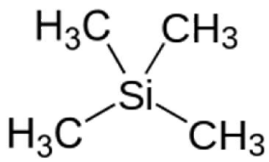


Figure 2.39 – *TMS*

Simple NMR spectra are recorded in solution, and solvent protons must not be allowed to interfere. Deuterated (deuterium = 2H, often symbolized as D) solvents especially for use in NMR are preferred, e.g. deuterated water, D_2O, deuterated acetone, $(CD_3)2CO$, deuterated methanol, CD_3OD, deuterated dimethyl sulfoxide, $(CD_3)_2SO$, and deuterated chloroform, $CDCl_3$. However, a solvent without hydrogen, such as carbon tetrachloride, CCl_4 or carbon disulphide, CS_2, may also be used.

Historically, deuterated solvents were supplied with a small amount (typically 0.1%) of tetramethylsilane (TMS) as an internal standard for calibrating the chemical shifts of each proton. TMS is a tetrahedral molecule, with all protons being chemically equivalent, giving one single signal, used to define a chemical shift = 0 ppm. It is volatile, making sample recovery easy as well. Modern spectrometers are able to refer to spectra based on the residual proton in the solvent (e.g. the $CHCl_3$, 0.01% in 99.99% $CDCl_3$). Deuterated solvents are now commonly supplied without TMS.

Proton NMR spectra of most organic compounds are characterized by chemical shifts in the range +14 to -4 ppm and by spin-spin coupling between protons. The integration curve for each proton reflects the abundance of the individual protons.

Chemical shift is the resonant frequency of a nucleus relative to a standard. Often the position and number of chemical shifts are diagnostic of the structure of a molecule. Important factors influencing chemical shift are electron density and electronegativity of neighboring groups. Hydrogen nuclei are sensitive to the hybridization of the atom to which the hydrogen atom is attached and to electronic effects. Nuclei tend to be shielded by groups which withdraw electron density. Deshielded nuclei resonate at higher δ values, whereas shielded nuclei resonate at lower δ values **[57]** .

Considering TMS as reference value, we can assume to read the different groups in the spectrum as follows:

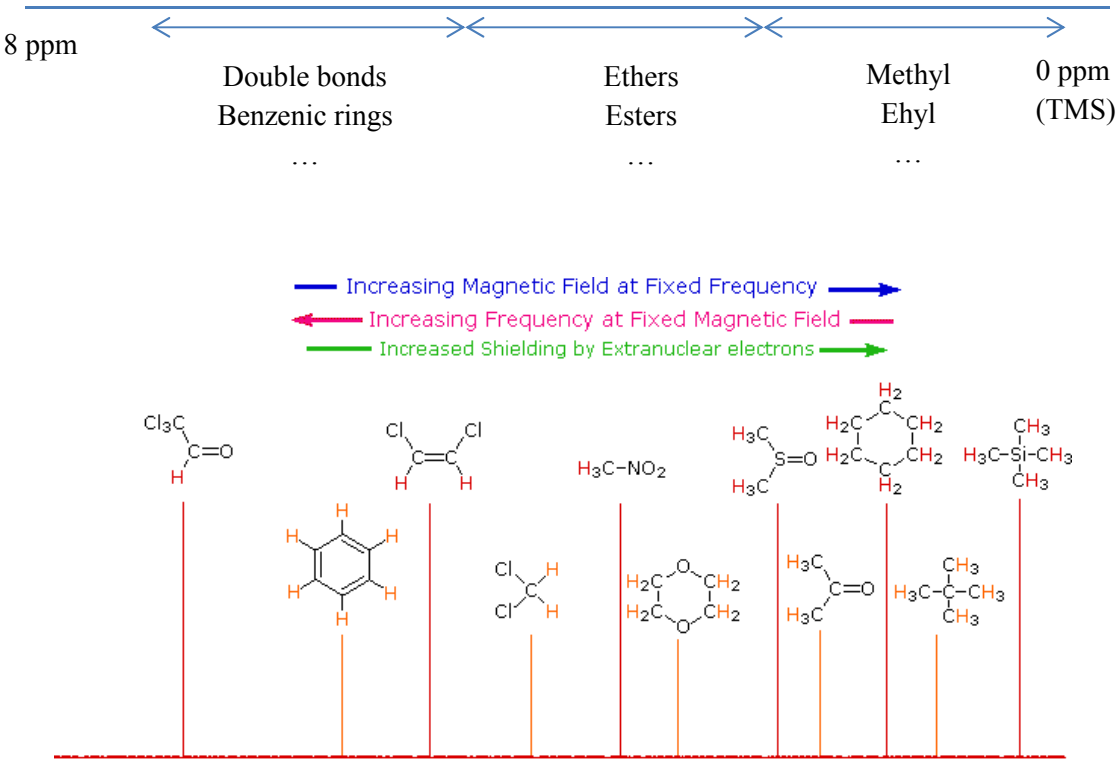


Figure 2.40 – *HNMR Resonance Signals for some different compounds*

Ppm stands for parts per million, and it is the unit used to measure chemical shift. The proton chemical shifts range from 0 ppm to 15 ppm. The chemical shift is identical for a specific proton regardless of the spectrometer used. The formula for the chemical shift is:

$$\delta = \frac{distance\ downfield\ from\ TMS\ (Hz)}{operation\ frequency\ of\ the\ spectrometer\ (MHz)} = ppm \qquad \textbf{(2.8)}$$

In this case H-NMR has been used to confirm the structure of the macromonomers and to determine reactants conversion.

Similar functional groups have similar chemical shifts. However shift numbers cannot be used to assign proton types to NMR signals. On the characteristic

proton NMR chemical shifts table, two molecules with the same functional group may have different chemical shifts. This could be due to many factors, such as being positioned near an electronegative atom.

2.6.2 Dynamic Light scattering

Light scattering represents a consequence of the interaction of light with the electric field of a small particle or molecule. Scattering of light depends on the wavelength or frequency of the light being scattered **[58]** .

We can discriminate between **Static light scattering and dynamic light scattering**:

In static light scattering, the experimental variable is the time-average intensity of scattered light, whereas in dynamic light scattering it is the fluctuations in light intensity that are studied.

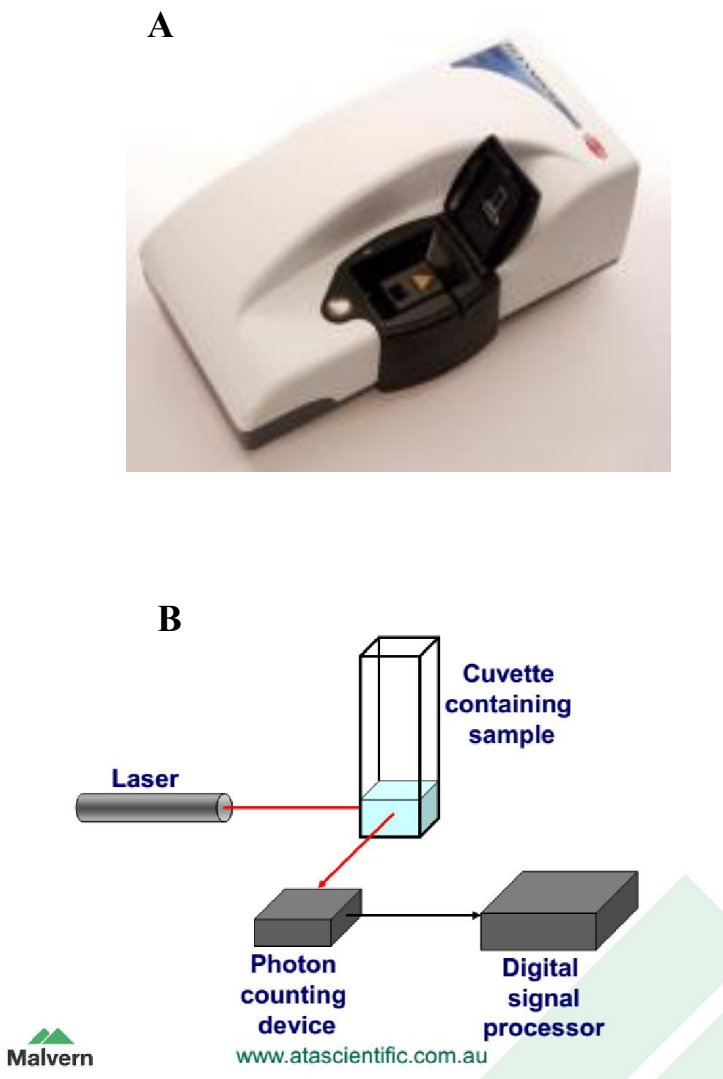


Figure 2.41 – *Dynamic Light scattering (Malvern): A) external structure, B) inner mechanism:interaction between laser and cuvette.*

Dynamic light scattering is a technique that can be used to determine the size distribution profile of small particles in suspension or polymers in solution.

When light hits small particles, it scatters in all directions (**Rayleigh scattering**) as long as the particles are small compared to the wavelength (below 250 nm).

If the light source is a laser, and thus is monochromatic, the scattering intensity fluctuates over time. This fluctuation is due to the fact that the small molecules in solutions are undergoing Brownian motion. For this reason the distance between the scatters in the solution is constantly changing. Scattered light undergoes then either constructive or destructive interference by the surrounding particles, and within this intensity fluctuation, information is contained about the time scale of movement of the scatterers. The dynamic information of the particles is derived from an autocorrelation of the intensity trace recorded during the experiment. The second order autocorrelation curve is generated from the intensity trace as follows **[58]**:

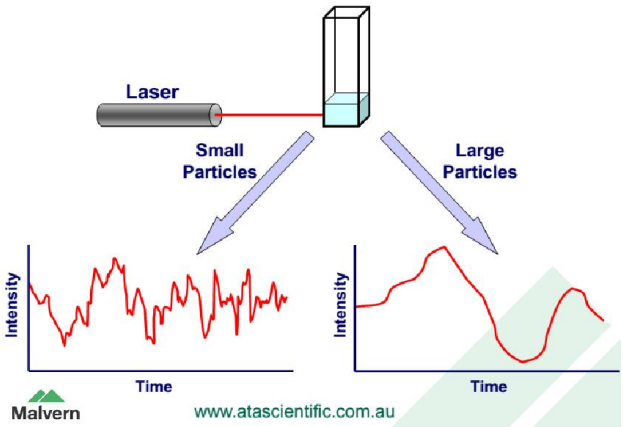


Figure 2.42– *Intensity signals received by the DLS*

$$g^2(q;\tau) = \frac{<I(t)I(t+\tau)>}{<I(t)>^2} \quad \textbf{(2.9)}$$

where $g^2(q;\tau)$ is the autocorrelation function at a particular wave vector, q, and delay time, τ, and I is the intensity. The angular brackets <> denote the expected value operator, which in some texts is denoted by a capital E.

At short time delays, the correlation is high because the particles do not have the chance to move to a great extent from the initial state that they were in. The two signals are thus essentially unchanged when compared after only a very short time interval. As the time delays become longer, the correlation decays exponentially, meaning that, after a long time period has elapsed, there is no correlation between the scattered intensity of the initial and final states. This exponential decay is related to the motion of the particles. To fit the decay (i.e., the autocorrelation function), numerical methods are used, based on calculations of assumed distributions. If the sample is monodisperse then the decay is simply a single exponential. The **Siegert equation** relates the second-order

autocorrelation function with the first-order autocorrelation function $g^1(q;\tau)$ as follows **[58]:**

$$g^2(q;\tau) = 1 + \beta[g^1(q;\tau)]^2 \quad \textbf{(2.10)}$$

where the parameter **β** is a correction factor that depends on the geometry and alignment of the laser beam in the light scattering setup. It is roughly equal to the inverse of the number of speckle from which light is collected. A smaller focus of the laser beam yields a coarser speckle pattern, a lower number of speckle on the detector, and thus a larger second order autocorrelation.

The most important use of the autocorrelation function is its use for size determination.

DLS is used to characterize size of various particles including proteins, polymers, micelles, carbohydrates, and nanoparticles. If the system is monodisperse, the mean effective diameter of the particles can be determined. This measurement depends on the size of the particle core, the size of surface structures, particle concentration, and the type of ions in the medium.

Since DLS essentially measures fluctuations in scattered light intensity due to diffusing particles, the diffusion coefficient of the particles can be determined. DLS software of commercial instruments typically displays the particle population at different diameters. If the system is monodisperse, there should only be one population, whereas a polydisperse system would show multiple particle populations.

Stability studies can be done conveniently using DLS. Periodical DLS measurements of a sample can show whether the particles aggregate over time by seeing whether the hydrodynamic radius of the particle increases. If particles aggregate, there will be a larger population of particles with a larger radius **[58]** .

3. Results

All the results that allowed the characterization of the synthetized materials are described in the following Chapter. The way the sections are organized reflects the sequential steps in which the work has been developed.

Investigation techniques and general meaning of the information recorded can be summarized as follows:

- **H-NMR** permits to gather information about samples composition and reactions conversion, thus investigating the procedures executed to obtain the macromonomers;
- **Dynamic Light Scattering (DLS)** allows evaluating both NPs Synthesis and Degradation phases, by giving an estimate of Particle Size Distribution, Polydispersity Index and Count Rate.
- **PH variations** give additional information about NPs degradation environment.

3.1 H- NMR Results

General guidelines to comment H-NMR spectra can be summarized as :

- Enumerating the peaks: each one corresponds to physically different Hydrogen atoms within the molecule.
- Calculation of peaks corresponding area and comparison with the number of H contained in the formula, in order to understand how many H are related to every single peak.
- Analysis of **Chemical Shift** data: it refers to the position on the δ scale (in ppm), where the peak occurs. Different position are a sign of different chemical environments (see **Figure 3.1**)
- Analysis of **Multiplicity** (m): the closeness of other "n" H atoms, brings about the signals to be split into "n+1" lines indeed.

$$number\ of\ adjacent\ hydrogens\ = m - 1$$

- Definition of the configuration to which the molecule could be associated.

- Verifying Chemical Schift, Multiplicity and Peaks area compatibility.

All the synthetized macromonomers were analyzed with H-NMR .$CDCl_3$ was used as solvent. Before considering macromonomers spectra, the way the different groups should be identified in the spectrum is schematically reminded in **Figure 3.1**.

Results are reported below.

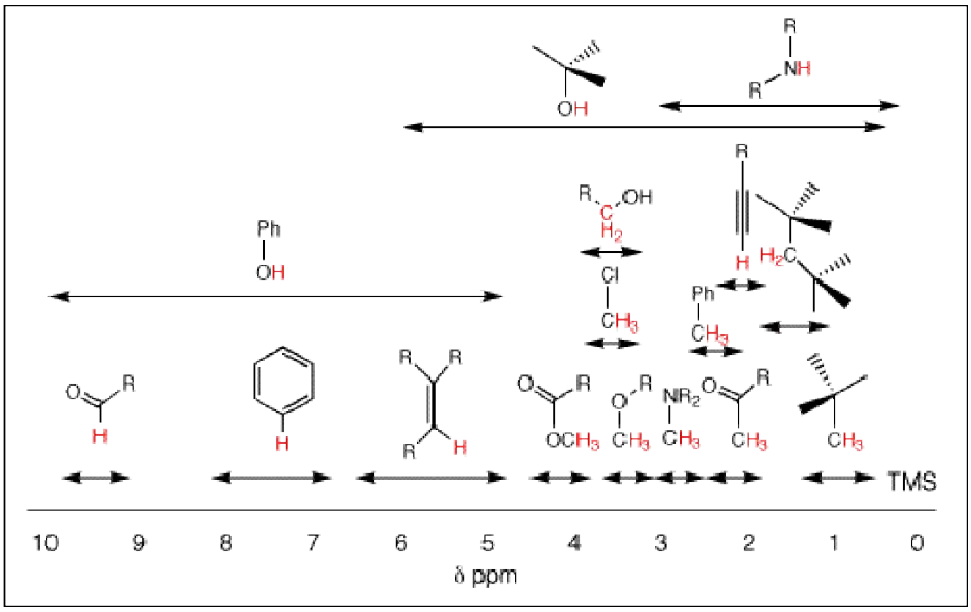


Figure 3.1 – *HNMR Resonance Signals for some different compounds*

3.1.1 ROP of Lactide: HNMR spectra analysis

With the purpose of explaining in detail the results obtained, ETL LA_4 spectrum is reported.

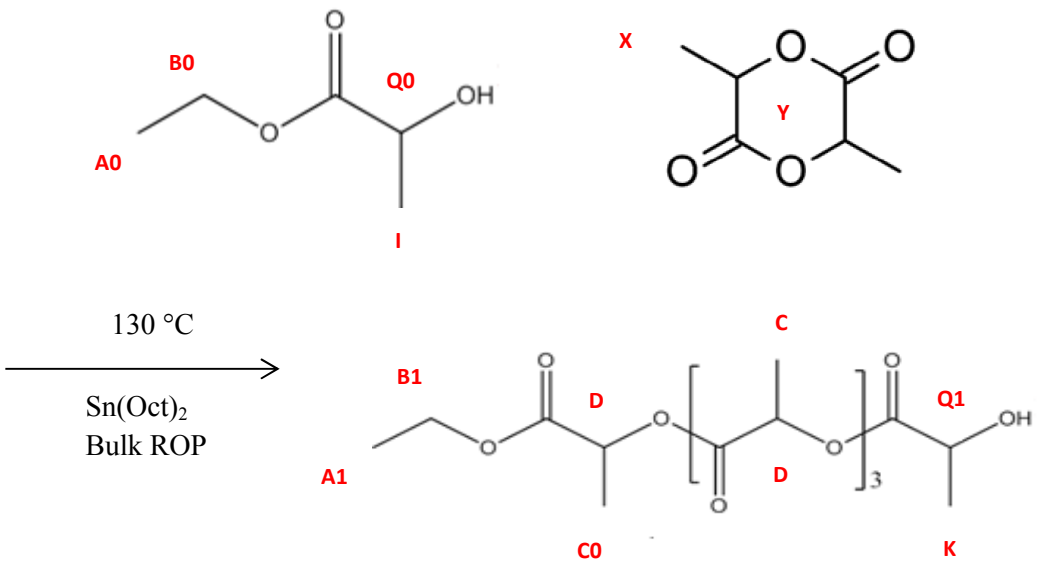


Figure 3.2 – *ROP ETL LA_4*

Figure 3.2 reports reactants and products of the ROP of Lactide. H-NMR spectrum is referred here to a confirmation tool of the chemical structure of the macromonomers obtained.

In order to set relative ratios that correlate the different protons, a unit of reference needs to be fixed.In this case Hydrogen in position **Q1** results conveniently usable, since in Q1 we expect 1 H only. According to the NMR chart, which reports the different chemical shifts, Q1 is expected to be around 4 ppm. Integration corresponding to Q1 goes from 4.39 to 4.31 ppm. **Figure 3.3** represents an enlargement of the examined spectrum.

Multeplicity can now be considered. **Multiplicity** refers indeed to the number of neighboring hydrogen atoms. It represents one of the most important pieces of information, since it allows to identify molecular structures. In this case $m = 4$

$$number\ of\ adjacent\ hydrogens\ to\ Q1 = m - 1 = 3$$

Q1 position is now completely identified. The spectrum can be examined on the base of Q1 relative integration.

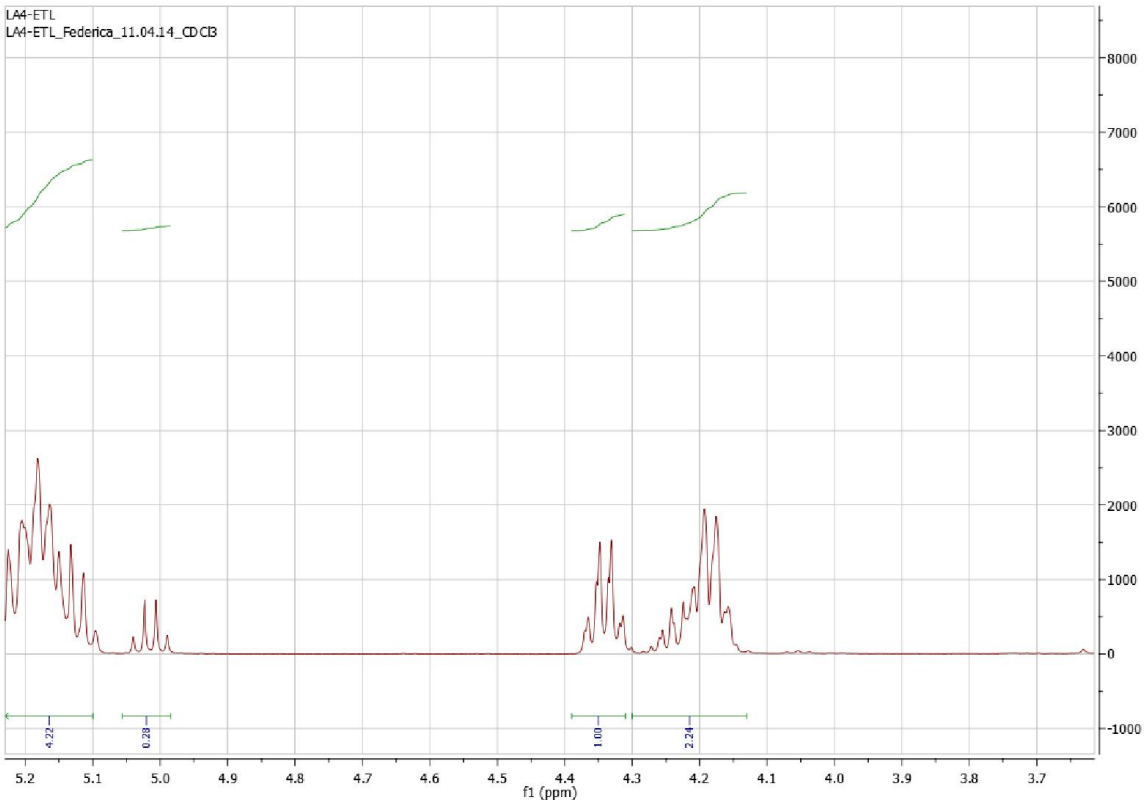


Figure 3.3 – *Enlargement of ETL LA_4 spectrum. Q1 Multiplicity highlights 4 neighboring hydrogen atoms, m=4*

Considering now all the integrations related to ETL LA_4 macromonomer, reported in **Figure 3.4**, it is possible to draw the following conclusions:

$$Conversion\ of\ Lactide: \frac{Peak\ \frac{D}{2}}{Peak\ \frac{D}{2} + Peak\ Y} \sim 90\%$$

$$where\ Peak\ Y = Peak\ X/3$$

Note:

- $Peak\ \frac{D}{2}$ takes into consideration the contribution given by the dimer structure of Lactide. It corresponds to the reactant converted.
- $Peak\ Y$ refers to H belonging to residual, non-converted Lactide.
- $(Peak\ \frac{D}{2} + Peak\ Y)$ indicates the whole initial amount of hydrogen atoms introduced as the reaction started.

$$Average\ Lactic\ Acid\ units\ added\ to\ ETL: \frac{Peak\ D}{Peak\ Q1} = 4.22$$

Note:

Average Lactic Acid units added to ETL are identified by considering the relation between the number of hydrogen atoms contained in the repeating elements and the reference unit Q1 (corresponding to H=1).

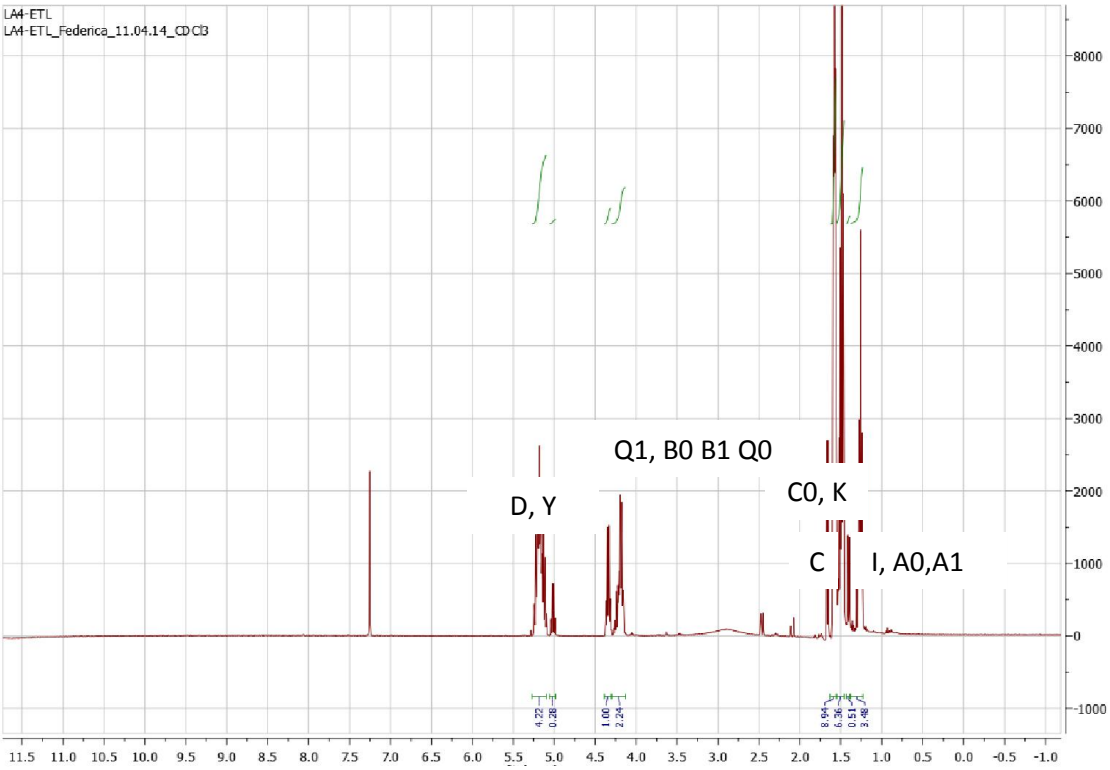


Figure 3.4 –*ETL LA_4 spectrum starting from Q1 relative integration.*

The same method can be applied to characterize all the other spectra obtained.

In **Table 3.1** conversion data and Average Lactic Acid Units added to ETL are reported:

Macromonomer	Conversion	Average Lactic Acid Units added to ETL
ETL LA_4	90 %	4.22
ETL LA_6	82 %	6.15
ETL LA_8	86 %	8.31

Table 3.1 –*ETL LA_X conversion data and average repeating units*

3.1.2 ROP of Lactide and Glycolide: HNMR spectra analysis

As already mentioned in the preceding case, it is possible to characterize ETL $PLGA_X$ macromonomers, starting from the information available from the H-NMR spectra. ETL $PLGA_4$ is taken as representative example:

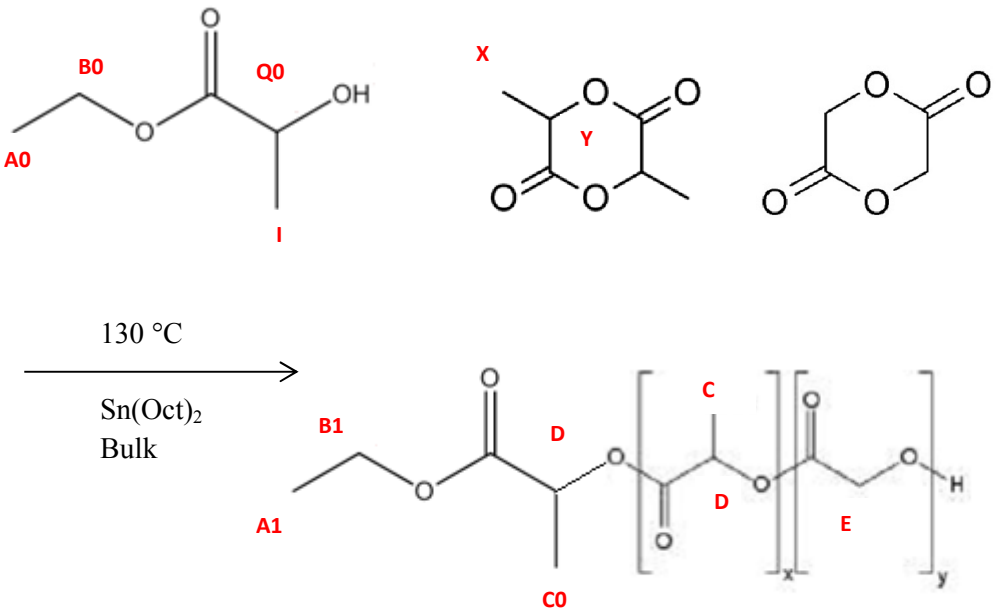


Figure 3.5 – *ROP ETL $PLGA_4$*

In this case a reference unit is more difficult to isolate.
Calculations are based on the Hydrogen atom present in position **I,** although it has to be considered that in I a methyl group is located (3H).

Figure 3.3 reports peaks integration for ETL $PLGA_4$

$$Conversion\ of\ Lactide: \frac{Peak\ \frac{D}{2}}{Peak\ \frac{D}{2} + Peak\ Y} \sim 86\%$$

$$where\ Peak\ Y = Peak\ X/3$$

Note:

- $Peak\ \frac{D}{2}$ takes into consideration the contribution given by the dimer structure of Lactide. It corresponds to the reactant converted.
- $Peak\ Y$ refers to H belonging to residual, non-converted Lactide.
- $(Peak\ \frac{D}{2} + Peak\ Y)$ indicates the whole initial amount of hydrogen atoms introduced as the reaction started.

In **Table 3.2** conversion data are reported:

Macromonomer	Lactide Conversion
ETL $PLGA_4$	86%
ETL $PLGA_6$	84%
ETL $PLGA_8$	80%

Table 3.2 –*ETL $PLGA_X$ conversion data*

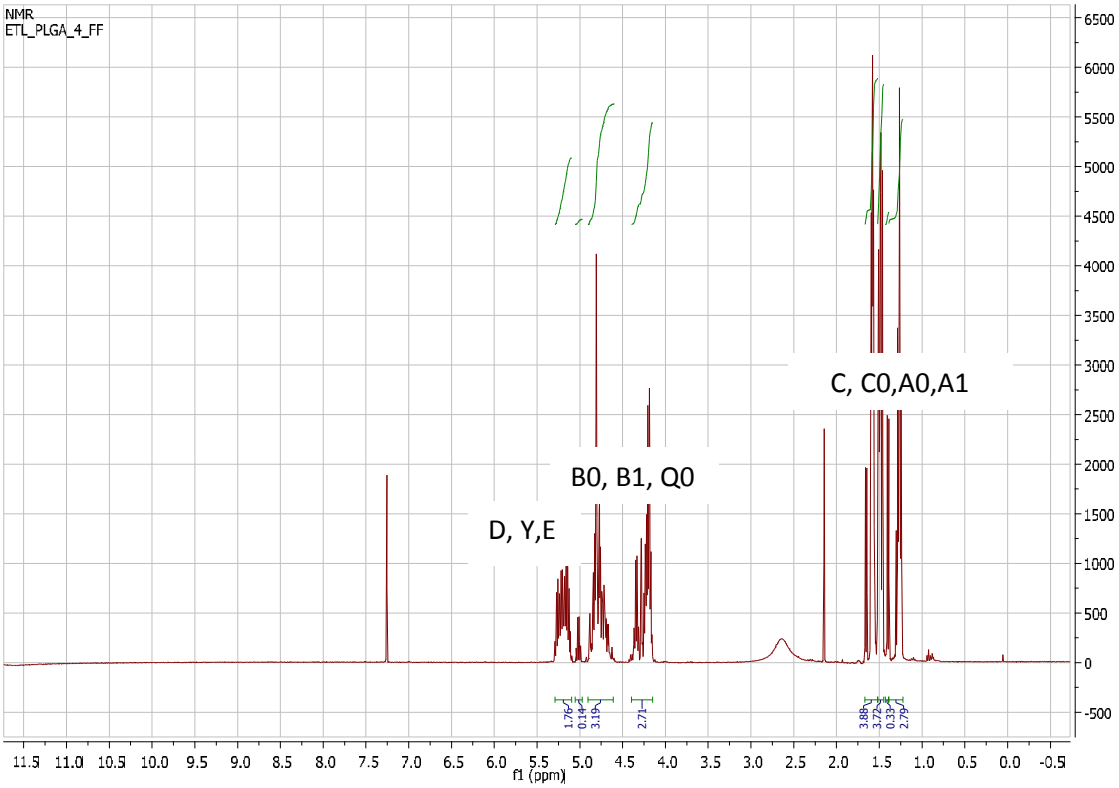


Figure 3.6 –*ETL PLGA$_4$ NMR spectrum.*

3.1.3 PLA-based macromonomers: Coupling Reaction

In this case Hema ETL LA_4 can be assumed as an example, to characterize PLA-based macromonomers coupling reactions:

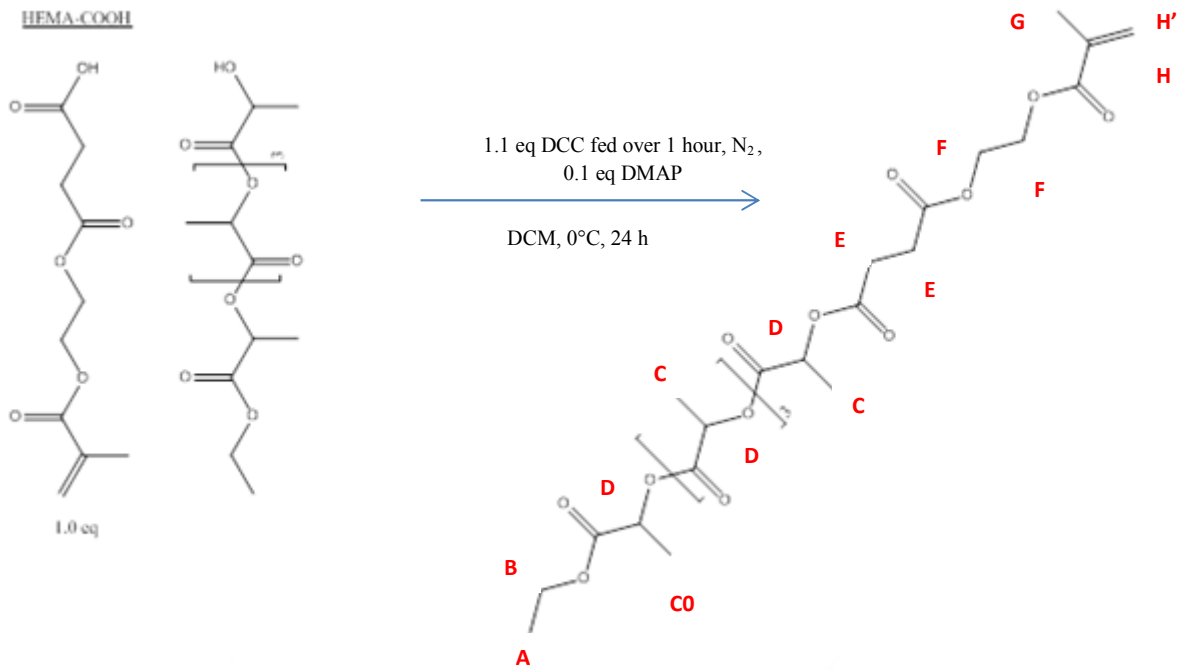


Figure 3.7 – *ETL LA_4 coupling reaction*

In this case Hydrogen atom in position **H** result conveniently usable as a reference. According to the NMR chart, which reports the different chemical shifts, **H** is expected to be around 6 ppm. **Figure 3.8** represents an enlargement of the examined spectrum.

In this case $m = 1$

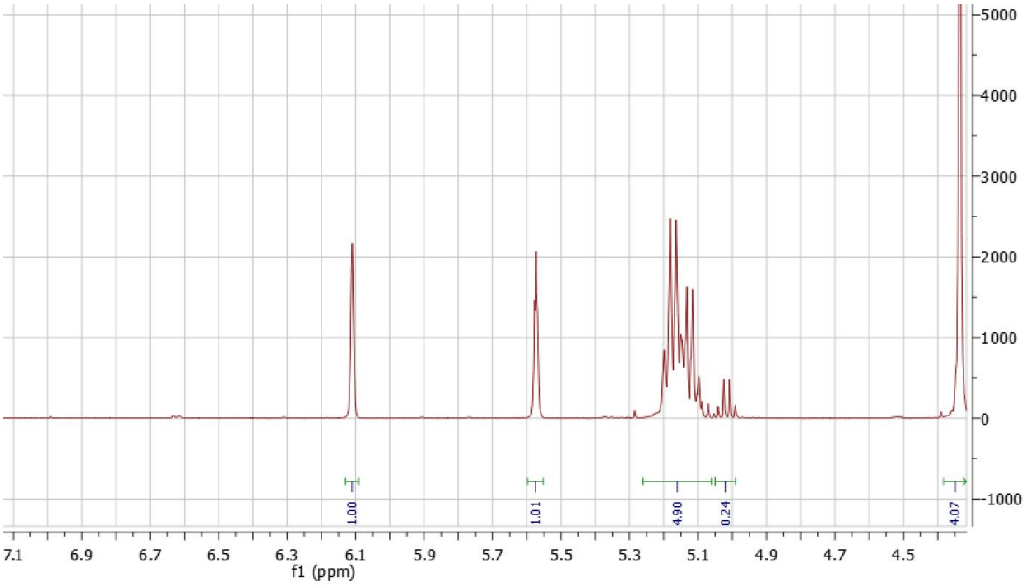


Figure 3.8 – *Enlargement of HEMA ETL LA_4 NMR spectrum . H Multiplicity highlights1 neighboring hydrogen atom, m=1*

Considering now peaks integration, Hema ETL LA_4 structure can be highlighted:

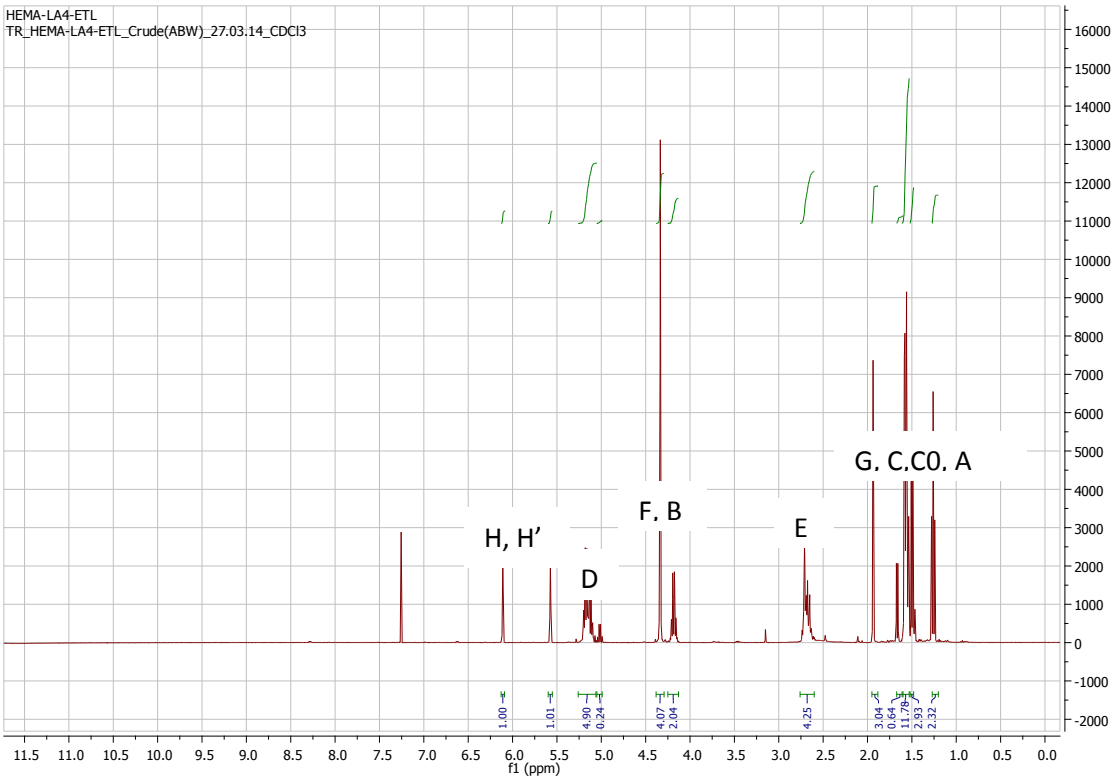
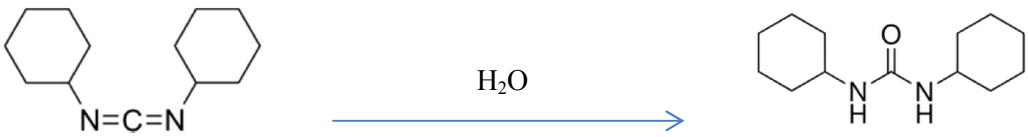


Figure 3.9 –*HEMA ETL LA$_4$ NMR spectrum*

It is possible to give a first estimate of the conversion associated to the coupling reactions, on the base of experimental data.DCU is formed by DCC hydration:



H_2O comes from the functionalized macromonomers, that is to say that an indirect evaluation of the reacted macromonomers is obtained by calculating the DCU conversion:

Macromonomer	Conversion
Hema ETL LA_0	87 %
Hema ETL LA_4	95 %
Hema ETL LA_6	88 %
Hema ETL LA_8	93 %

Table 3.3– *Hema ETL LA_X macromonomers synthesis conversion data*

Alternatively, **DMSO** could be used as a solvent in H-NMR, in order to better point out all the -OH groups and directly verify the macromonomers functionalization.

3.1.4 PLGA- based macromonomers: Coupling Reaction

In this case Hema ETL $PLGA_4$ can be considered representative to characterize PLGA-based macromonomers coupling reactions:

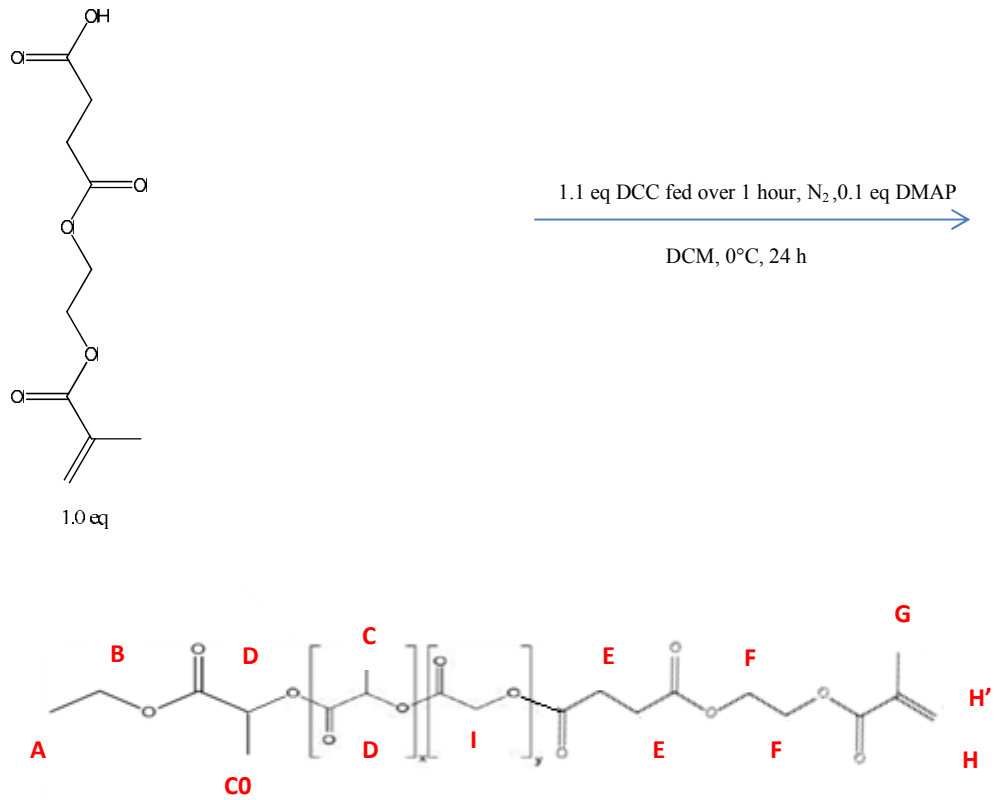


Figure 3.10 –*ETL $PLGA_4$ coupling reaction*

Hydrogen atom in position **H** results conveniently usable as reference in this situation as well. According to the NMR chart, which reports the different chemical shifts, **H** is expected to be around 6 ppm. **Figure 3.11** represents an enlargement of the examined spectrum. In this case $m = 1$

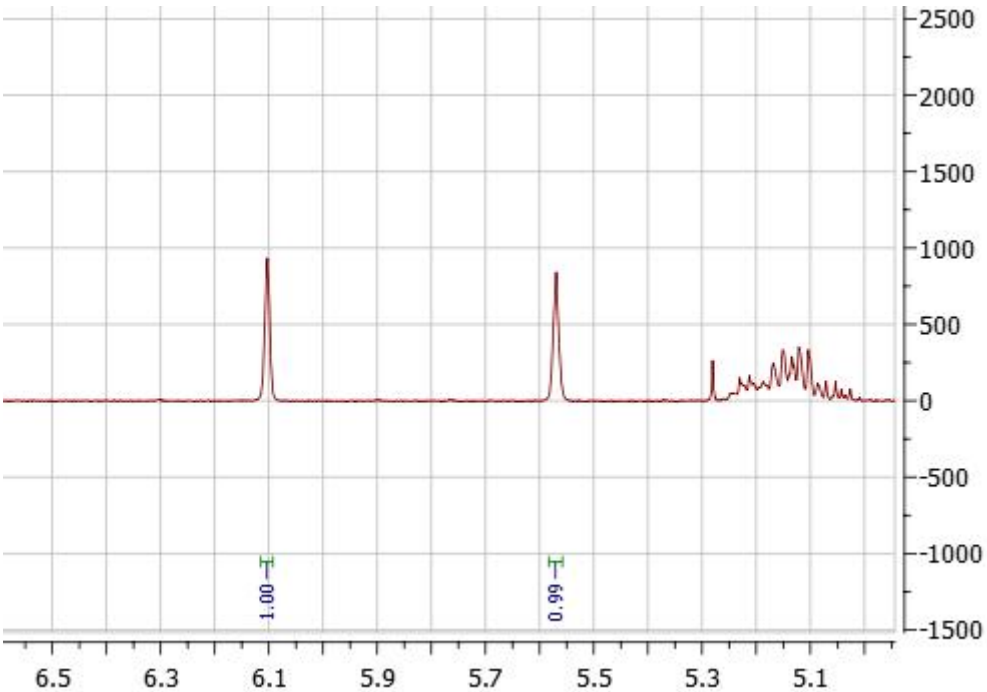


Figure 3.11– *Enlargement of HEMA ETL PLGA$_4$ NMR spectrum*

Considering now peaks integration, Hema ETL PLGA$_4$ structure can be highlighted:

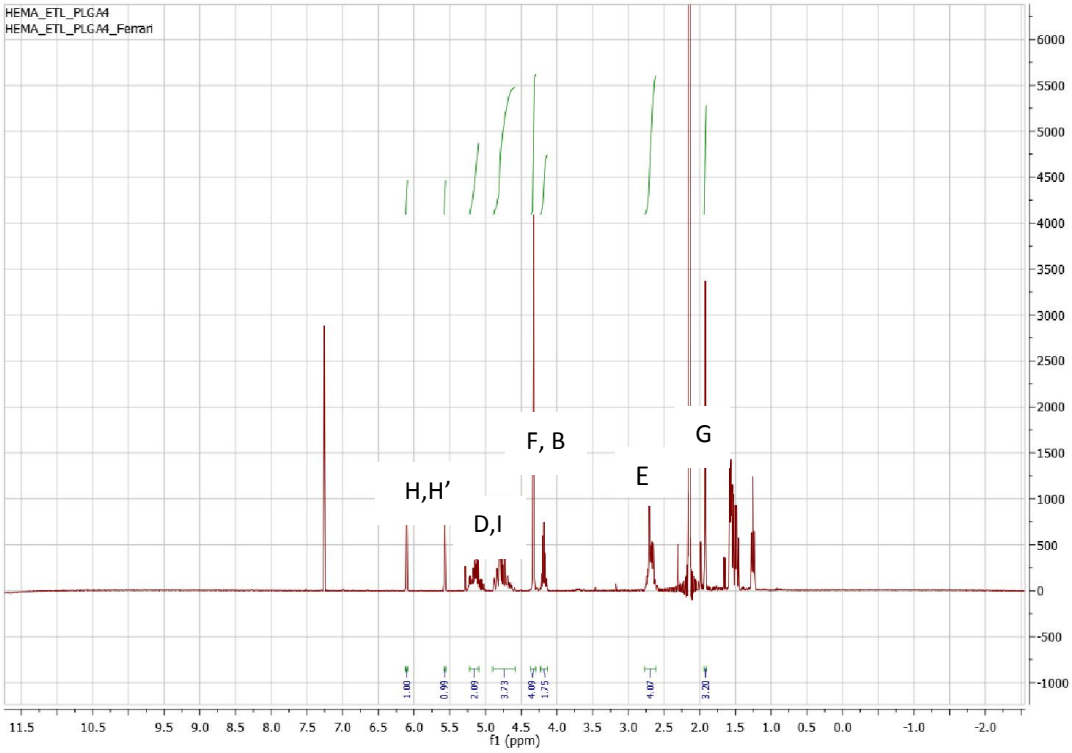


Figure 3.12–*H-NMR analysis Hema ETL PLGA$_4$, coupling reaction*

Effective chain lengths are now determined in readiness: taking Hydrogen atoms in position **H** and **H'** (double bond) as a reference, it is possible to assess average PLGA units added to Hema succinate.
Each Lactide unit is expected to give 1 Hydrogen atom in position **D**, whereas each Glycolide unit is expected to contribute with 2 H in position **I**.

$$Average\ Lactic\ Acid\ units\ added\ to\ Hema\ succinate: \frac{Peak\ D}{Peak\ H} = 2.09$$

$$Average\ Glycolic\ Acid\ units\ added\ to\ Hema\ succinate: \frac{(Peak\ I)/2}{Peak\ H} = 1.87$$

$$Average\ PLGA\ units\ added\ to\ Hema\ succinate \sim 4$$

It is also possible to give a first estimate of the conversion associated to the coupling reactions, on the base of experimental data.
As already noticed, H_2O comes from the functionalized macromonomers, that is to say that an indirect evaluation of the reacted macromonomers is obtained by calculating the DCU conversion :

Macromonomer	**Conversion**	**Average PLGA Units added to Hema succinate**
Hema ETL $PLGA_4$	90 %	~4
Hema ETL $PLGA_6$	94 %	~6
Hema ETL $PLGA_8$	92 %	~8

Table 3.4– *Hema ETL $PLGA_X$ macromonomers synthesis conversion data and average repeating units*

Alternatively, **DMSO** could be used as a solvent in H-NMR, in order to better point out all the -OH groups and directly verify the macromonomers functionalization.

3.2 NPs synthesis

NPs were produced from the synthesized macromonomers described in the previous section, using SDS as surfactant. Final particle size and polydispersity index (PDI) have been measured and reported below.

3.2.1 PLA based NPs synthesis

Hema ETL LA_0

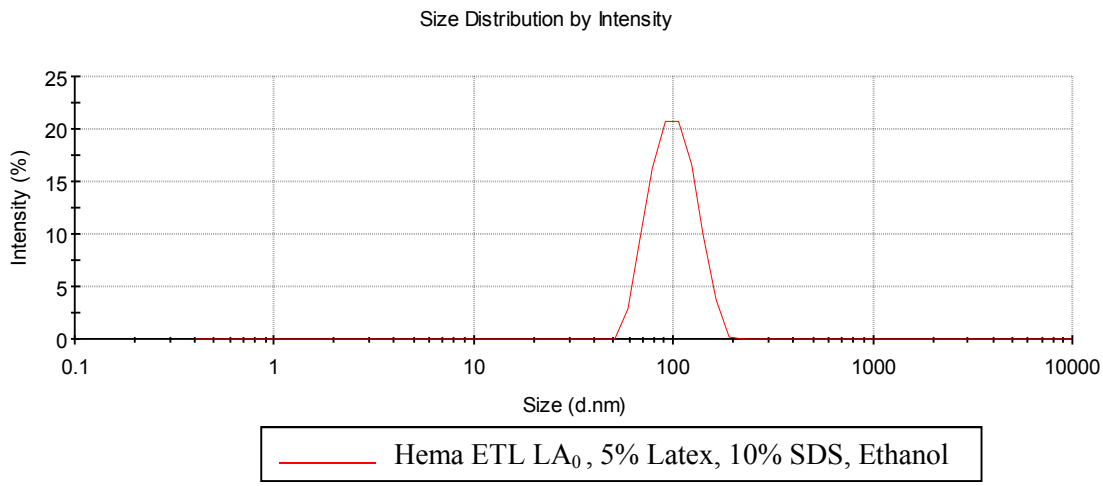


Figure 3.13–*Hema ETL LA_0 NPs*

Hema ETL LA_4

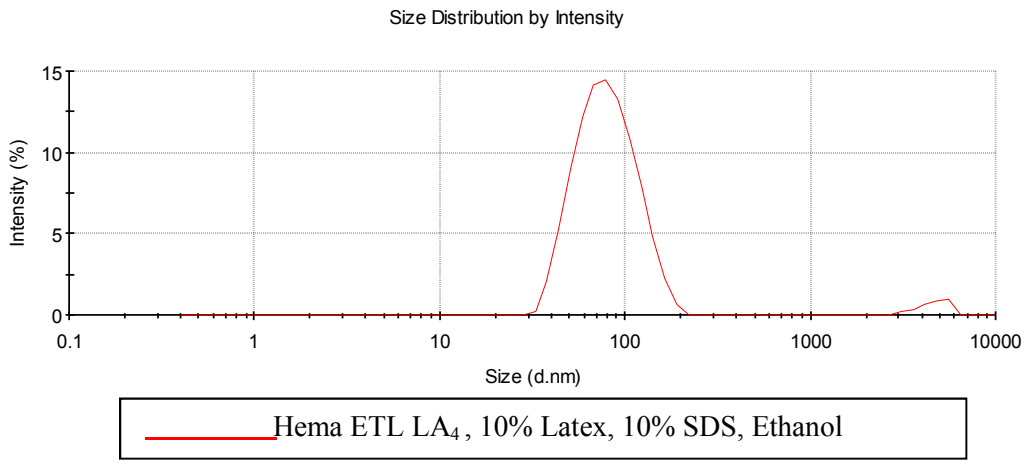


Figure 3.14–*Hema ETL LA_4 NPs*

Figure 3.13 and **Figure 3.14** illustrate respectively Hema ETL LA_0 and Hema ETL LA_4 NPs synthesis. In both cases a monodisperse latex is obtained, by using Ethanol as solvent. Regarding Hema ETL LA_0 no indication of aggregates can be outlined.On the contrary Hema ETL LA_4 show aggregates contribution, which is quantified as less than 5% sample anyhow. Considering their no significant amount and their dimensions (μm), aggregates can be removed with ease through a filtration step.

Hema ETL LA_6

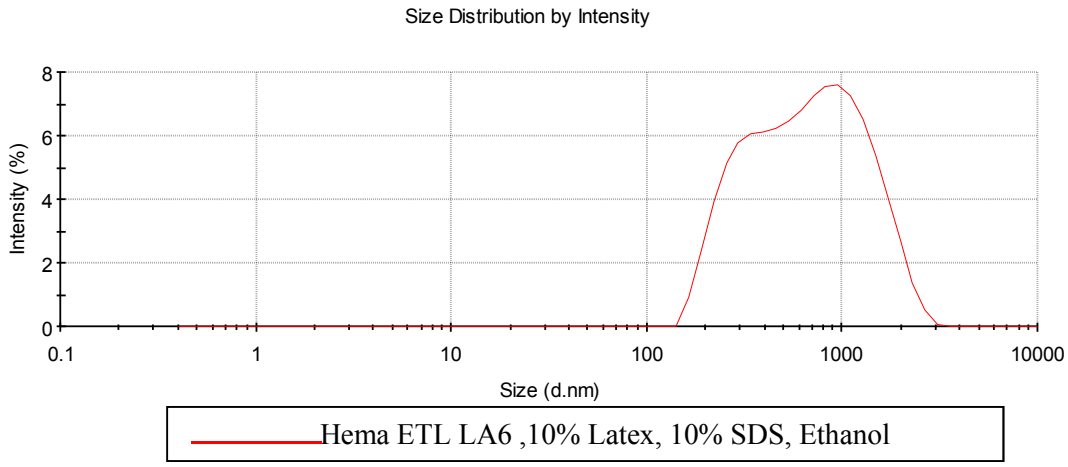


Figure 3.15 a–*Hema ETL LA_6 NPs*

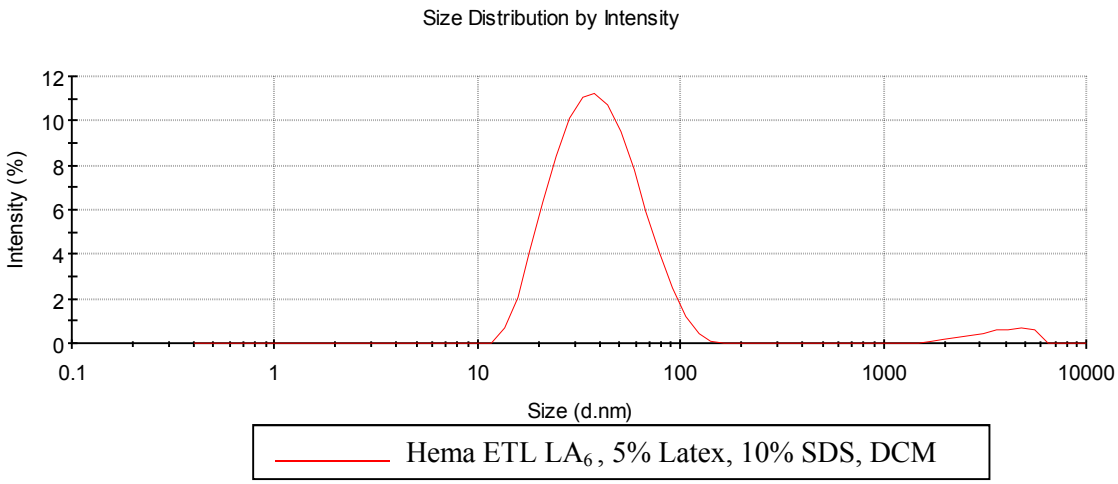


Figure 3.15 b–*Hema ETL LA_6 NPs*

As to Hema ETL LA_6 , initial difficulties in synthesis have been observed, due to the increasing hydrophobic features. The NPs synthetized by using Ethanol as solvent for the macromonomer injection demonstrated unreliable for further studies. For this reason DCM was proposed. Improving emulsion polymerization is possible by working with a non-water-soluble solvent, which promote the interaction between the macromonomer and the reaction environment.

The solution suggested revealed effective, as shown by the narrower size distribution in **Figure 3.16 b**.PDI values are near the acceptability limit (which is fixed to 0.2). We can accept them in this preliminary phase of study, since only a small percentage of the NPs sample (3%) is represented by aggregates, which can be easily removed through filtration.

Hema ETL LA_8

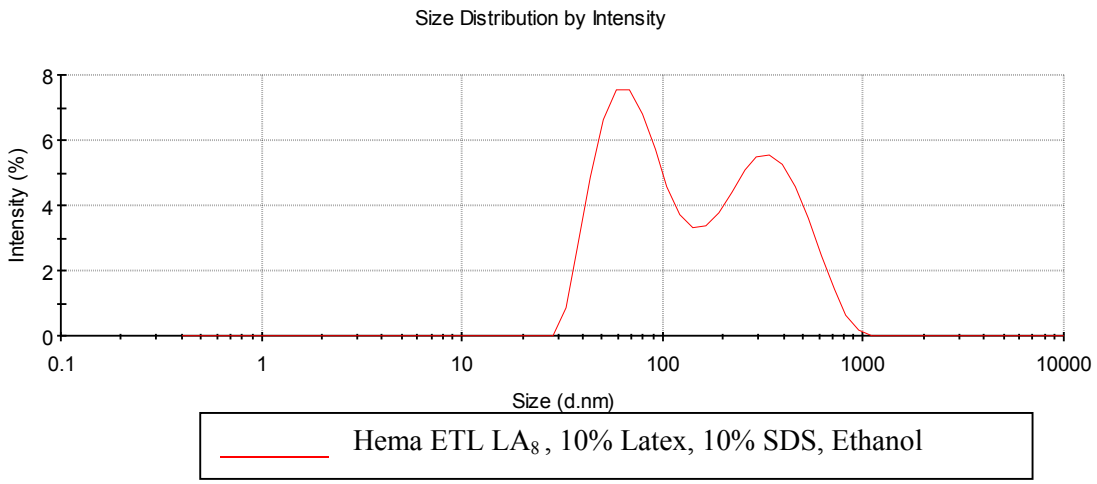


Figure 3.16 a–*Hema ETL LA_8 NPs*

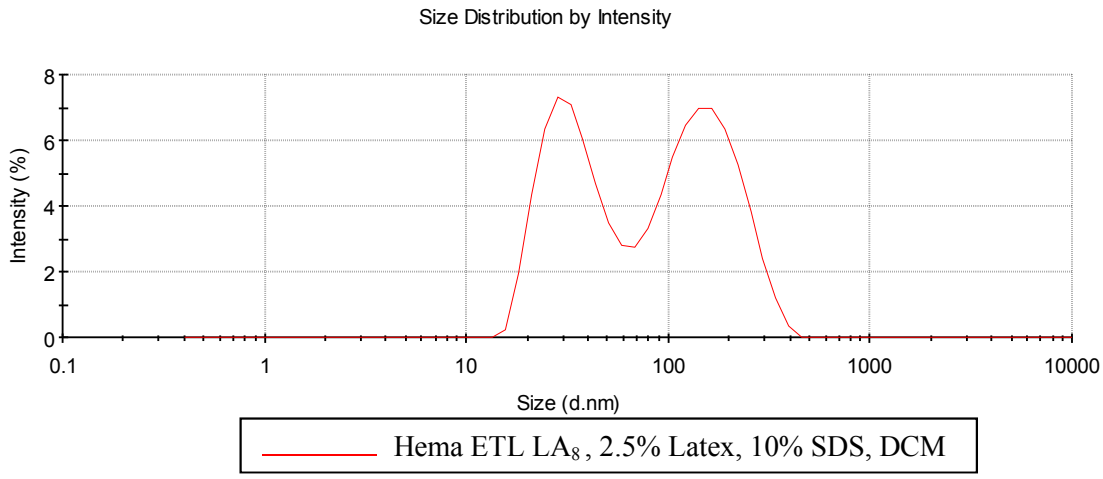


Figure 3.16 b–*Hema ETL LA_8 NPs*

On the contrary the problems occurred during NPs synthesis with Hema ETL LA_8 have not been solved by changing solvent, because of a too high hydrophobicity. Size distribution has not been improved by a lower latex concentration neither, which was introduced in order to increase dilution. Thus Hema ETL LA_8 based NPs were retained unsuitable at this stage of the study.

We can now sum up the **average characteristics** highlighted by Hema ETL LA_x based NPs. Dynamic Light Scattering (DLS) operating conditions were set to T= 25°C and water as reference dispersant.

Hema ETL LA_X		
Chain Length	**d (nm)**	**PDI**
0	103	0.028
4	81.04	0.191
6	42.39	0.225
8	-	-

T = 25 °C
Dispersant : Water

Table 3.5– *Hema ETL LA_X NPs features*

Figure 3.17 plots the average diameter of the NPs as a function of macromonomers chain length.

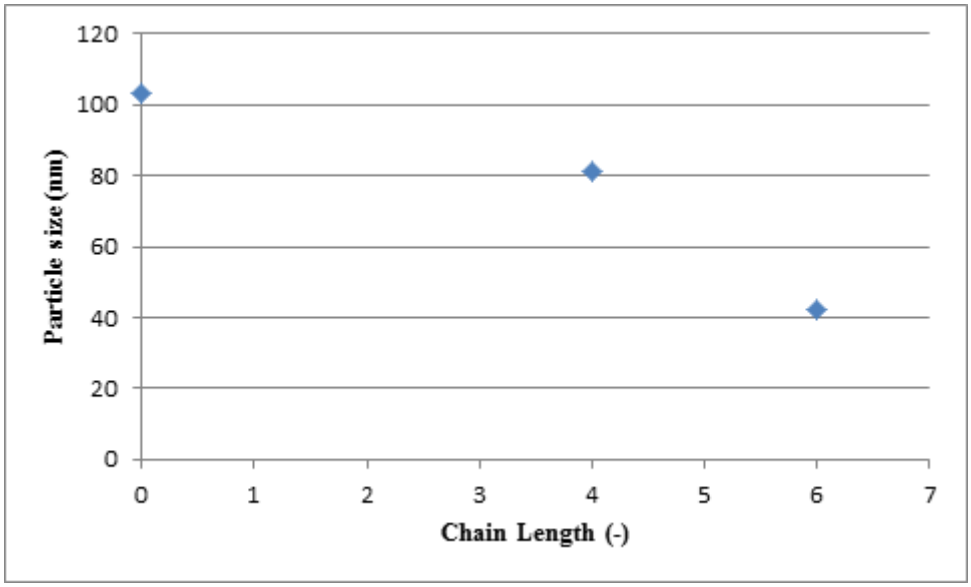


Figure 3.17–*Hema ETL LA_X NPs*

As we can see Particle size decreases when Chain Length rises. A similar behavior will be underlined regarding Hema ETL $PLGA_X$, as follows.

3.2.2 PLGA based NPs synthesis

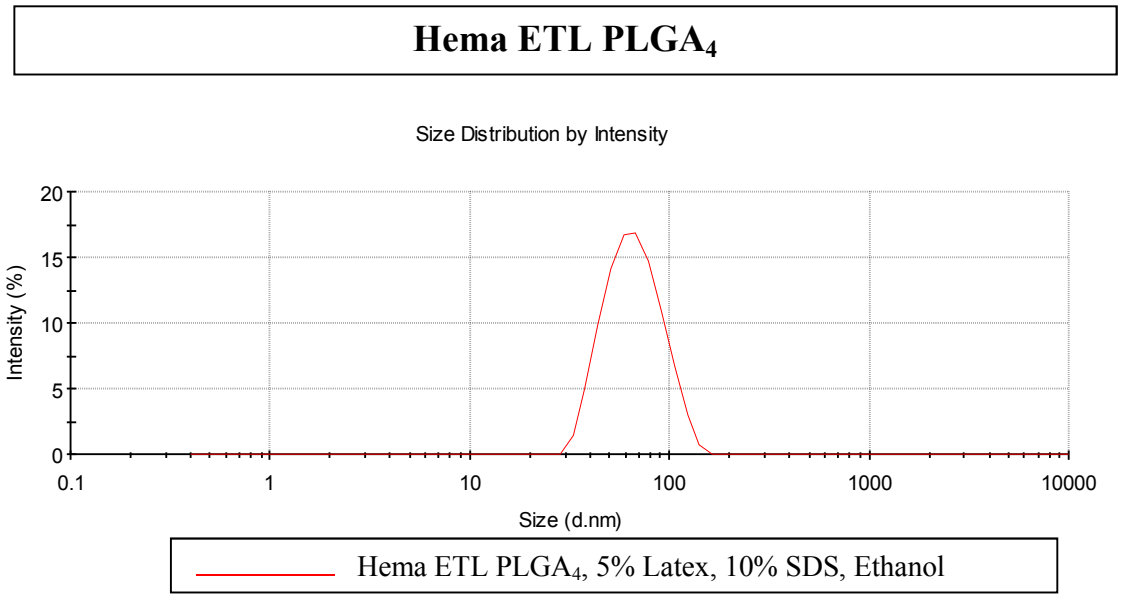


Figure 3.18–*Hema ETL $PLGA_4$ NPs*

Even in the matter of Hema ETL $PLGA_4$ NPs synthesis a monodisperse latex is obtained, by using Ethanol as solvent. The situation observed here in **Figure 3.18** is similar to what highlighted in **Figure 3.14**, when PLA-based NPs with Chain Lenghts = 4were considered.

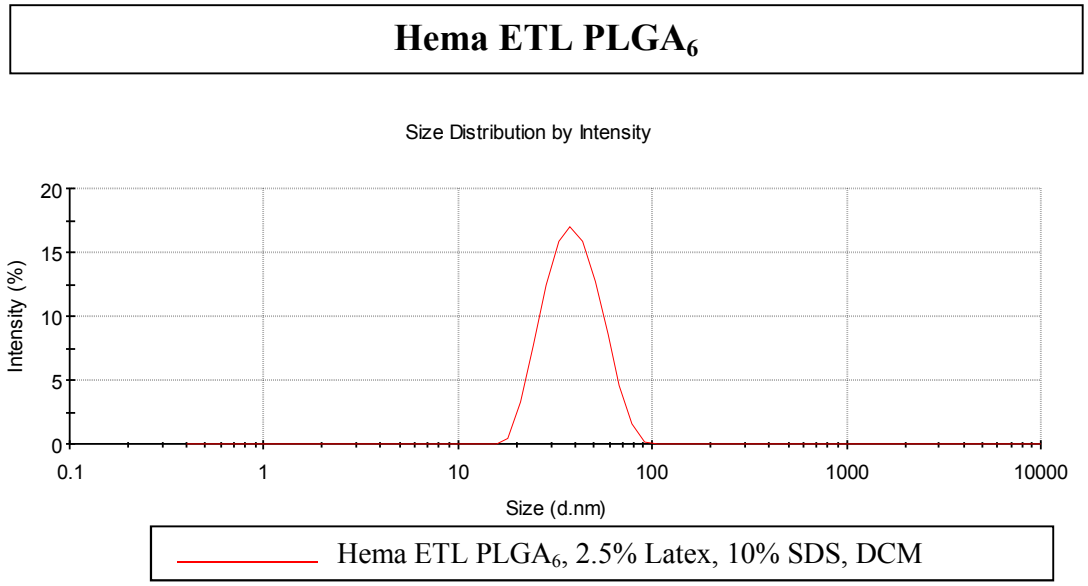


Figure 3.19 –*Hema ETL PLGA$_6$ NPs*

As Chain Length increases a non-water-soluble solvent needs to be exploited. Ethanol was then substituted with DCM in this case as well. Referring to Hema ETL LA$_6$ NPs, a similar result can be detected, consisting in a monodisperse latex with no aggregates sign.

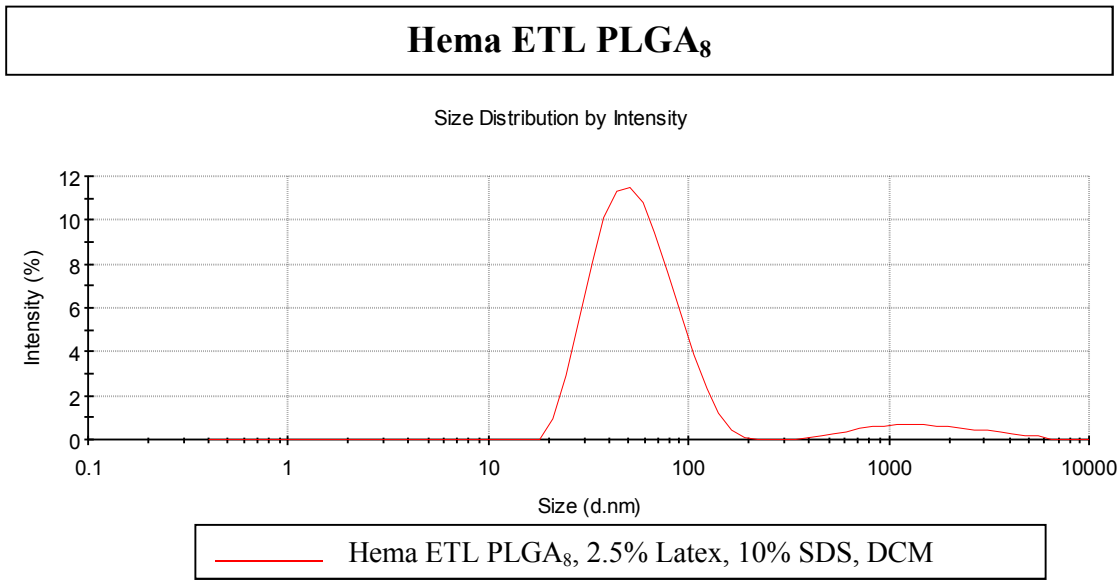


Figure 3.20 –*Hema ETL PLGA$_8$ NPs*

Hema ETL $PLGA_8$ does not confirm what was remarked in the Hema ETL LA_8 situation. Here the two macromonomers differ significantly, that means the distribution shown in this case appears narrower and in line with the trend followed by the previous macromonomers. The reason why it happens needs to be found in a more hydrophilic character, typical of the chemical structure considered.

Aggregates are about 10%. They need to be removed, in order to permit Hema ETL $PLGA_8$ NPs application in further procedures.

We can now sum up the **average characteristics** highlighted by Hema ETL $PLGA_x$ NPs.Light Scattering (DLS) operating conditions were set to T= 25°C and water as reference dispersant.

Hema ETL PLGAx		
Chain Length	**d (nm)**	**PDI**
4	68.45	0.138
6	40.56	0.145
8	58.03	0.245

T = 25 °CT (°C)
Dispersant : Water

Table 3.6– *Hema ETL $PLGA_X$ NPs features*

Hema $PLGA_8$ Size Measure and PDI deserve particular consideration: the values recorded are affected by aggregates presence indeed, which reflects on an increased average diameter and an increased polydispersity index.

Figure 3.21 below plots the average diameter of the NPs as a function of macromonomers chain length.

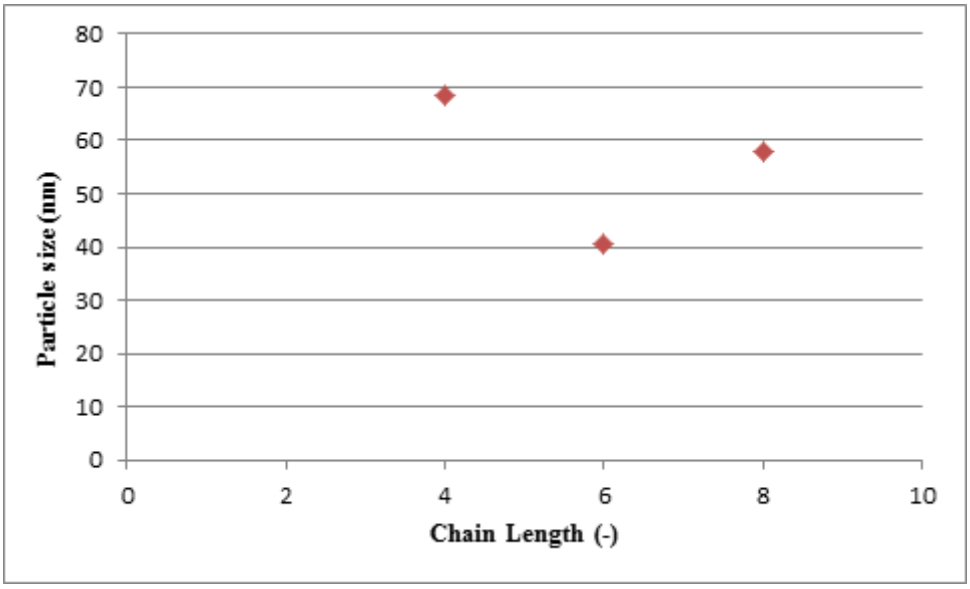


Figure 3.21–*Hema ETL PLGA$_X$ NPs*

Even in this case Particle size decreases while Chain Length increases. Comparing the results discussed, in **Figure 3.22**:

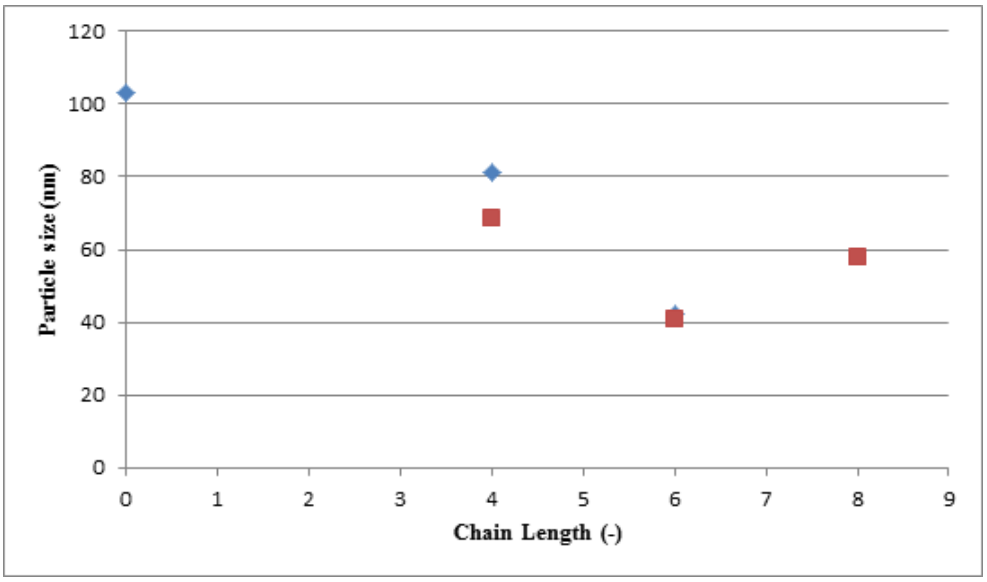


Figure 3.22–*Hema ETL LA$_X$ vs Hema ETL PLGA$_X$ NPs*

Both materials show the same behavior, in reference to Chain Length = 4 and Chain Length = 6 in particular. To report entirely the trends pointed out in Literature, **[32]** expresses a similar result obtained by PCL NPs, performed through batch emulsion polymerization (**Figure 3.23**):

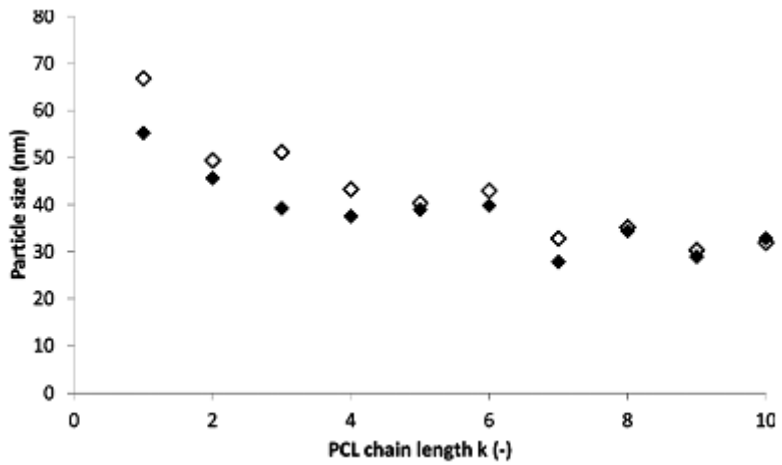


Figure 3.23–*PCL NPs*

To summarize what has been just highlighted, by using water as dispersant and SDS as surfactant:

- Small NPs with narrow particle size distribution were produced, as expected by performing emulsion polymerization;
- In both cases the largest NPs are defined by the macromonomers with the shortest Chain Length (i.e. Hema ETL La_0 and Hema ETL $PLGA_4$);
- Hema ETL La_8 and Hema ETL $PLGA_8$ exhibit non-negligible aggregates formation. They need to be removed before performing any other procedure;

3.3 PEGylated NPs Synthesis

PEGylated NPs were produced from the synthesized macromonomers described in the previous section, using TWEEN 80 as surfactant. Final particle size and polydispersity index (PDI) have been measured and reported below.

DCM was used as solvent, since it revealed effective for both short and long chains.

3.3.1 PEGylated PLA based NPs synthesis

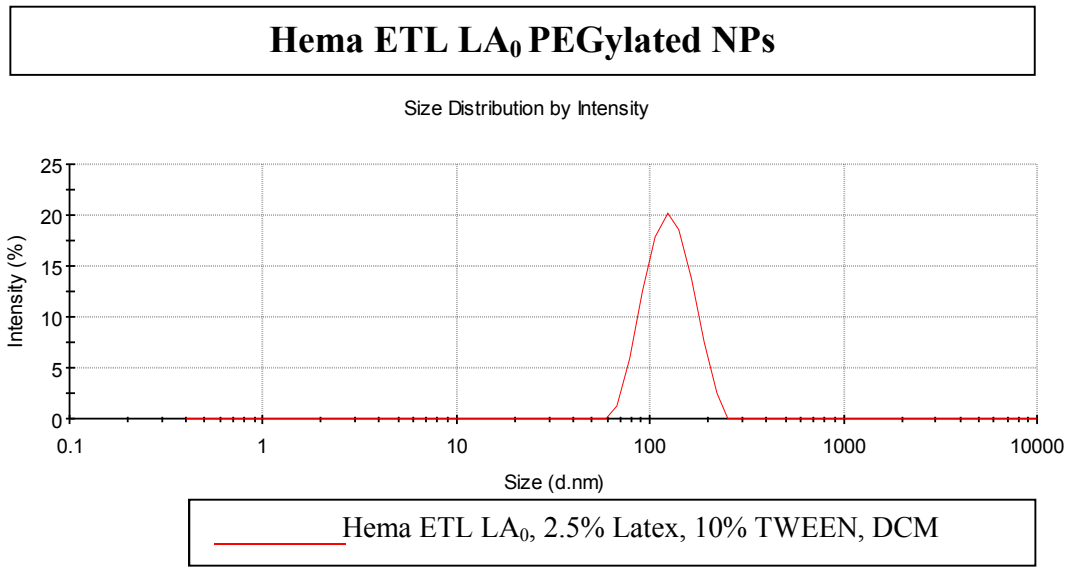


Figure 3.24 – PEG *Hema ETL LA_0 NPs*

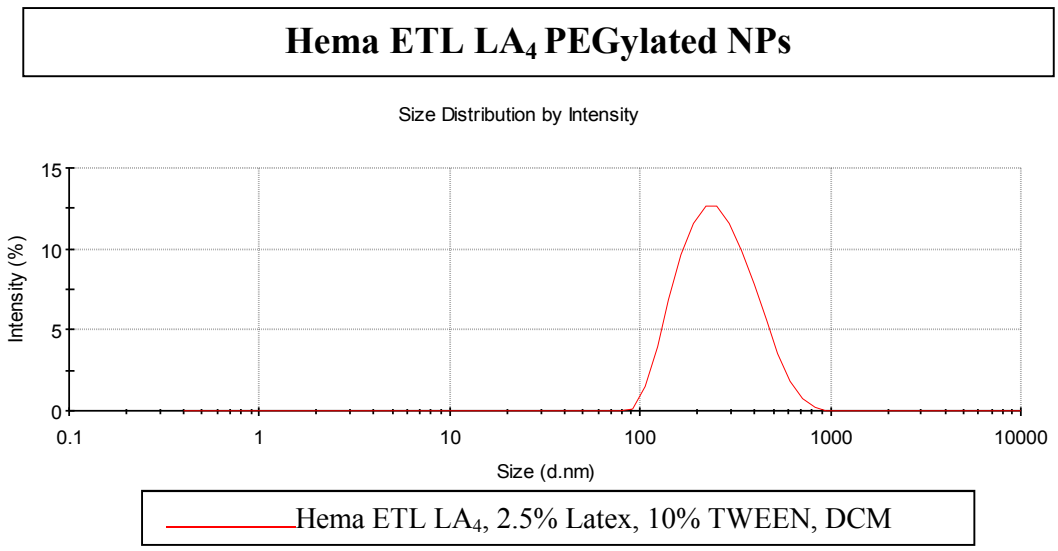


Figure 3.25 – PEG *Hema ETL LA_4 NPs*

In **Figure 3.24** and **3.25** PEG Hema ETL LA_0 and PEG Hema ETL LA_4 NPs are reported.
In both cases monodisperse latex with no aggregates was obtained. DCM was used as solvent.

Hema ETL LA_6 PEGylated NPs

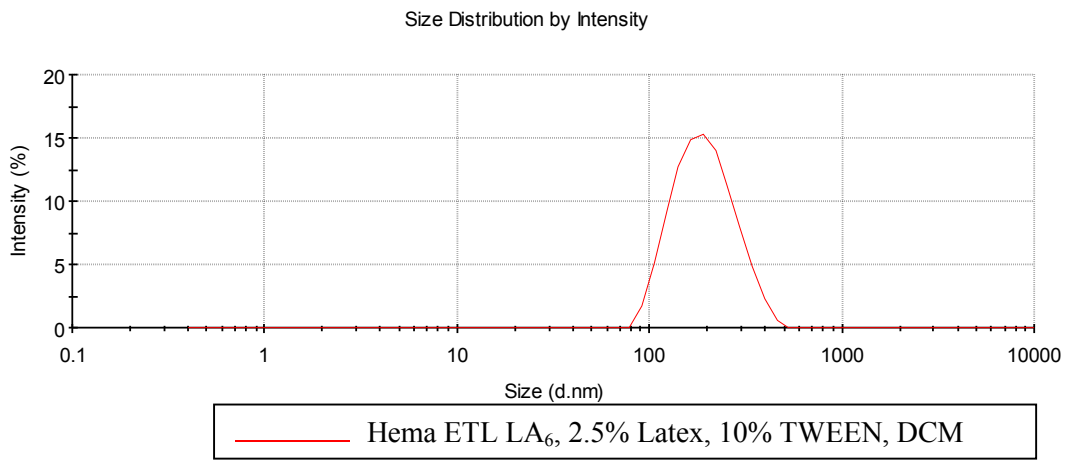


Figure 3.26 – PEG *Hema ETL LA_6 NPs*

PEG Hema ETL LA_6 NPs are characterized by monodisperse Size measures as well. No aggregates indication is detected.

Hema ETL LA_8 PEGylated NPs

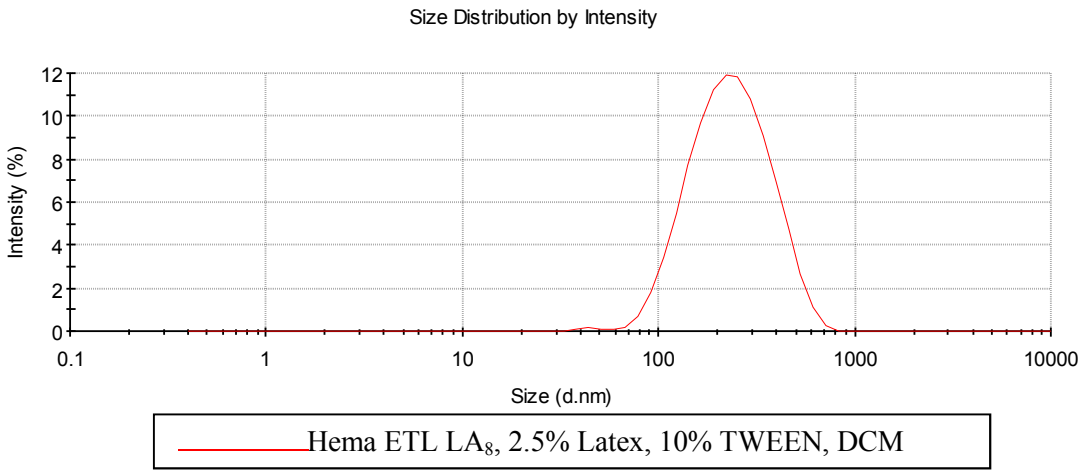


Figure 3.27 –PEG *Hema ETL LA_8 NPs*

Hema ETL LA_8 PEGylated NPs show features in agreement with what pointed out in the preceding cases, that is to say monodispersity and no aggregates formation

What can be observed from a general point of view is that PEG improves significantly NPs structures, solving problems in NPs synthesis for short- and long-chain macromonomers as well.

We can now sum up the **average characteristics** highlighted by Hema ETL LA_x PEGylated NPs. Dynamic Light Scattering (DLS) operating conditions were set to T= 25°C and water as reference dispersant.

Hema ETL LA_X		
Chain Length	**d (nm)**	**PDI**
0	120.06	0.052
4	236.5	0.157
6	181.7	0.132
8	207.8	0.167

T = 25 °C
Dispersant : Water

Table 3.7– PEG *Hema ETL LA_X NPs features*

Figure 3.28 below plots the average diameter of the NPs as a function of macromonomers chain length.

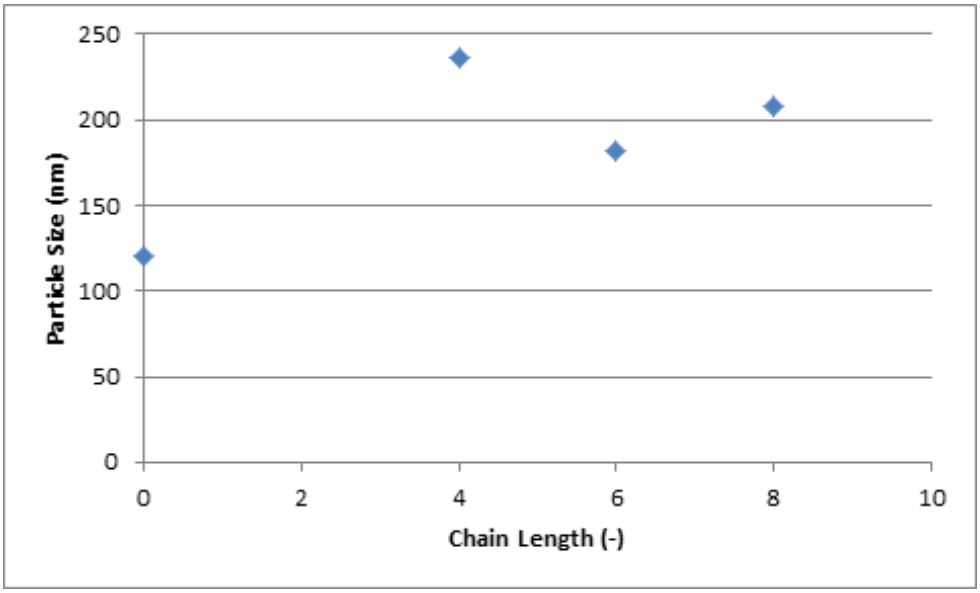


Figure 3.29–PEG *Hema ETL* LA_X *NPs*

In this case Particle Size increases with Chain Length. A similar behavior will be highlighted for PEGylated PLGA based NPs.

3.3.2 PEGylated PLGA based NPs synthesis

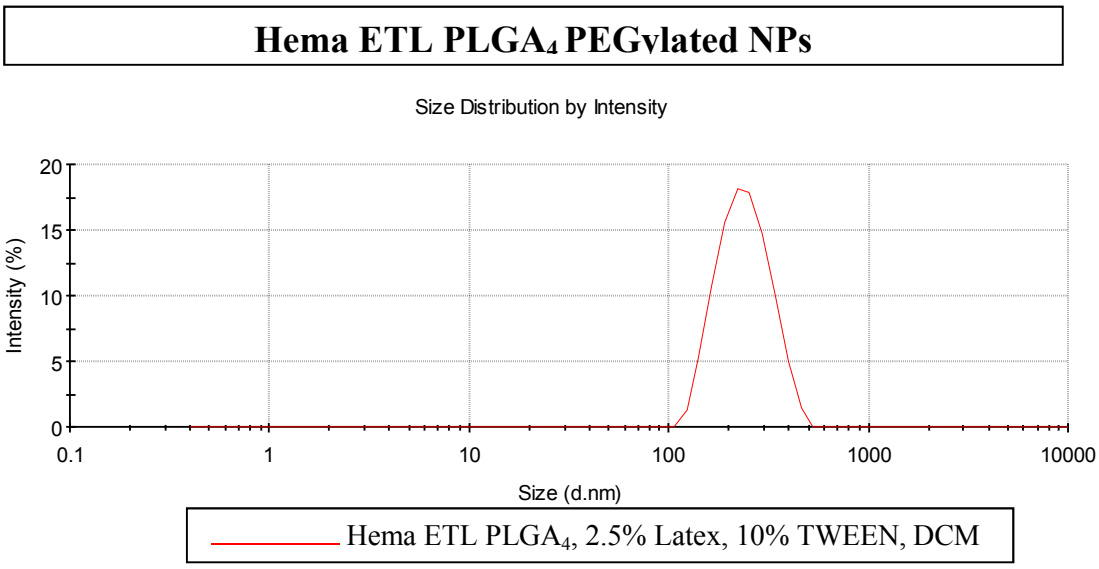


Figure 3.29 –PEG *Hema ETL* $PLGA_4$ *NPs*

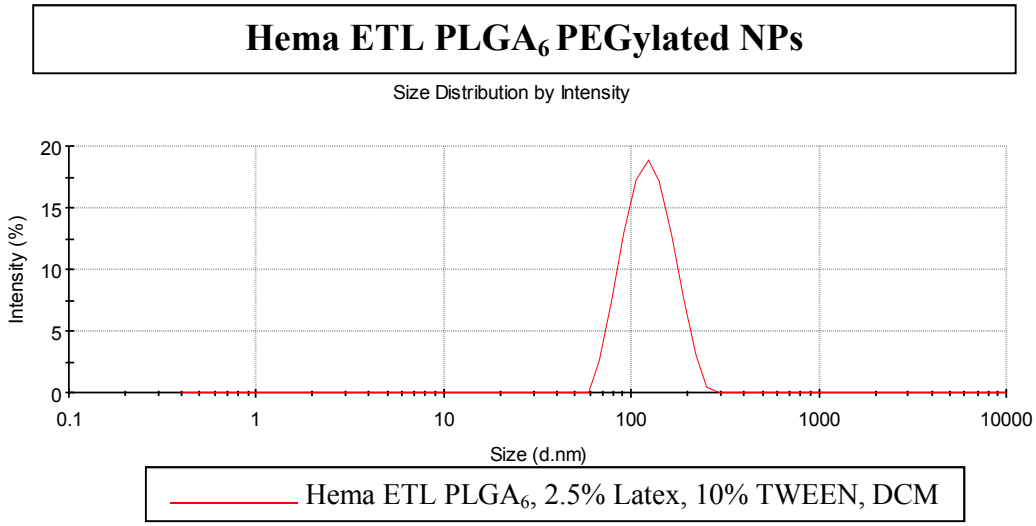


Figure 3.30 –PEG *Hema ETL PLGA$_6$ NPs*

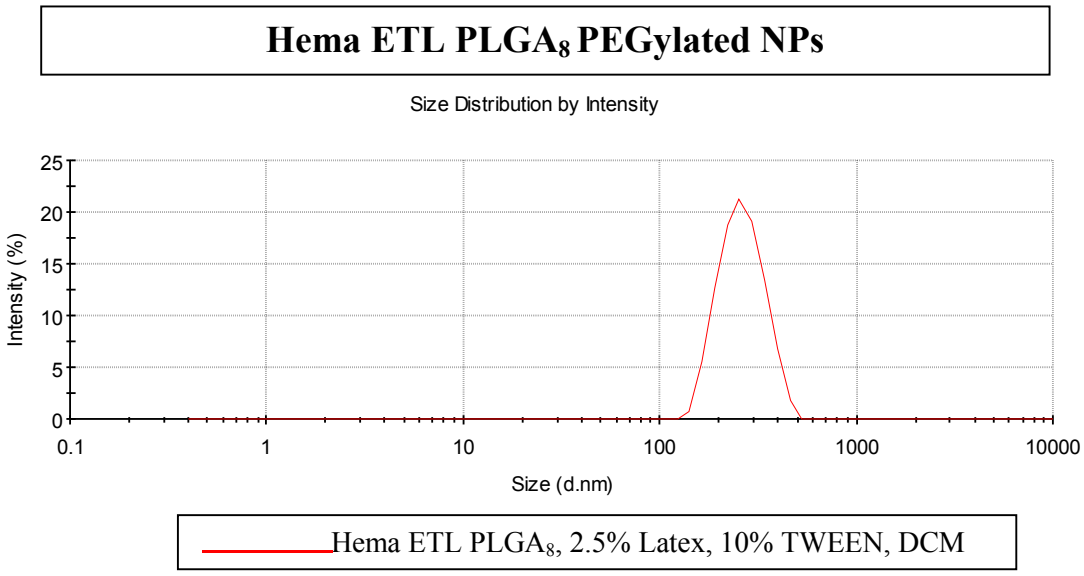


Figure 3.31 –PEG *Hema ETL PLGA$_8$ NPs*

As discussed in the previous sections, also in PEG Hema ETL PLGA$_X$ NPs the same trends can be emphasized, that is to say PEGylation permitted to obtain monodisperse latexes and no aggregates signals. In **Figure 3.29**, **3.30** and **3.31** PEG Hema ETL PLGA$_X$ NPs are illustrated.

Hema ETL $PLGA_x$ PEGylated NPs **average characteristics** can be represented. Dynamic Light Scattering (DLS) operating conditions were set to T= 25°C and water as reference dispersant.

Hema ETL PLGAx		
Chain Length	**d (nm)**	**PDI**
4	227.2	0.065
6	118.9	0.056
8	252.3	0.029

T = 25 °C
Dispersant : Water

Table 3.8– PEG *Hema ETL $PLGA_X$ NPs features*

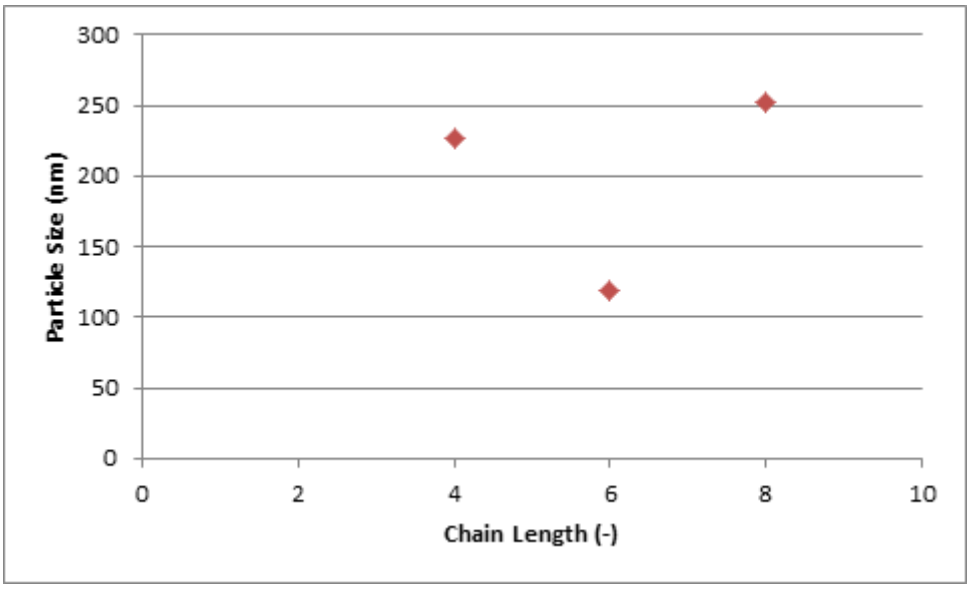


Figure 3.32– PEG *Hema ETL $PLGA_X$ NPs*

Comparing the results discussed, in **Figure 3.33**:

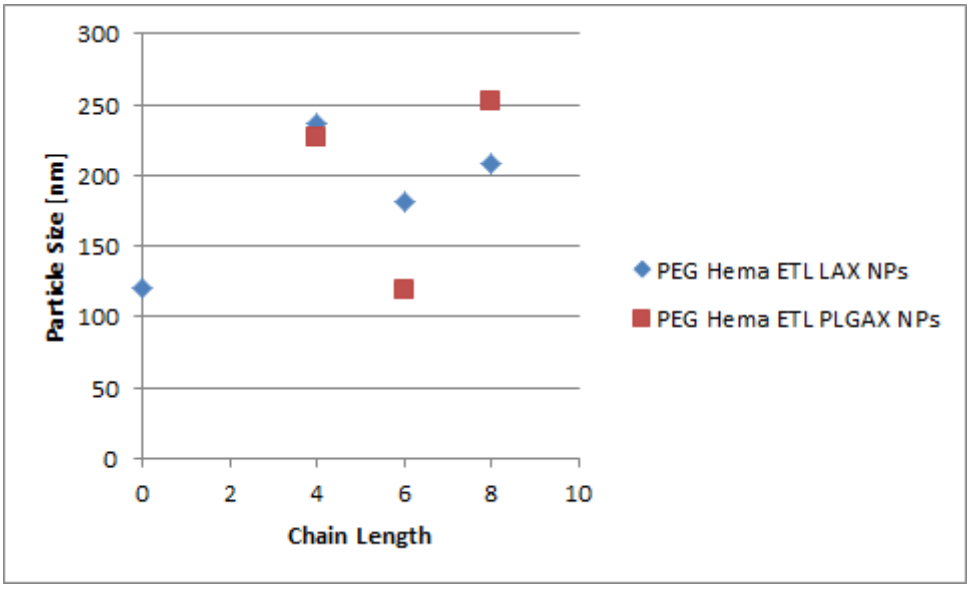


Figure 3.33–PEG *Hema ETL* LA_X *vs PEG Hema ETL* $PLGA_X$ *NPs*

Even in this situation PEGylated PLA based and PLGA based NPs show comparable results for Chain Lentgh= 4, 6 and 8 as well.

3.4 Degradation Study

As final part of this work, a preliminary degradation study of these materials was performed. In order to verify macromonomers properties, degradation in Water was performed at first.
PBS Stability Study and Cell Medium degradation were realized to demonstrate PEGylated NPs stability and degradation features.

Degradation study was carried out as:

- Degradation in H_2O;
- Degradation in PBS (stability study);
- Degradation in Cell medium.

NPs characterization focused in particular on:

- **Size [nm]**
- **PdI [-]**
- **Count Rate [kcps]**

These three parameters were considered indicative of the stability of the samples examined.

Size: It is measured in [nm]. As time passes we expect particles to vary initial Size measure. If Size decreases Micelles are formed, as NPs are degrading.

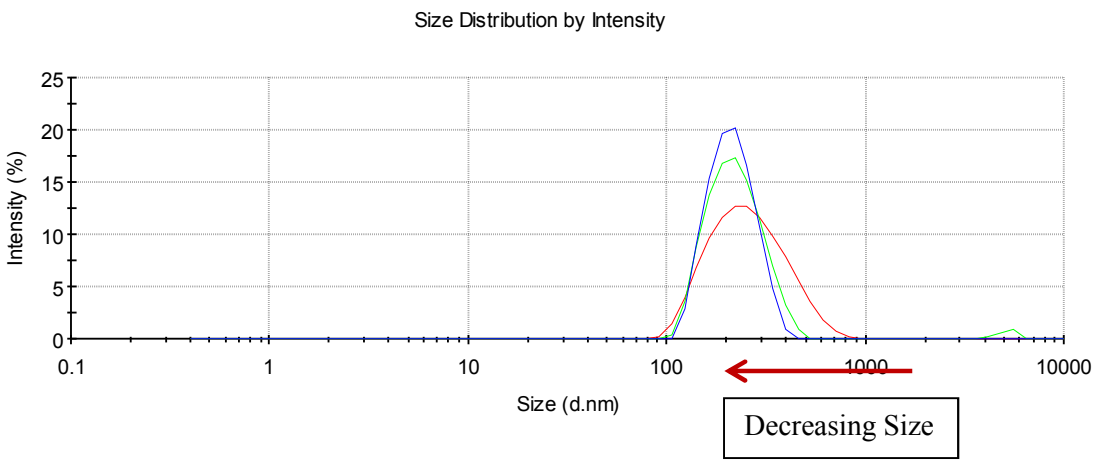


Figure 3.34–*Size theorical variation*

PDI: Polidispersity index is dimensionless. It represents a measure of the broadness of size distribution. It ranges from 0 to 1. PDI values near to 0 indicate NPs have the same average characteristics. As degradation proceeds PDI can be influenced by aggregates formation and non-uniform degradation of the particles.

Count Rate (or Photon Count Rate): Its measure is based on the number of photons detected in a "per second" basis. This is useful for determining the sample quality, by monitoring its stability as a function of time. The count rate needs to be above some minimum value in order to have enough signal for analysis.

3.4.1 Water Degradation results

In the following section all the gathered results, relative to water degradation study, are discussed. For each material only cumulative figures are reported, in order to have a general representation of the trends and to have a more critical point of view.

Degradation study was first lead in H_2O. It revealed a valuable way to better understand NPs properties and degradation time.
NPs produced have been placed in cuvettes and heated up to 50 $\pm$ 1°C. Degradation grade was characterized at different times. Particles size measurements were performed through dynamic light scattering (Malvern, Zetanano ZS) **[42]** over one month.
NPs characterization focused in particular on:

- **Size [nm]**
- **PdI [-]**

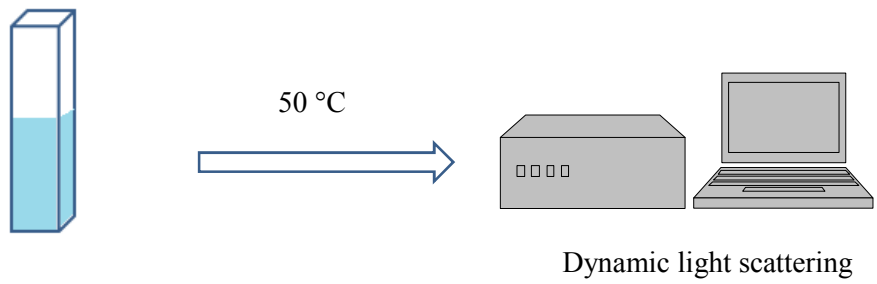


Figure 3.35 – *Schematic representation of Dynamic Light scattering used during the degradation study*

3.4.1.1. Hema ETL LA_X NPs

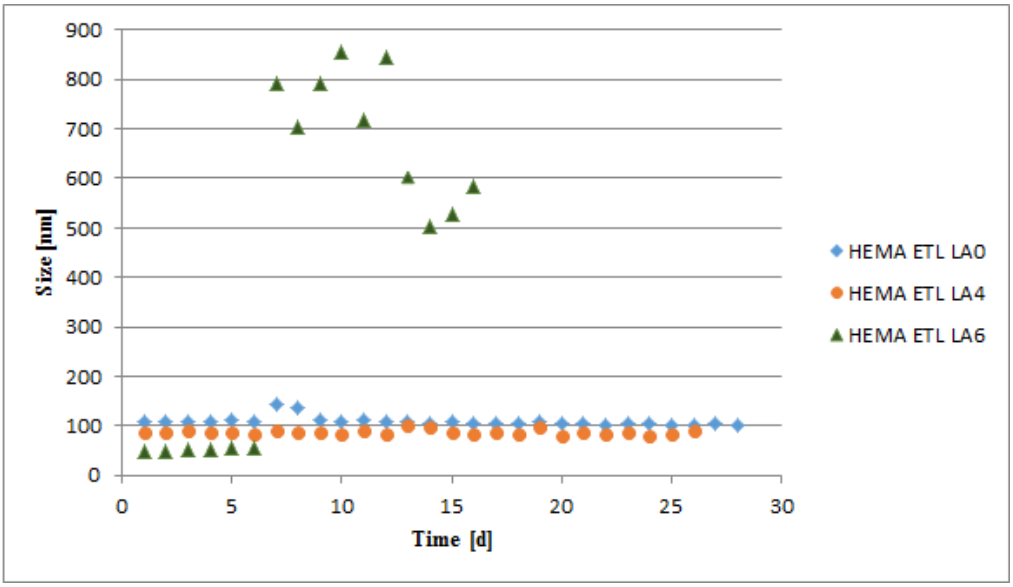


Figure 3.36 – *Size Hema ETL LA_X NPs water degradation*

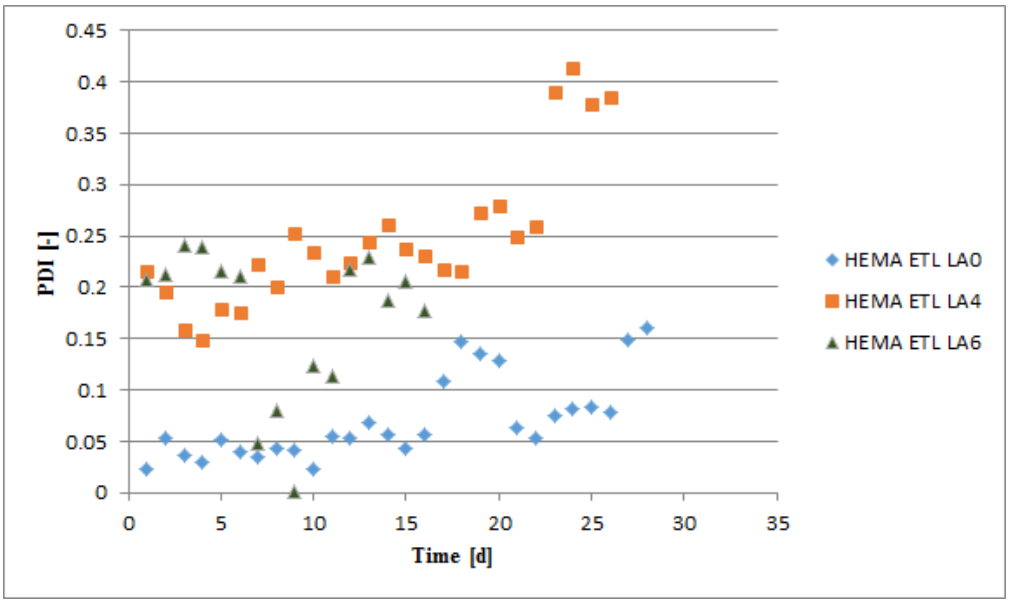


Figure 3.37 – *PDI Hema ETL LA_X NPs water degradation*

In **Figure 3.36** and **3.37** Hema ETL LA_X NPs Size and PDI are reported:

Hema ETL LA_0 NPs dimensions remain basically constant.
On the other hand PDI trend appears oscillatory. However it stays lower than 0.2, that means its variations are not relevant. In any case Hema ETL LA_0 NPs behavior has to be considered notable, since it does not show sensible degradation over one month.

In Hema ETL LA_4 NPs degradation in water, Size remains almost constant, though Hema ETL LA_0 NPs appear more stable. PDI trend appears crescent, that points out an increasing degradation.

Hema ETL LA_6 NPs show a less stable behavior, compared to LA_0 and LA_4. The longer chain offers more chances of interaction with the environment indeed. This brings about a faster degradation, whose signs become visible after day 10. Though oscillating PDI trend presents acceptable values (~ 0.2), which let us suppose no significative NPs aggregation occurs.

3.4.1.2 Hema ETL $PLGA_X$ NPs

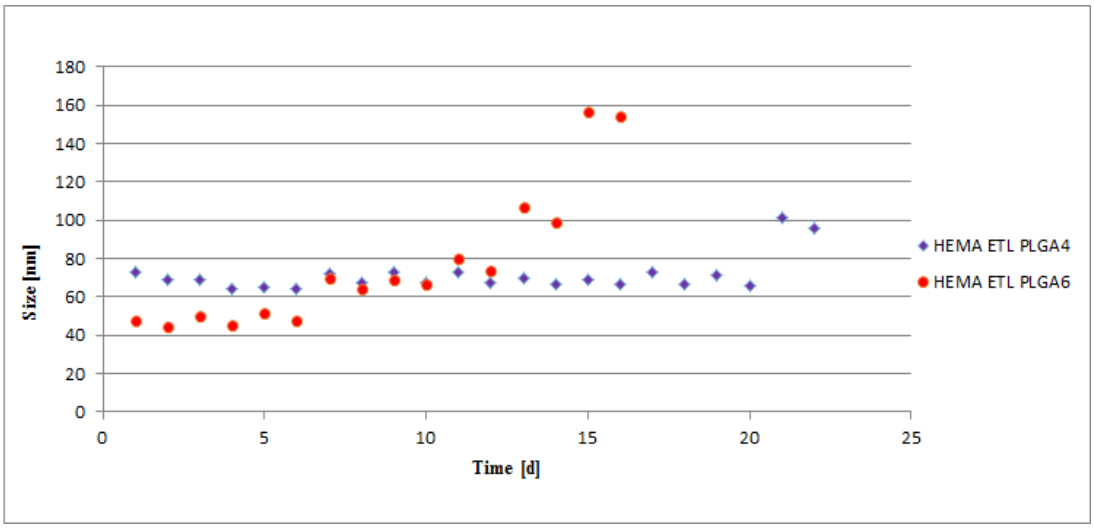


Figure 3.38– *Size Hema ETL $PLGA_X$ NPs water degradation*

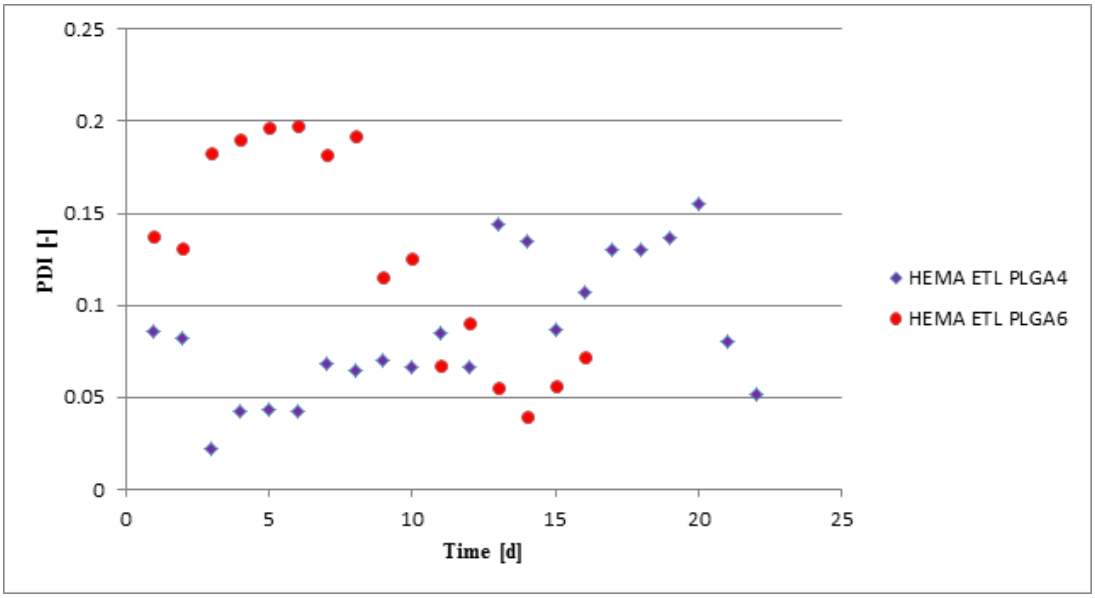


Figure 3.39 –PDI *Hema ETL PLGA$_X$ NPs water degradation*

In **Figure 3.38** and **3.39** Hema ETL PLGA$_X$ NPs Size and PDI are reported:

Hema ETL PLGA$_4$ NPs show stable features during degradation in water. Sensible variations in Size appear around the 15th day. It is comparable to Hema ETL LA$_4$ behavior. PDI trend appears oscillatory, but it lays always in the acceptability range (which means 0<PDI<0.2). Secondary peaks data appear non relevant.

Hema ETL PLGA$_6$NPs show similar characteristics to those highlighted for LA$_6$ NPs. A faster degradation was noticed, whose signs become visible after the first day. PDI trend lays in the acceptability range. Secondary peaks data play non relevant role.

To summarize the non-PEGylated NPs behavior:

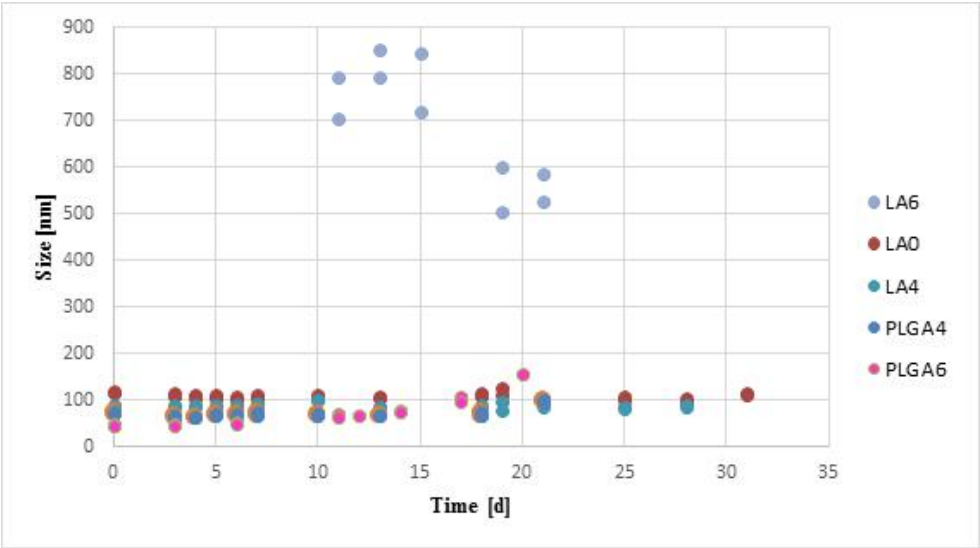


Figure 3.45–*Hema ETL LA_X and $PLGA_X$ water degradation, Size*

- Hema ETL LA_0 NPs show the most stable features, among the synthetized NPs.
- Hema ETL LA_4 and LA_0 NPs degradations are comparable, in the sense that they are stable for the duration of the test.
- Hema ETL $PLGA_X$ NPs degrade faster than PLA-based ones. It can be due to a more hydrophilic structure, as confirmed in **[30]**.

3.4.2 PBS Stability Study

PEGylated NPs were subjected to PBS Stability study. This particular investigation could not be lead for non-PEGylated NPs, since measures would not be reliable. This is to be attributed to the NPs precipitation phenomenon in Phosphate buffered saline.

For PEGylated NPs we are expecting comparable results to what has been obtained with the degradation study in H_2O, that means NPs resistance over at least a two-week long period.
Degradation grade has been characterized at different times. Particles size measurements were performed through dynamic light scattering (Malvern, Zetanano ZS).
It was performed over a 2-week period. NPs characterization focused in particular on:

- **Size [nm]**
- **Pdl [-]**
- **Count Rate [kcps]**

These three parameters were considered indicative of the stability of the samples examined.

DLS measurements were carried out at 37°C using 137° backscatter angle. After the first measure, values of attenuator and measure position have been kept constant. This permitted to perform the analysis with standardized operating condition, thus optimizing particles characterization:

NPs	Attenuator	Measure position
PEG Hema ETL LA_0	6	4,65
PEG Hema ETL LA_4	6	4,65
PEG Hema ETL LA_6	7	4,65
PEG Hema ETL LA_8	7	4,65
PEG Hema ETL $PLGA_4$	5	4,65
PEG Hema ETL $PLGA_6$	7	4,65
PEG Hema ETL $PLGA_8$	7	1,25

Table 3.9–*Attenuator and Measure position, PBS stability study*

3.4.2.1. PEG Hema ETL LA_X NPs

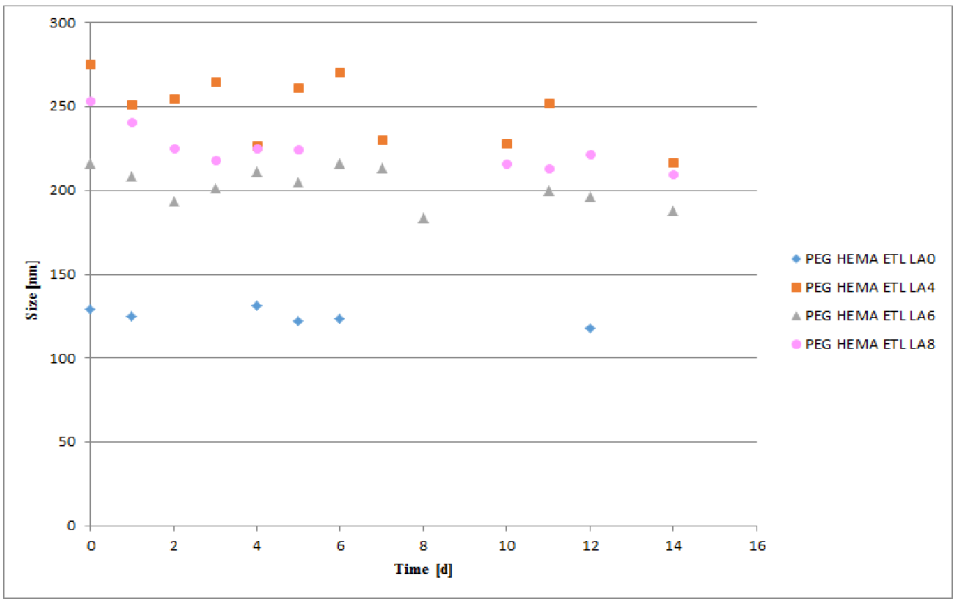


Figure 3.41 –*Size PEG Hema ETL LA_X NPs PBS stability study*

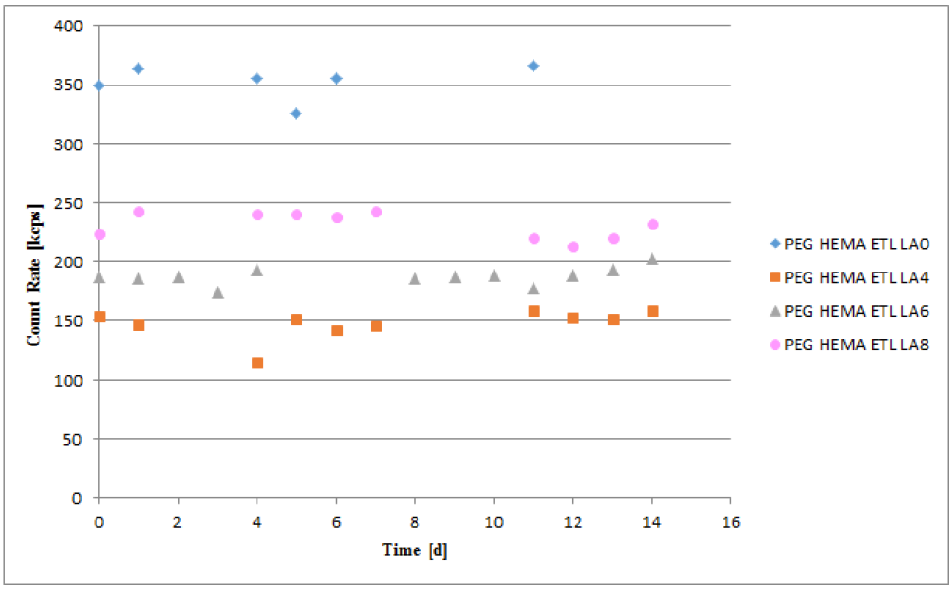


Figure 3.42 –*Count Rate PEG Hema ETL LA_X NPs PBS stability study*

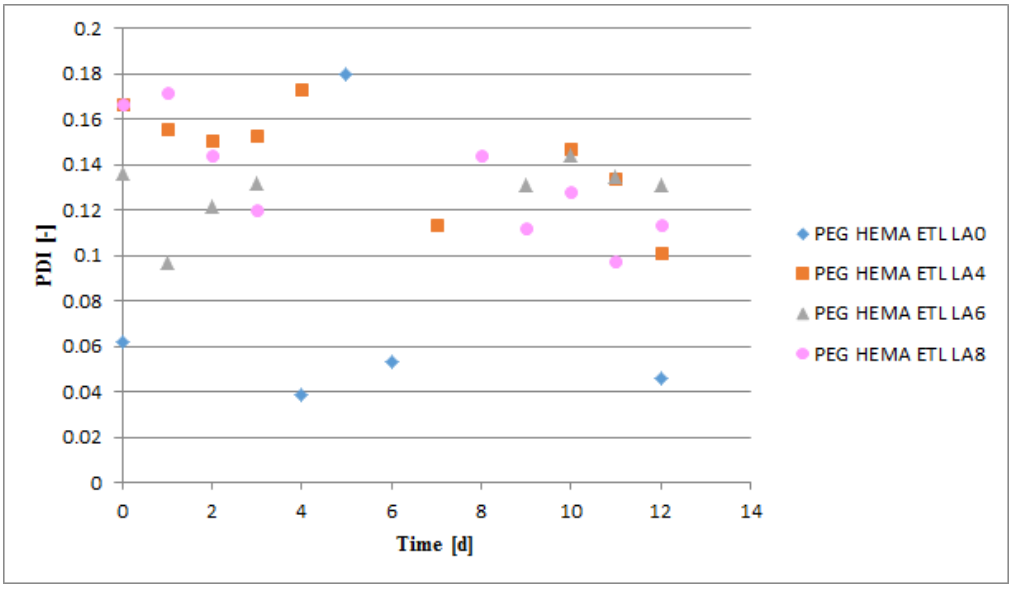


Figure 3.43 –*PDI PEG Hema ETL LA_X NPs PBS stability study*

In **Figure 3.41, 3.42** and **3.43** PEG Hema ETL LA_X NPs Size, Count Rate and PDI are reported:

Hema ETL LA_0 PEGylated NPs show a uniform degradation over 12 days. All the parameters considered do not vary significantly during the period of study, therefore confirming the stability of these NPs.

Hema ETL LA_4 PEGylated NPs show an oscillatory behavior. However from Size distribution trend an average measure of 250 nm over the first 10 days can be seen. After that, values start decreasing. Secondary peak formation is observed; however both measures refer to less than 5% of the NPs examined.

Hema ETL LA_6 PEGylated NPs degradation confirms a reduction in Size measurement after the 10th day. In this case Size data are oscillating around an average value (200 nm) before degradation becomes notable.

Hema ETL LA_8 PEGylated NPs degradation follows the trend already highlighted. Size reduction is recognizable after the 10th day. PDI values can

state an indication of a broad distribution, that means particles are not degrading homogenously.

3.4.2.2 PEG Hema ETL PLGA$_X$ NPs

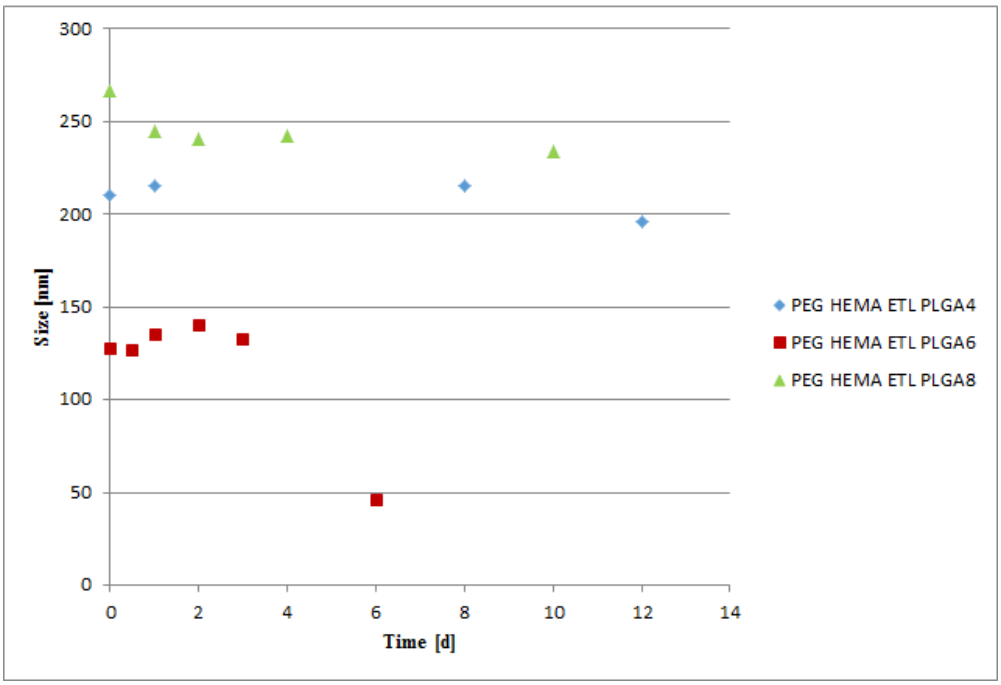


Figure 3.44 –*Size PEG Hema ETL PLGA$_X$ NPs PBS stability study*

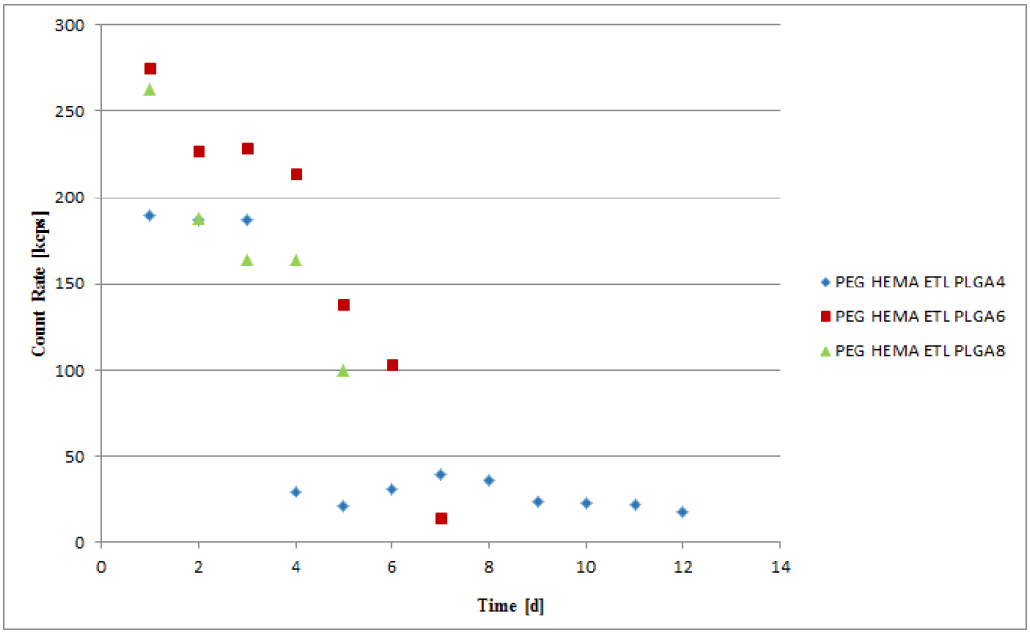


Figure 3.45 –*Count Rate PEG Hema ETL PLGA$_X$ NPs PBS stability study*

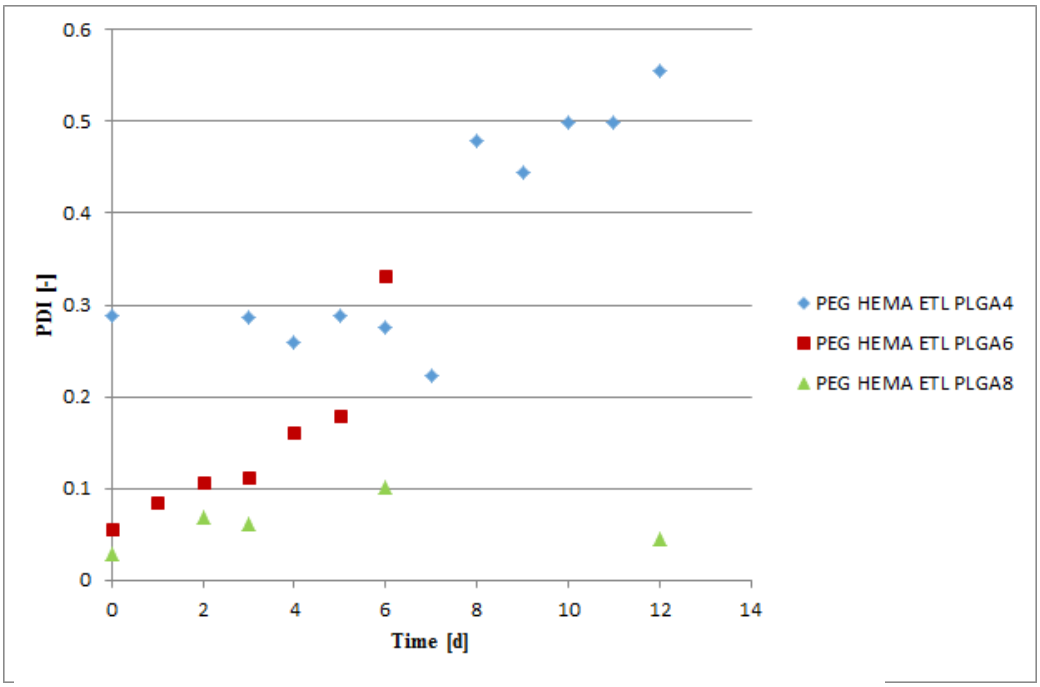


Figure 3.46 –*PDI PEG Hema ETL PLGA$_X$ NPs PBS stability study*

In **Figure 3.44, 3.45** and **3.46** PEG Hema ETL $PLGA_X$ NPs Size, Count Rate and PDI are reported:

Hema ETL $PLGA_4$ PEGylated NPs degrade faster than Hema ETL LA_4, as expected. In 13 days, Size reduces from 210 to 195 nm. Significant decrease is observed after the 8th day. No relevant secondary peaks have been detected.

Hema ETL $PLGA_6$ PEGylated NPs degradation is faster than $PLGA_4$. In this case, after the third day, Size reduction is significant (it ranges from132.6 nm to 45.99 nm) . As the degradation in PBS proceeds, PDI values increase. In this occasion a 2-week period of study revealed unnecessary. On the 6th day a second peak is registered: the measure corresponds to 18%, therefore it is not negligible. This can help explain the increasing PDI values.

Hema ETL $PLGA_8$ PEGylated NPs confirm what already underlined. Degradation proceeds after the first days. It could be due to the more hydrophilic features.

To sum up PEGylated NPs behavior during PBS Stability Study:

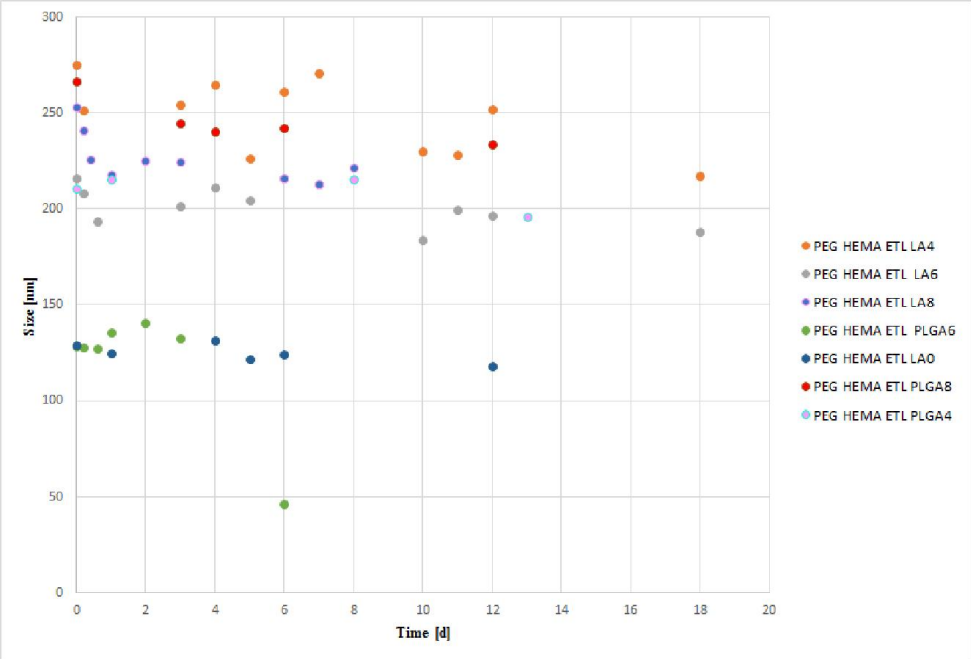


Figure 3.47 –*PEG Hema ETL LA_X vs $PLGA_X$ NPs , Size PBS stability study*

- Hema ETL $PLGA_X$ PEGylated particles degrade faster than Hema ETL LA_X NPs, with a more oscillatory behavior.
- Hema ETL LA_0 NPs demonstrate to be the most stable. This could be considered further proof of the fact that, having less ester bonds, Hema ETL LA_0 NPs chemical structure cannot offer as many points of interaction as PLA-based and PLGA-based NPs with longer Chain Lenghts can.
- On the other hand Hema ETL $PLGA_6$ and Hema ETL $PLGA_8$ revealed the particles with less stability in PBS. This can be due both to a more hydrophilic structure and a longer chain, which offer more contact points with the surrounding system.
- Also considering Count Rate data, $PLGA_X$ NPs show fluctuating trends, due to the sensible degradation.
- LA_X Count Rate trends are comparable.

3.4.3 Cell Medium Degradation

PEGylated NPs degradation rate was also tested in Cell medium. It represents a degradation study. We are expecting a quicker degradation compared to H_2O degradation study.
Degradation grade has been characterized at different times: particles size measurements were performed through dynamic light scattering (Malvern, Zetanano ZS) 3 times a day.
It was performed over a 2-week period. NPs characterization focused in particular on:

- **Size [nm]**
- **Pdl [-]**
- **Count Rate [kcps]**

These three parameters were considered indicative of the state of degradation of the samples examined.

DLS measurements were carried out at 37°C using 137° backscatter angle. After the first measure, values of attenuator and measure position have been kept constant. This permitted to perform the analysis with standardized operating condition, thus optimizing particles characterization:

NPs	Attenuator	Measure position
PEG Hema ETL LA_0	6	4,65
PEG Hema ETL LA_4	6	1,25
PEG Hema ETL LA_6	6	4,65
PEG Hema ETL LA_8	7	4,65
PEG Hema ETL $PLGA_4$	7	1,25
PEG Hema ETL $PLGA_6$	7	4,65
PEG Hema ETL $PLGA_8$	6	4,65

Table 3.10–*Attenuator and Measure position, Cell medium degradation*

3.4.3.1 PEG Hema ETL LA_X NPs

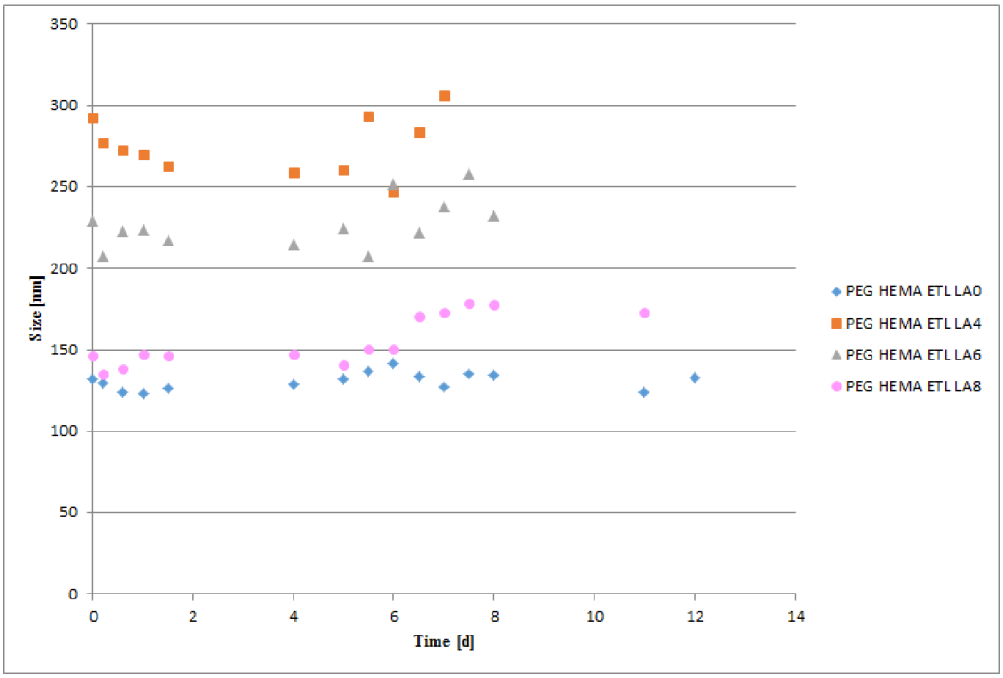


Figure 3.48 –*Size PEG Hema ETL LA_X NPs Cell Medium Degradation*

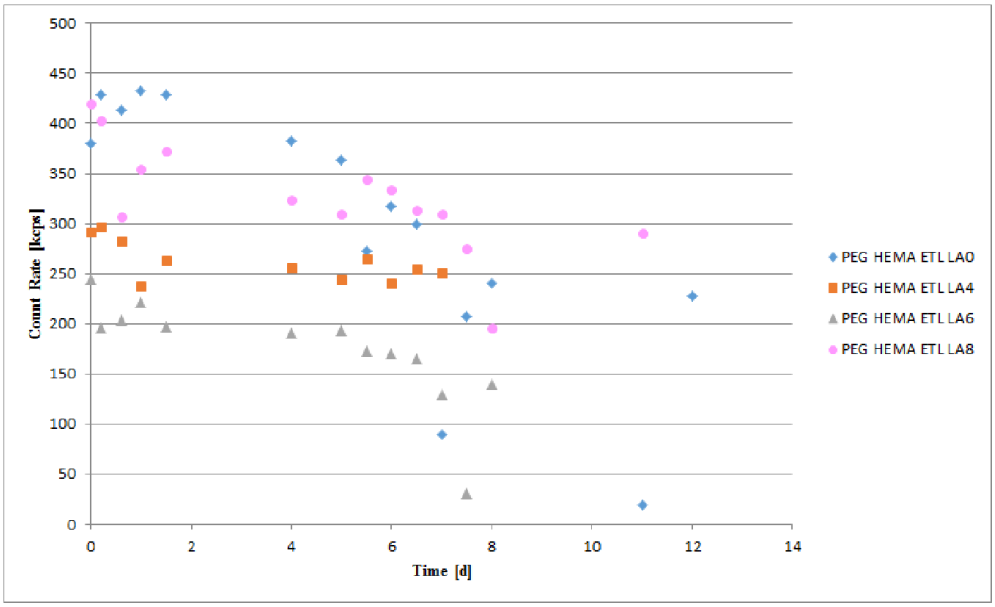


Figure 3.49 –*Count Rate PEG Hema ETL LA_X NPs Cell Medium Degradation*

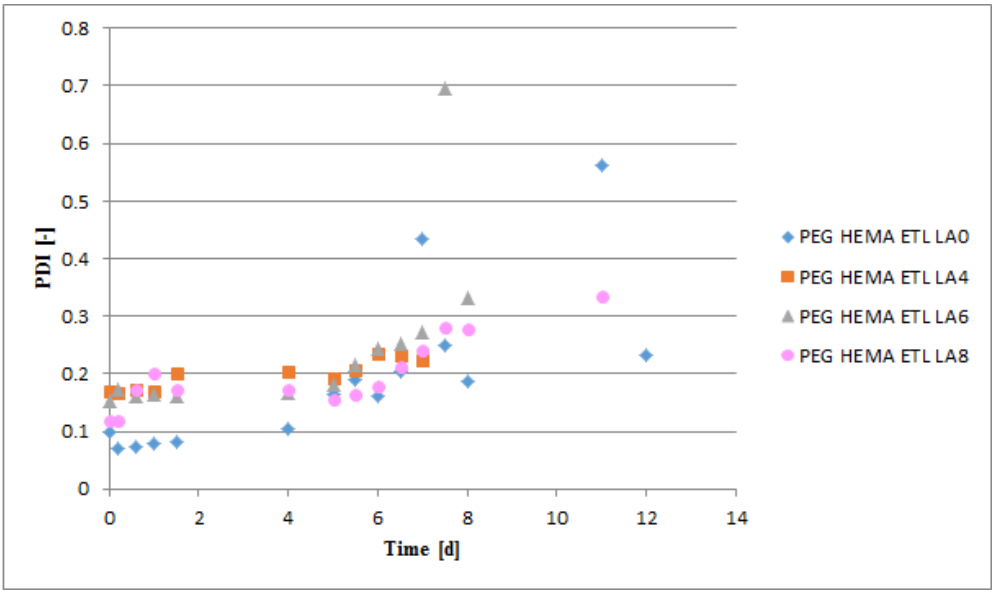


Figure 3.50 –*PDI PEG Hema ETL LA_X NPs Cell Medium Degradation*

In **Figure 3.48, 3.49** and **3.50** PEG Hema ETL $PLGA_X$ NPs Size,Count Rate and PDI are reported:

Hema ETL LA_0 PEGylated NPs degradation in cell medium confirms the stability verified during PBS stability study and water degradation as well. Size measures do not change sensitively during the 2-week experiment. Polydispersity index varies. From the 6th day, data exceed the acceptability limits (PDI >0.2). Count Rate shows a decreasing trend.

Hema ETL LA_4 PEGylated NPs degradation in cell medium also confirms the stability verified during PBS stability study and water degradation. PDI results oscillating around the acceptability limits. A secondary peak is observed since the 1st day of measures. In any case, percentage pointed out are not relevant, laying under 5% of the examined NPs. Count Rate measures do not change sensitively, in line with the other measures.

Hema ETL LA_6 PEGylated NPs degradation appears faster, in comparison to the results seen in La_4 and La_0 cases. This statement can be justified considering Count Rate data: the signal which characterizes the sample gets weaker and tends to 0 after 1 week. Count Rate decreasing trend could be

related to the formation of Micelles, that indicates NPs degradation. It also shows a plateau between the 3rd and 6th day. Size trend results oscillatory. PDI exceed the suitability range after the 3rd day, as a sign of proceeding degradation.

Hema ETL LA_8 PEGylated NPs demonstrate an increasing Size since the very first day of measures. As it can be seen, the longer the chain gets, the faster the degradation is registered. PDI exceeds the acceptability range as degradation proceeds. A relevant secondary peak is evaluated since day 6. As reported, it increases with time. Increasing Count Rate indicates aggregates formation.

3.4.3.2 PEG Hema ETL $PLGA_X$ NPs

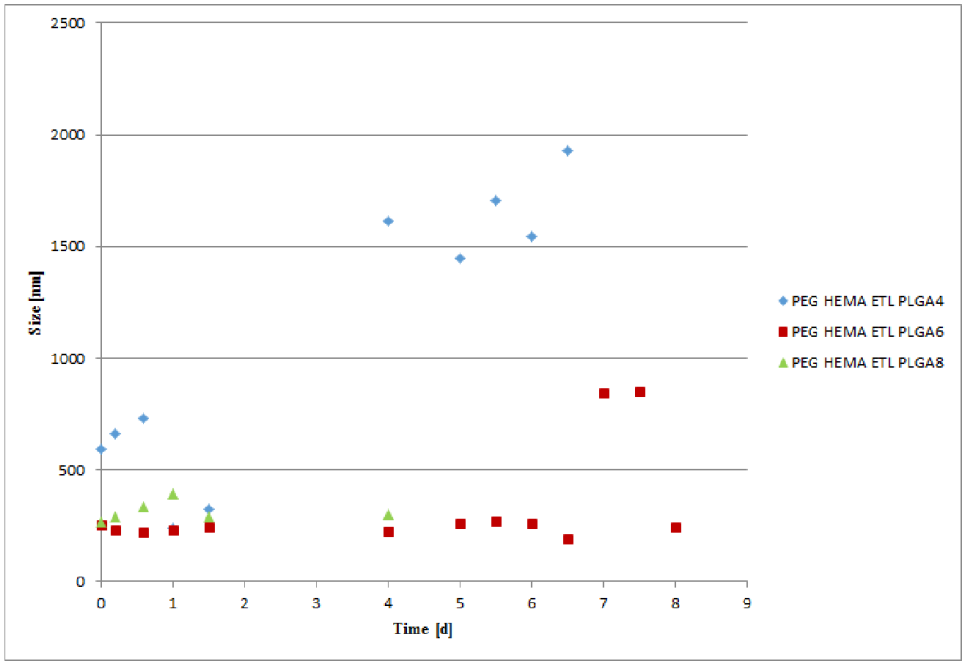


Figure 3.51 –*Size PEG Hema ETL $PLGA_X$ NPs Cell Medium Degradation*

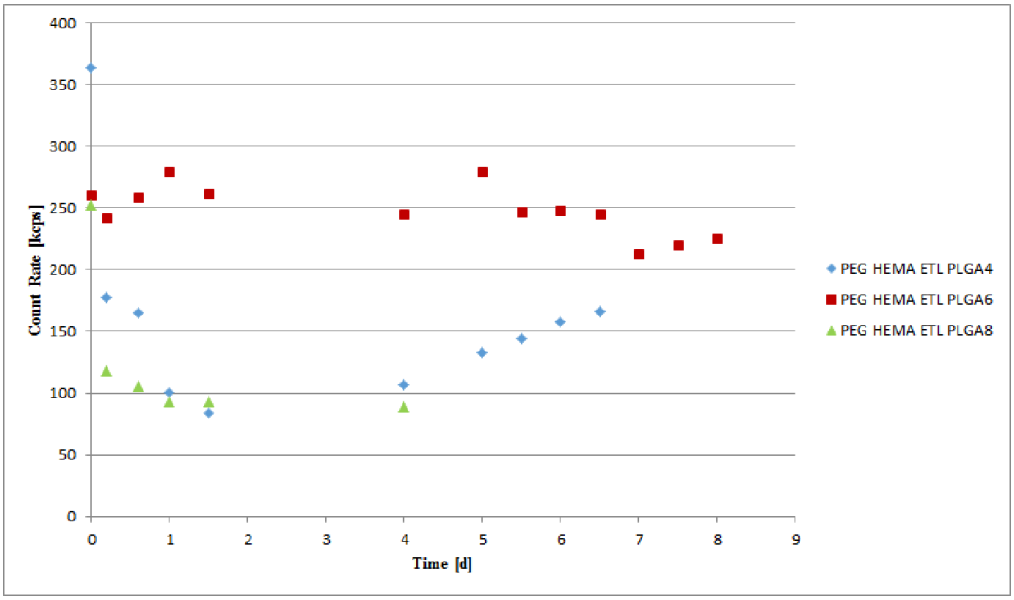


Figure 3.52 –*Count Rate PEG Hema ETL PLGA$_X$ NPs Cell Medium Degradation*

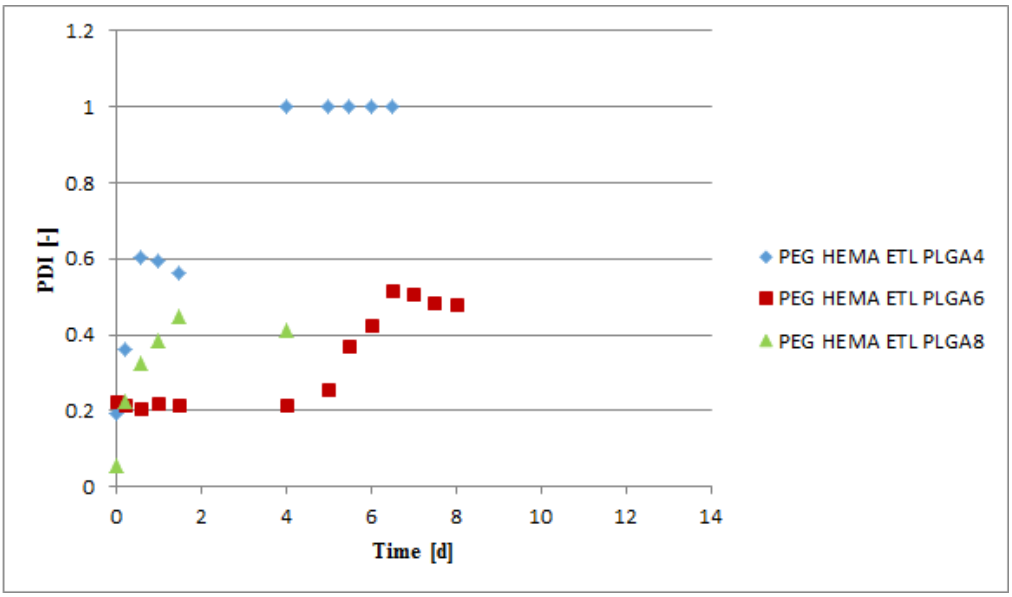


Figure 3.53 –*PDI PEG Hema ETL PLGA$_X$ NPs Cell Medium Degradation*

In **Figure 3.51, 3.52** and **3.53** PEG Hema ETL PLGA$_X$ NPs Size, Count Rate and PDI are reported:

Hema ETL $PLGA_4$ PEGylated NPs in cell medium show fast degradation. Size increases sensitively since the first days. This trend differs significantly from the one pointed out for LA_4. It confirms that the more hydrophilic features make easier the degradation. PDI exceeds the acceptability limit since the 1st day. Secondary and Tertiary peaks detected are reported in negligible percentages. Count Rate appears to decrease during the first three days (Micelles formation). After that it starts to increase, that means aggregates start to form.

Hema ETL $PLGA_6$ PEGylated NPs are characterized by an oscillating Size trend, whose variations become sensible after the 6th day. It confirms all the data gathered. PDI exceeds significantly the acceptability limit after day 2. Count Rate appears to decrease during the first three days (Micelles formation).

Hema ETL $PLGA_8$ PEGylated NPs show degradations features, which increase with time. Size is constantly rising indeed. PDI confirms the trend, by exceeding the acceptability limit after day 2. Count Rate appears to decrease over the period of measures. This can be connected to Micelles formation, in relation with NPs degradation.

It is possible to sum up all the results gathered, in order to have a general overview:

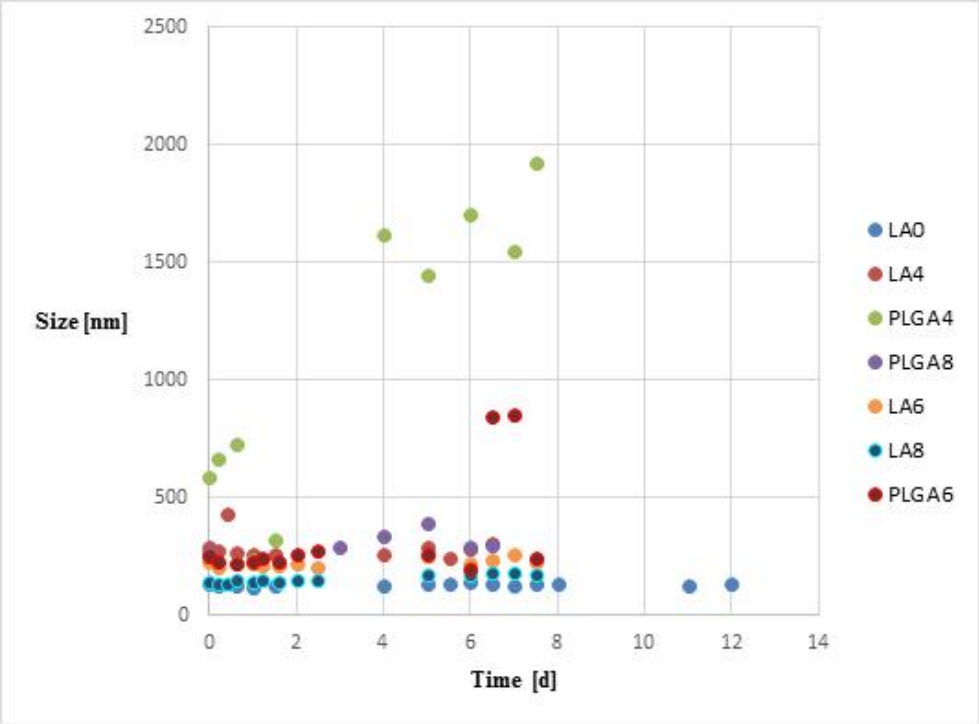


Figure 3.54 –*PEG Hema ETL LA_X vs $PLGA_X$ NPs , Size Cell Medium Degradation*

- Hema ETL LA_X PEGylated NPs measures are comparable. In particular LA_0 particles reveal the most stable behavior.
- Hema ETL $PLGA_X$ PEGylated NPs degrade faster than LA_X, as confirmed in the other studies.
- Count Rate trends appear oscillating for all the particles examined. No significant difference has to be highlighted between Hema ETL LA_X and Hema ETL $PLGA_X$ PEGylated NPs.

3.5 pH study

By virtue of the preliminary character of the study presented herein, a pH study was conducted to give additional information to what observed during Degradation studies, by means of DLS. In this sense was proposed the 5-day long investigation in Cell Medium (setting T=37°C) reported below, whose meaning is only qualitative and perceived to be strictly connected to DLS results.

pH is a measure of hydrogen ion concentration, that is to say a measure of the acidity or alkalinity of a solution . Aqueous solutions at 25°C with a pH less than 7 are acidic, while those with a pH greater than 7 are basic or alkaline . A pH level equal to 7.0 at 25°C is defined neutral **[59].**

In Figure 3.55 a pH Chart is given. pH measurements take on great importance in fields like medicine, biology and chemistry, in relation to different chemical environments associated to the different values.

When we think about biomedical applications in particular, we need to keep in mind that all the devices and all the chemical formulations developed have to be biocompatible and non-citotoxic in every stage of their activity. Thereby investigating the pH is essential to understand the way NPs could interact with neighboring cells during degradation.

Most living tissues prosper at a near-neutral pH, i.e. a pH close to 7. The pH of blood ranges from 7.35 to 7.45, for instance. An acid PH could represent a threat for cells or at least a suffering condition.

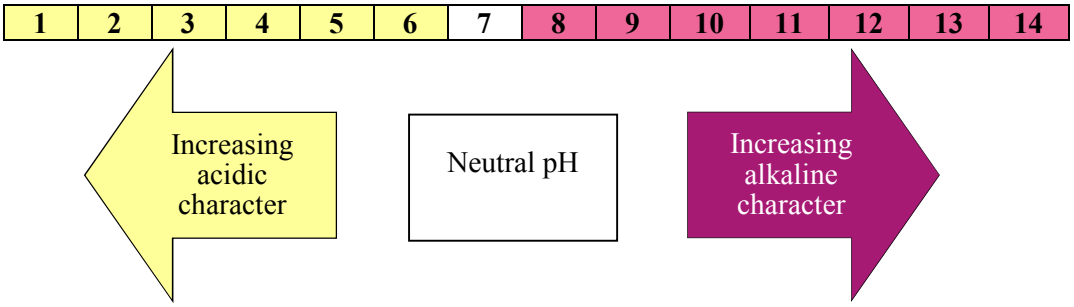


Figure 3.55–*pH Chart*

As pointed out in the previous Chapter, Cell medium contains Phenol red which is a good pH indicator. Coloration is therefore an important factor to evaluate how the degradation is proceeding and to characterize the surrounding environment. In **Figure 3.57** Pure Cell Medium before Investigation start is illustrated.

Its color exhibits a gradual transition from red to yellow over the pH range 8.2 to 6.8 **[60]** .

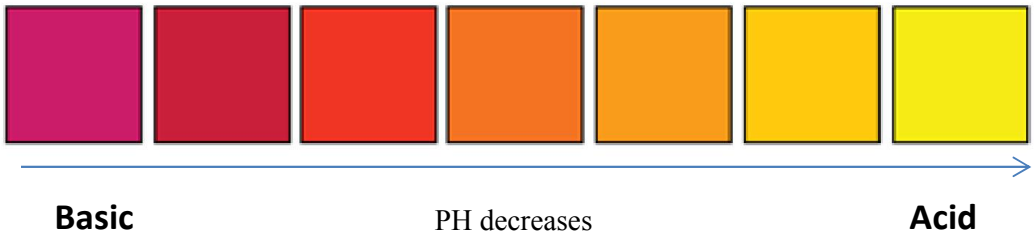


Figure 3.56–*Phenol red PH Chart*

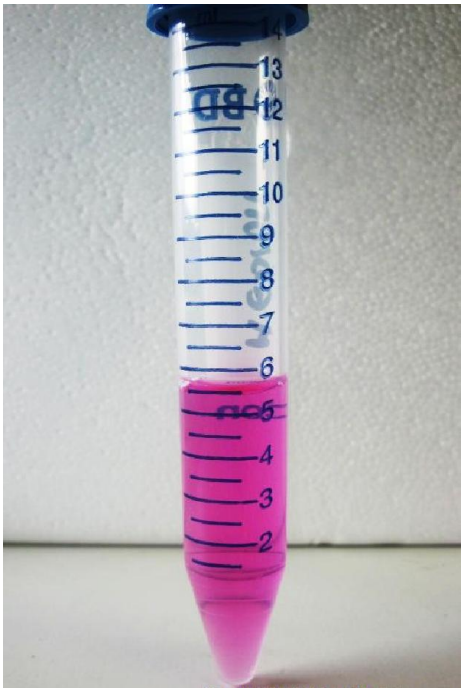


Figure 3.57– *Cell Medium Sample*

3.5.1 Day 1:

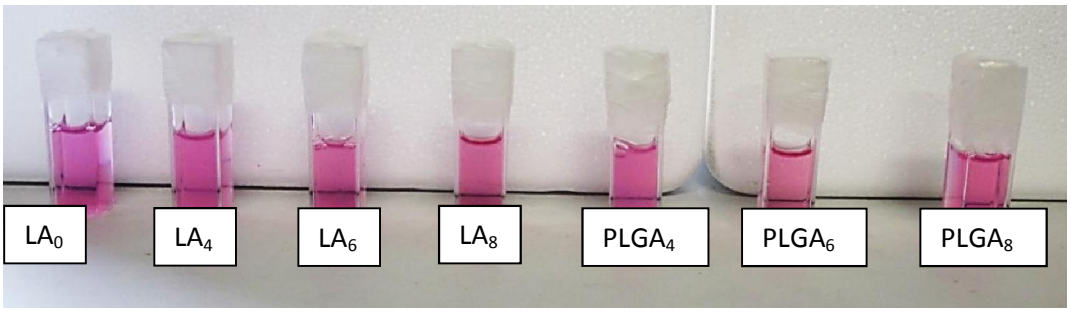


Figure 3.58- *NPs samples, Day 1*

All NPs show the coloration typical for pure Cell medium. No differences can be highlighted among the samples.

3.5.2 Day 4:

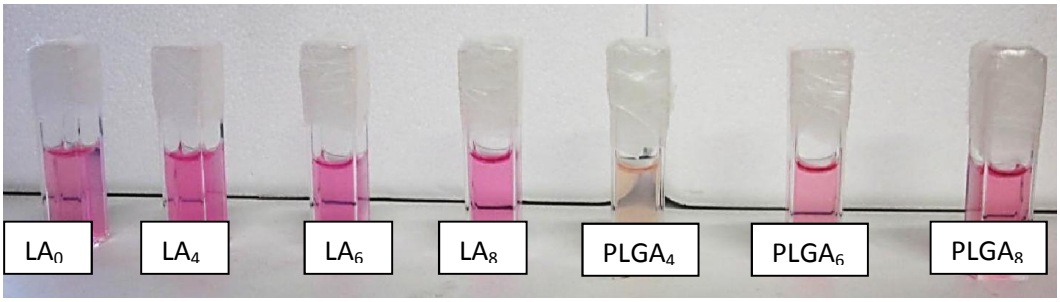


Figure 3.59- *NPs samples, Day 4*

A change in colour becomes sensible on the 4th day. $PLGA_4$ based NPs are degrading faster than the others, in line with what has been shown by Light Scatteting investigation. Besides, Hema ETL LA_X NPs are evidently degrading slower than Hema ETL $PLGA_X$. The more hydrophilic features already discussed confirm the trend evaluated. **Figure 3.60** and **3.61** show respectively an enlargement of PEG Hema LA_X and $PLGA_X$ NPs in Cell Medium.

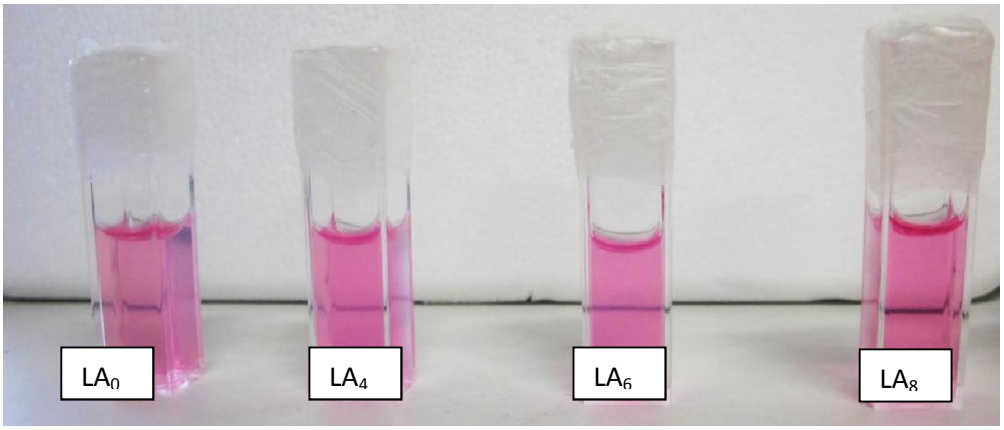


Figure 3.60- *LA_x NPs samples, Day 4*

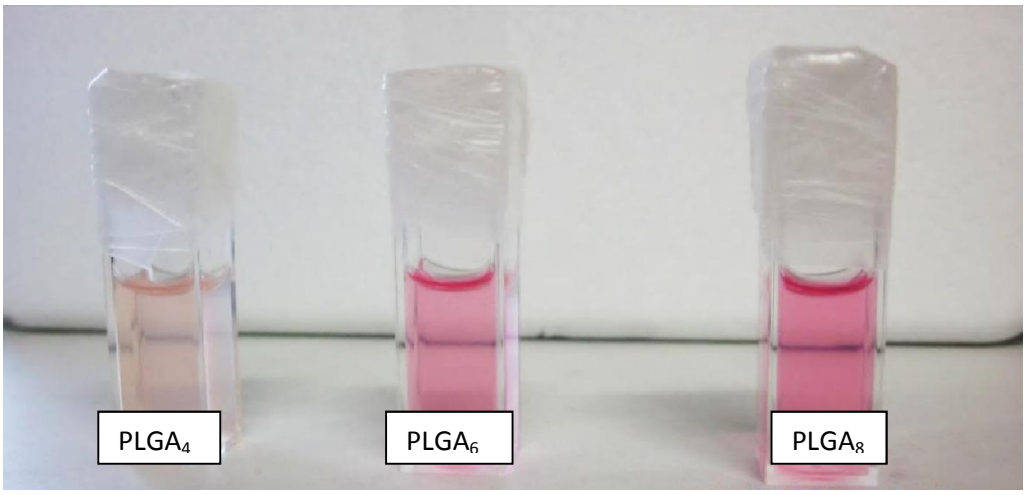


Figure 3.61- *$PLGA_x$ NPs samples, Day 4*

3.5.4 Day 5:

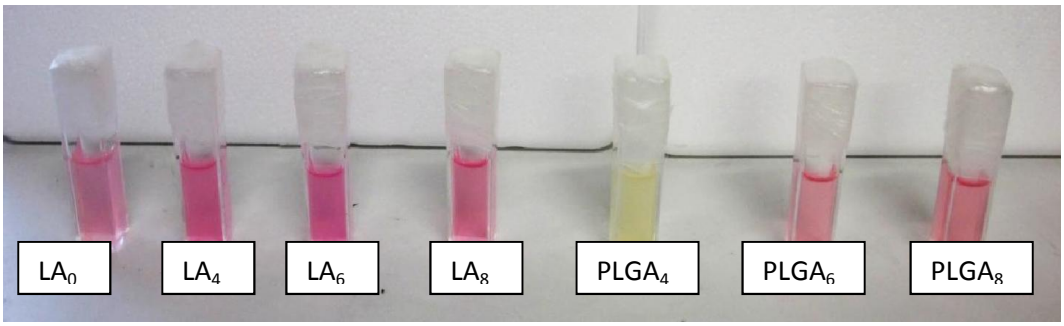


Figure 3.62- *NPs samples, Day 5*

Day 5 verify the faster degradation of $PLGA_4$ NPs (noticeable both in **Figure 3.62** and **3.64**), which now exhibit an acidic degradation environment. Hema LA_X are degrading slower, as can be seen by the still purple-like coloration visible in **Figure 3.62** and **3.63**.

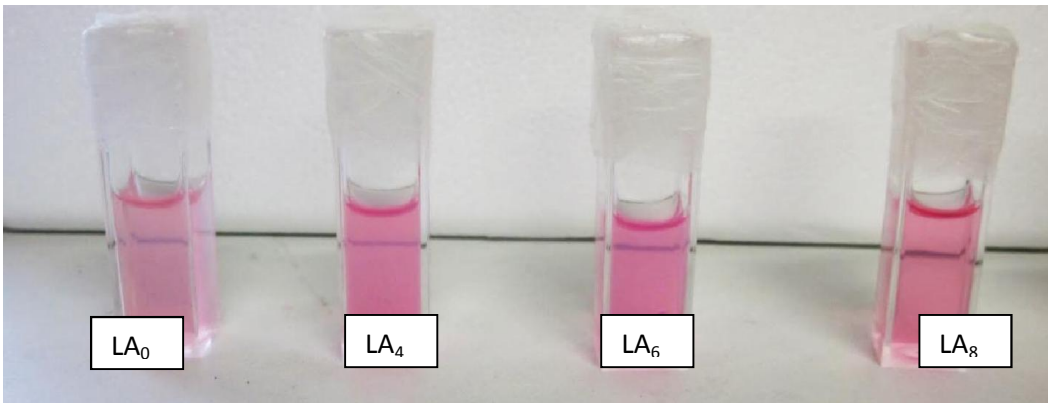


Figure 3.63- *LA_x NPs samples, Day 5*

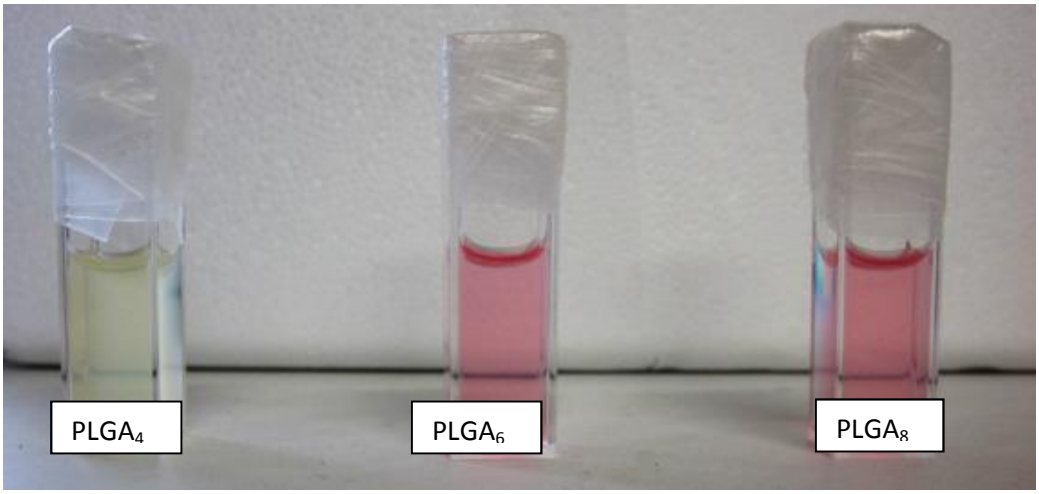


Figure 3.64- *$PLGA_x$ NPs samples, Day 5*

To conclude this section an in depth study in necessary, in order to have the optimal operating conditions. Further applications of these NPs have to take into consideration the degradation environment developed in short-term time. The change in properties during polymer biodegradation influences the release and degradation rates of incorporated drug molecules indeed.

4. Conclusions

An efficient Drug Delivery Device can ideally provide for high dose of therapeutic agents specifically to the diseased cells, thus minimizing side effects. Among the different classes, polymeric NPs have gained much attention thanks to several attractive properties, including:

- biodegradability and biocompatibility;
- controlled release of payload;
- no severe synthesis conditions;

Polymeric NPs are able to dissolve hydrophobic drugs in polymeric matrix, solving in this way the drug solubility problem as well as possess advantages such as **[30]**:

- high stability;
- efficient drug load;
- sustained drug release;

In this work of Thesis a preliminary study of PLA and PLGA based macromonomers is hinted, intended to synthetize NPs for biomedical applications. Their main feature is intended to be a $-CH_3$ termination, whose purpose lays in increasing their hydrophobicity. Methyl groups facilitate also the addition of targeting agents on specific places on NPs surfaces.

Therefore a two-step procedure was proposed, consisting in the synthesis of macromonomers through ROP and successive coupling reaction with HEMA succinate.

Macromonomers with different chain lengths were then polymerized through FRP to produce NPs with narrow particle size distribution. As final step of the Thesis, material properties were verified with Degradation and Stability studies.

On the basis of what highlighted in the previous Chapters, it is possible to draw the following conclusions:

- The synthesis of PLA and PLGA macromonomers result reproducible in each step, with acceptable yield values for both ROP and coupling reactions;

- PEGylated NPs shown longer degradation times than what is reported in Literature for –OH terminating macromonomers (about 8 days at 50°C) in agreement with what expected, regarding their structure and their more hydrophobic characteristics. Complete degradation is shown between 8 and 12 days at 37°C in Cell Medium and after 20 days at 50°C in water. In particular:

Hema ETL LA_X based NPs exhibited the highest stability. Especially Hema ETL LA_0 NPs displayed remarkable results;

- Tuning Degradation time is also possible, according to the number of repeating units contained in the macromonomers.

Nevertheless some aspects need an in depth study, with the intention of facing the problems raised during the synthesis:

- Hema ETL LA_8 and Hema ETL $PLGA_8$ NPs synthesis have to be improved, in order to avoid aggregates formation. Additional filtration steps are required to remove undesired aggregates.

- Optimization of reaction conditions, to achieve higher yields and guarantee pureness of the final product.

In the light of what has been pointed out, the macromonomers obtained result suitable for biomedical applications.

Guidelines for future studies could include:

- In vitro and in vivo trials, to confirm the conclusions introduced;
- Drugs loading studies;
- Optimization of degradation times, in order to find the best combination of drug activity and controlled release.

Further applications of these NPs have to take into consideration the degradation environment developed. The change in properties during polymer biodegradation influences the release and degradation rates of incorporated drug molecules indeed.

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