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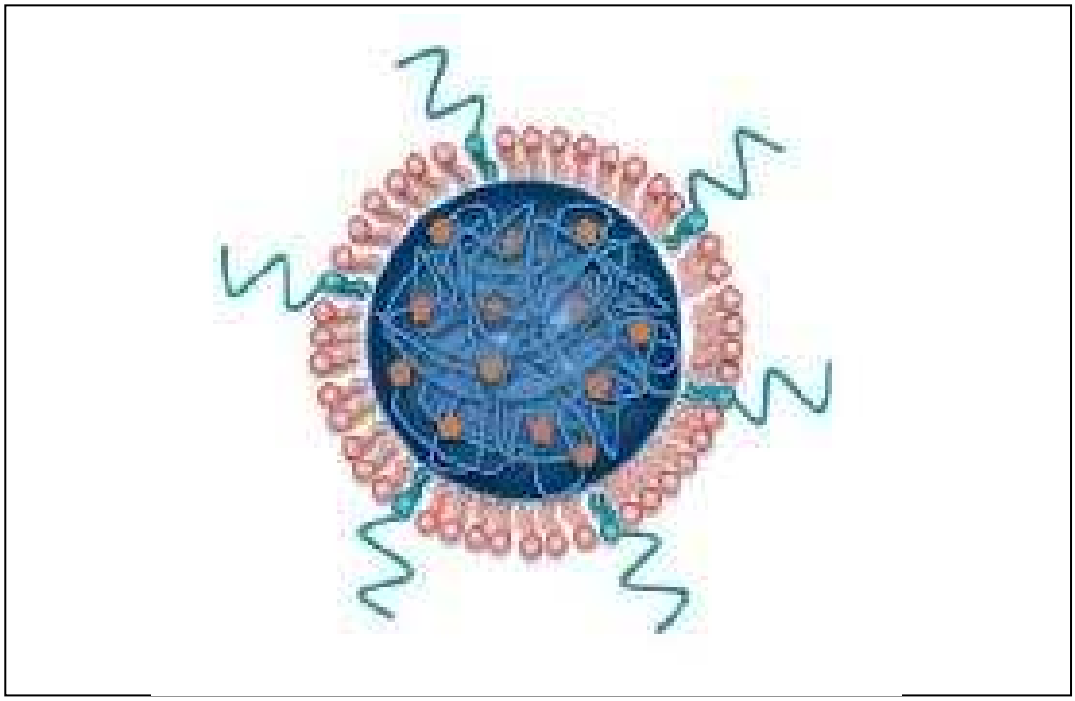

Formation of polymeric nanoparticles encapsulating and releasing a new hydrophobic cancer drug

Master of Science Thesis in Materials and Nanotechnology

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Department of Chemical and Biological Engineering
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Cover:
[Image showing a lipid-pegylated nanoparticle with drug. Taken from Zhang L., Lipid-polymer hybrid nanoparticles: synthesis, characterization and applications (2010), World Scientific Publishing Compagny, Vol.1, 163-173.]

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SUMMARY

Two anti-solvent precipitation methods have been used to encapsulate a new hydrophobic cancer called MCHB. Biocompatible and biodegradable polymeric nanoparticles were used for this purpose, as the drug must be delivered into cancerous cells. The size, the surface charge, the encapsulation efficiency and the release were the determinant factors for these particles. Nanoprecipitation was the first technique used in this project and 34% of the initial amount of drug was successfully incorporated in poly(lactic-co-glycolic acid) (PLGA) nanoparticles. This formulation showed a good dispersity and a particle size of 80 nm, but a fast release of a few hours was obtained in a two-phase water/octanol system. As this drug is highly hydrophobic, the release study could not be done in only water and octanol was therefore added in order to always have the drug dissolve. This two-phase system gives an easy way to determine the released amount of drug, as the nanoparticles stay in the aqueous phase and the drug diffuses to the organic phase. The second anti-solvent technique was Solution Enhanced Dispersion by Supercritical fluids (SEDS) and 80% of the injected amount MCHB was encapsulated in pegylated L-poly(lactic acid) (L-PLA-PEG) particles. The release was longer than for the particles made by nanoprecipitation, as it lasted 3-4 days in the same water/octanol system. However the particles obtained were bigger (200-500 nm) and less dispersed than for the nanoprecipitation method. L-PLA particles were also made using supercritical fluids and showed a lower encapsulation efficiency, but also a much slower release. Finally, the polymeric nanoparticles were lipid coated using sonication and the efficiency was analyzed by confocal microscopy. The coating worked for both samples, but was more successful for the non-pegylated particles due to their higher hydrophobicity.

Keywords: drug delivery, MCHB, PLGA, PLA, polymeric nanoparticles, nanoprecipitation, SEDS, lipid coating, pegylation, release.

ABBREVIATIONS

CHF: Chloroform

CNOB: 6-chloro-9-nitro-5-oxo-5H-benzo(a)phenoxazine

DCM: Dichloromethane

DLS: Dynamic Light Scattering

DPPC: DiPalmitoylPhosphatidylCholine

DSPE-PEG: 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)

FDA: Food and Drug Administration

FITC: Fluorescein IsoThiocyanate

MCHB: 9-Amino-6-chloro-5H-benzo(a)phenoxazine-5-one

NHS: N-Hydroxysuccinimide

PBS: Phosphate Buffered Saline

PEG: PolyEthylene Glycol

PDI: PolyDispersity Index

PF68: Pluronic® F68

PLGA: Poly(Lactid-co-Glycolic Acid)

PLA: Poly(Lactid Acid)

RES: ReticuloEndothelial System

SEDS: Solution Enhanced Dispersion by Supercritical Fluid

SEM: Scanning Electron Microscopy

SCF: SuperCritical Fluid

Contents

1. INTRODUCTION

Nanotechnology has brought new possibilities to many fields such as in electronics or biomaterials production, but also in the pharmaceutical industry. Indeed, new ways of delivering a drug have been developed recently. But what is in fact nanotechnology? There is a confusion around this term concerning the limitation of the size, what is counted as nano and what is not. In general, the definition of a nanostructure is a structure with a size between 1 and 100 nm, in at least one dimension.[1] However, one spatial dimension in the size up to 1000 nm (equal to 1 μm) is considered as a nanomaterial, as it still has physical and chemical properties different from the bulk.[2] Forming these types of structure enables a new approach for the delivery of a drug and new formulations can be made with different techniques. In this master thesis, nanoparticles have been prepared using two different techniques, in order to encapsulate a new hydrophobic cancer drug.

2. BACKGROUND

2.1. A new drug against prostate cancer

Prostate cancer is one of the most common types of cancer for men. In the United States, it is the most common one and it is the second largest cause of cancer deaths. When this disease is in an advanced state, it is essentially incurable. Some clinical trials are currently going on, but the results of these treatments have unfortunately given mixed results. Chemotherapeutic treatment is used to kill the cancerous cell, but does not always eradicate all these cells and its toxicity to healthy cells is another disadvantage. There is therefore a need of finding a new treatment able to efficiently kill prostate cancerous cell.[3]

In 2009, a new prodrug, called CNOB (6-chloro-9-nitro-5-oxo-5H-benzo(a)phenoxazine) was discovered.[4] It is a non-toxic prodrug which is transformed to a toxic form, called MCHB (9-Amino-6-chloro-5H-benzo(a)phenoxazine-5-one) when it comes in contact with its activating enzyme ChrR6 (Figure 1). MCHB is the only product of this two-electron reduction by ChrR6. Nicotinamide adenine dinucleotide phosphate (NADP+) is the coenzyme of this reaction and is produced in the mammalian cells through its reduced form NADPH.

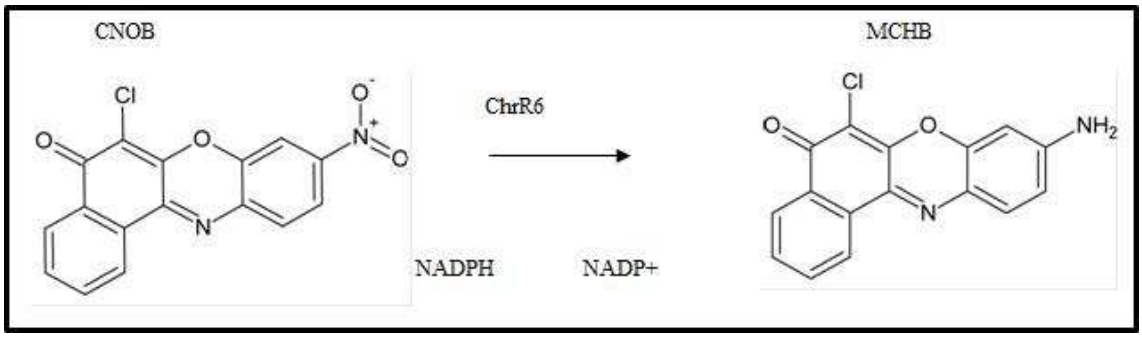

Figure 1. Structure of CNOB and MCHB.

CNOB have nitrosubstituted benzene rings that have already been seen in another cancer drug, 5-aziridinyl-2,4-dinitrobenzamide (CB1954). This drug is currently in clinical trial and is converted by a bacterial enzyme into a toxic form, which is able to kill cells by the formation of DNA cross-links.[4][5] It has been shown that CNOB/ChrR6 is more effective killing cancer cells than the CB1954/ChrR6, but it also has another more important advantage. Indeed, CNOB and MCHB are two fluorescent components and this enables a direct visualization of the drugs activation in the cells. A better understanding during the *in vitro* and *in vivo* analysis can therefore be obtained.

With the arrival of the nanotechnology, new ways of delivering drugs have been possible to develop. CB1954 uses such a new delivery way, called Virus-directed enzyme–prodrug therapy (VDEPT).[6] It consists of delivering the drug and the enzyme separately into the cancerous cell. When the contact is made, the toxic form is formed directly into the cell and can kill it. This new prodrug, CNOB, has been thought to be delivered similarly, but bacteria and viruses are not the wanted drug carriers. In fact, the use of these components can be problematic as they

can give an immunological response by the human body, especially when the cancer patients immune systems are already weakened.

CNOB and its activating enzyme, ChrR6, will therefore be delivered using another carrier. Nanoparticles are planned to be used for that purpose and Gene-delivered enzyme prodrug therapy (GDEPT) is the right term for this method. As it is seen in Figure 2, the gene-encoding enzyme has to be delivered into the nucleus of the cancerous cell (Box 1). After transcription of the gene, the enzyme can activate the non-toxic prodrug delivered into the cell (Box 2). The formed toxic drug can thereafter kill the cell in which it is present or diffuse to the neighboring cells (Box 3). This phenomenon is called the bystander effect and is very important for the GDEPT, as all cells will not be able to transform the prodrug into its toxic form.

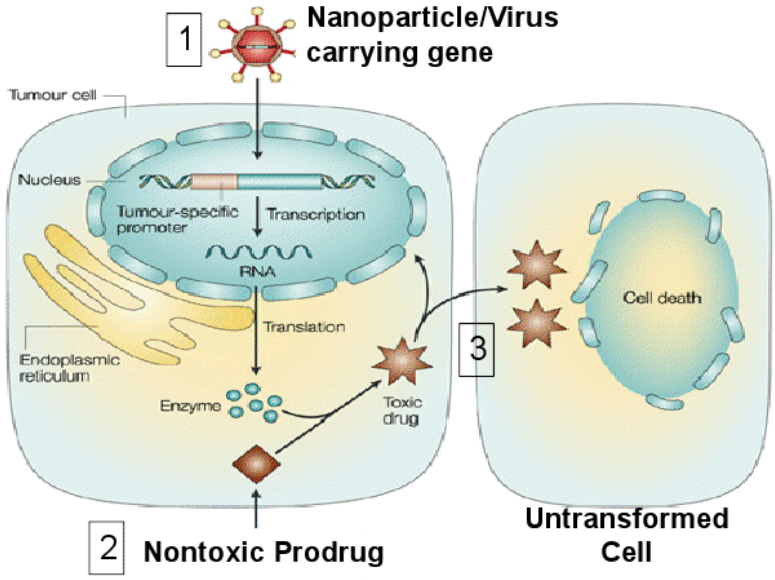

Figure 2. Schematic description of GDEPT. Modified from [5].

Studies done with MCHB *in vitro* and *in vivo* have shown that this drug is effective in killing cancer cells, through mitochondrial DNA intercalation. The fluorescence intensity of MCHB was found to be directly proportional to its cell killing activity and an important parameter for the GDEPT is that MCHB has shown to have an impressive bystander effect.

To summarize, nanoparticles containing CNOB will be transported through the blood circulation. Using a specific targeting, these particles will go to the prostate region into the cancerous cells. Some of these will express the gene (green cells in Figure 3) and some will not (blue cells in Figure 3). CNOB will be activated to MCHB in the cells containing the gene (red cells in Figure 3) and by the bystander effect, the surrounding cells that are not activated, will also get MCHB.

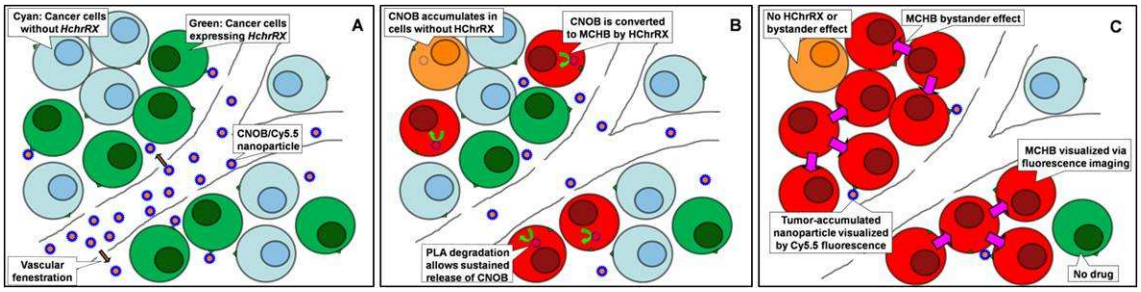

Figure 3. Activation mechanism of CNOB.

This master thesis is a part of the bigger project in which the goal is to form nanoparticles capable of specifically targeting and killing prostate cancer cells. It is a project where two research groups at Stanford University collaborate. The laboratory of Professor AC Matin has discovered the prodrug/active enzyme and is responsible for the selection of targeting ligand as well as *in vitro* analysis. The laboratory of Professor RN Zare is responsible for the nanoparticle formulations and coupling of the targeting ligand.

It was decided to use the toxic drug MCHB for this formulation study. This was done in order to simplify the following step in the project. In fact, when suitable nanoparticles have been formulated, the targeting ligand will be attached to them and they will be used in an *in vitro* analysis. In presence of cancerous and non-cancerous cells, it will be possible to see if the synthesized drug carriers enter the cells, how efficient the targeting is and if the cancerous cells are killed. MCHB and CNOB are both fluorescent and have similar properties. Therefore, if the *in vitro* analysis gives positive results, the formulation will be applied to the non-toxic prodrug.

2.2. Use of nanotechnology in drug delivery

The use of nanotechnology for drug delivery systems is an actual subject today. A lot of research is taking place at different universities in the world in order to find new formulations capable of delivering drugs to specific areas of the body. As it has been described, the next step in the formulation of this CNOB/MCHB drug is to find a way to deliver the prodrug and its activating enzyme specifically to the cancer cells. Nanoparticles were chosen as carrier because of their good properties. Indeed, they are able to protect the drug from degradation, increase its solubility in the body, give a sustained delivery and give the possibility of specific targeting. Specific targeting to the cancer cells will be achieved by using selected antibodies and attaching them to the nanoparticles.

When formulating the nanoparticles, some parameters are important to have in mind: the size, the surface charge, the drug release and of course the encapsulation efficiency. In fact, the size is crucial in order for the nanoparticles to reach the cancer cells as well as penetrate them. This physical property influences the lifetime inside the human body and according to Zhang et al., the nanoparticles should be smaller than 150 nm.[7]

Polymers, long chains of repeating structural units, have gained a lot of interest these last years as drug carrier and many advantages have been shown over lipid vesicles. Therefore polymeric nanoparticles have been investigated to carry the CNOB drug to the target cancer cells.
However all polymers are not capable of being drug carrier, as they need to be biocompatible and biodegradable. The first term "biocompatible" refers to a material which can be present in a body for a long time without giving any immune response (or very little response) and this property is fundamental when using a material in a body as an implant or a drug carrier. There is also one big difference between polymers used for implants and ones used to encapsulate and deliver a drug in the body: the biodegradability. This term refers to the ability to degrade/disintegrate in the organism. We don't want the polymer to stay and accumulate in the cells after that the delivery of the drug. Instead it should be cleared from the body by hydrolysis and the products should not be toxic. The degradation time must not be too long and not too short, but it should last some months.

Two conformations of polymeric nanoparticles are known to exist. The term "nanocapsule" is used when the polymer forms a core, in which the drug can be entrapped. The other common structure is called nanosphere and refers to a nanoparticle made of entangled polymer chains. For this conformation, the drug is present between the chains and it is the most truly conformation for the prepared nanoparticles in this project. It is although important to notice that the drug can also be absorb on the surface of the nanoparticles, as it is shown in the following Figure 4.

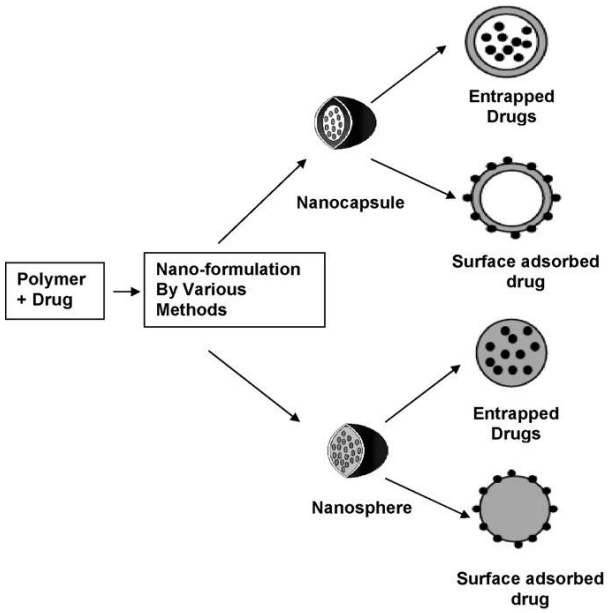

Figure 4. Type of biodegradable nanoparticles.[8]

3. FORMULATION METHODS

The encapsulation of MCHB was investigated using two different anti-solvent precipitation methods along with the use of either a biodegradable polymer or lipid. In all cases the MCHB and the polymer was dissolved in an organic solvent. This solution was then added to an anti-solvent which allowed the two components to precipitate into solid nanoparticles. The nanoprecipitation method uses an aqueous anti-solvent solution whereas the supercritical fluid process utilizes supercritical carbon dioxide. Both techniques are described in more details below, but it is first important to describe the components necessary to form the nanoparticles.

3.1. Components

3.1.1. MCHB

This toxic drug is very hydrophobic, which is due to the non-presence of leaving groups, free electrons and the presence of benzene rings which are highly hydrophobic. Its precipitation time is therefore very short in water and in order to encapsulate it, the precipitation time of the nanoparticle carrier should be the same as the drugs. MCHB is in form of a dark red powder and is soluble in many organic solvents, such as acetone, acetonitrile, dichloromethane, chloroform and octanol, but only to a certain level, which we will see had an impact on the encapsulation. Its fluorescence is characterized by an excitation wavelength at 575nm and an emission at 625nm.

3.1.2. Polymers

Two polymers have been used in this project. Poly(lactid acid) (PLA) is one of them and has been used as it is biodegradable, biocompatible and is Food and Drug Administration (FDA) approved. Its degradation is due to the hydrolysis of the ester bond (see the chemical structure in Figure 5), but its time depends on its degree of crystallinity. In fact, PLA can be made more or less crystalline due to the chirality of the lactid acid molecule (L-lactide and D-lactide). A semi-crystalline polymer is obtained from the polymerization of these monomers and the higher the percentage of D-lactide, the more amorphous the polymer is. L-Poly(lactide) (L-PLA), which has been highly used in this project, has a crystallinity of around 37%, which however also depends on the molecular weight of the polymer. L-PLA has a glass transition temperature of 60-65°C, which is important to notice in order for the prepared nanoparticles not to go into a rubber state and conserve their structure.

Poly(lactic-co-glycolic acid) (PLGA) is another biocompatible and biodegradable polymer, composed of lactid acid (left repetitive structure in Figure 5) and of glycolic acid (right repetitive structure in Figure 5). Indeed, PLGA hydrolyzes into lactid acid and glycolic acid in the presence of water, two compounds which are commonly present in the body because they are by-products of metabolic reactions in the body. PLGA is preferred to polylactic acid (PLA) and polyglycolic acid (PGA), as it has a better solubility in organic solvents and the degradation time of PLGA can be more easily controlled. This can be done by varying the ratio lactid acid: glycolic acid. The degradation time of polylactide is much longer than for polyglycolide due to

a higher hydrophobicity and a PLGA 50:50 will therefore degrade faster than PLGA 85:15 (1-2 months against 5-6 months).[9]

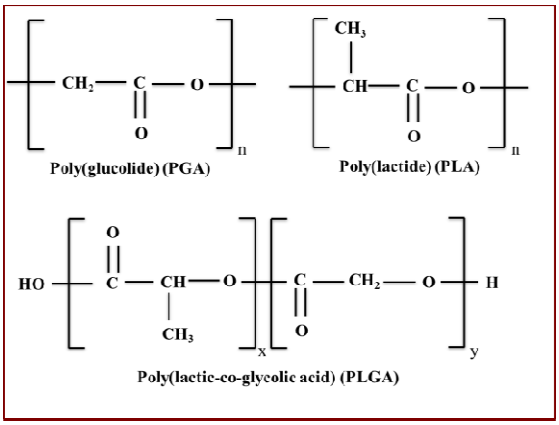

Figure 5. Chemical structure of PGA, PLA and PLGA.

It has been shown that PLGA is a surface eroding polymer and that these types of biomaterials have a good ability to give a sustained release, as well as to protect hydrolytically sensitive compounds. Poly(D,L-lactide) acid (PDLLA) has however shown a difficult sustained release.[8]

Other polymers can be used as nanoparticle carrier for drug delivery and is a parameter to control the release time. Polycaprolactone (PCL) is another biocompatible and biodegradable polymer, but it has not been used in this project because of its slow degradation time. It is usually used for delivering long-term drugs and vaccines.

3.1.3. Lipid and Polyethyleneglycol (PEG)

The formulated polymeric nanoparticles are highly hydrophobic and would not be able to reach the targeted cells in a high enough yield. The immune system has different mechanisms to recognize foreign components in the body and internalize these. Therefore, there is a necessity of modifying the surface of the nanoparticles and two widely used components were used for that purpose.

A homogeneous lipid layer surrounding the nanoparticle has shown some advantages. In fact, this coating can increase the biocompatibility of the nanoparticles in the body. It can also prevent the particles from drug retention as the lipid layer surrounds the particle, and it can by the same way decrease the water penetration rate. Finally, by having this component on the surface, the targeting ligand can easily be attached, as well as polyethylene glycol (PEG), which is the second component.[10]

PEG is a hydrophilic non-ionic polymer and is FDA approved. The process of attaching it onto nanoparticles is called pegylation and it has been proven to increase the systemic circulation lifetime and decrease the inflammatory responses by the body. The uptake of the nanoparticles by the Reticuloendothetial system (RES) is reduced. This process consists of sending foreign components to the liver, the spleen or the bone marrow where they can accumulate. Without the PEG shell layer, nanoparticles would aggregate in these organs and the

drug delivery would not be accomplished. PEG have decrease RES by different mechanisms. In fact, instead of having a hydrophobic surface, the nanoparticles will be covered by this hydrophilic chains and the water solubility will therefore be much higher. The surface charge of the polymeric drug carrier will similarly be hidden as PEG is non-ionic and less interaction with different blood components will occur. This will decrease the opsonization process, in which foreign components are marked by an antibody and are taken up by white blood cells, called phacocytes. The nanoparticles have a high surface energy, but by having long thin PEG chains forming a so called "mushroom" structure (see cover image), a steric stabilization is obtained. The attraction between the particles is decreased and the aggregation phenomenon is reduced. Aggregates are not formed and the size of the particles is minimized, which also prevents uptake from the phagocytosis.

PEG is largely used and no other polymers have been discovered to achieve these tasks as good. Although it is important to notice that this polymer is non-biodegradable, but a renal clearance is done if the concentration is sufficiently low.[11][12]

Lecithin is the lipid compound used in this project and is constituted of phosphoric acid, choline, fatty acids, glycerol, triglycerides and phospholipids. The pegylation has been done by using 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG (2000)). PEG is here covalently bonded to a lipid, DSPE and this is an easy method to attach its chains to the polymeric core. The pegylation has also been done by simply using block co-polymer with a covalently attached PEG.

3.1.4. Preparation components

Other components have been used during the formulation of the nanoparticles:

- Molecular grade water (Distilled, deionized DNAase, RNase and purase), in which the nanoparticles are finally dispersed.
- Organic solvents, such as acetone, acetonitrile, methanol, chloroform (CHF), dichloromethane (DCM) and octanol.
- Surfactant Pluronic® F68 (PF68): in order to get a better stability in the nanoparticle solution and less aggregation. (The concentration used has always been under the critical micelle concentration in order not to form micelles. This has been tested by analyzing the PF68 in water solution by Dynamic Light Scattering.)

3.2. Nanoprecipitation method

3.2.1. Description

In this liquid-liquid precipitation method the organic solution (dichloromethane, chloroform, acetone, or acetonitrile), containing the polymer and the drug, is added drop-wise (by a 200 µl pipette) to an aqueous water/methanol solution under stirring. The polymer and the MCHB are previously dissolved in the organic solvent with a known concentration (the polymer concentration was always set to 5mg/ml). These two organic solutions were stored in the freezer

(at -17°C) until use. At the time of formulation, predetermined amounts of polymer and MCHB solutions were mixed together before the addition to the receiver solution. The solutions were stirred for 2 hours and followed by the complete removal of all organic solvents on the rotary evaporator. The nanoparticles are obtained as dispersed in the water solution (see Appendix for complete protocols).[7][13]

This method is also called interfacial deposition method as the interfacial tension is decreased when the solvent is added to the aqueous phase. The solvent, which is water miscible, diffuses rapidly through the aqueous phase in form of nanodroplets. This allows the drug and the polymer to get into contact with the water in which they precipitate. Therefore, a nucleation point is created and the nanoparticles can grow.[14]

Some parameters can be varied in this preparation and are the factors controlling the efficiency of the precipitation. In fact, the choice of the solvent is an important factor in this method. It is also crucial that both the polymer and the drug are completely soluble. A mixture of organic solvents is also a possibility. A second criterion for the solvent is that its boiling point is lower as compared to water, permitting the easy removal of the organic solvent with the rotary evaporator.

The type of polymer is another factor, but in this project PLGA has mainly been used to encapsulate MCHB using the nanoprecipitation method. As it has been described, PLGA has a better solubility than other polymer and its degradation time can be easily controlled with its ratio lactic to glycolic acid as well as its molecular weight. DL-PLGA 50:50 (Mw= 5000-15 000 Dalton) and DL-PLGA 85:15 (Mw = 50 000-75 000 Dalton) have been used. Finally, the weight percentage of drug can also be varied; the concentration of the polymer and the drug in the solution, as well as the ratio of organic solvent to aqueous solution controls the nanoprecipitation.

This technique has been used as it is easy, energy-saving, as well as time saving compared to the normal double emulsion used to prepare nanoparticles and nanoprecipitation has previously shown encapsulation of hydrophobic drugs.

3.2.2. Previous studies

Nanoprecipitation has been widely used to encapsulate a drug and deliver it. The different studies have given different encapsulation efficiencies, mostly depending on the drug.
Paclitaxel ($C_{47}H_{51}NO_{14}$, M=853.9 g/mol) is a hydrophybic cancer drug and was incorporated in polymeric nanoparticles by Danhier et al. using two differents methods: nanoprecipitation and simple emulsion method. Acetone was the organic solvent used for the nanoprecipitation. The second method consisted of dissolving the drug and the polymer in dichloromethane and to add it to an aqueous solution containing a surfactant (sodium cholate). The polymer used was a mixture of PLGA, PLGA-PEG and PCL-PEG. The study showed a big difference in encapsulation between the two methods. The nanoprecipitation method resulted in an efficiency of 70%, compared to 40% for the simple emulsion method. It is although important to notice that the drug attached to the surface is taken into account in the determination of this efficiency

and an important initial burst is therefore visible in the release study results (almost 50% of the drug). After that, the paclitaxel was constantly released over more than 11 days.[15]

The same drug was studied some years before by Fonseca et al. and this laboratory group succeeded to encapsulate the drug to a higher extent (superior to 90%), also using the nanoprecipitation method. The incorporation was found to be very different depending on how the drug and the polymer were mixed. Indeed, a different encapsulation was obtained when mixing these two components as powder and then dissolving them, or first dissolving them separately, followed by their mixing. This variation was important as over 90% was encapsulated when mixing the dissolved drug with the polymer, compared to an efficiency of 15% for the other method. Acetone was also used in this study, but the polymer was only PLGA, therefore different from Danhier et al. Polymers with different molecular weights and ratios of lactic to glycolic acid were tried, but the efficiency of drug incorporation was found to be independent of these parameters. Similarly to the previous study, the release in vitro showed an important initial release the first hours (about 60% of the drug), followed by a more continuous release over 9 days.[16]

In another study, the authors have shown an encapsulation efficiency of 91 to 94% for an antipsychotic drug called risperidone ($C_{23}H_{27}FN_4O_2$, M=410.5 g/mol).[17] This drug is also hydrophobic and was encapsulated using D,L-PLA. In this article, it was shown that the presence of a stabilizer did not have an effect on the particle size or the encapsulation ratio. Throughout their experiments, the authors demonstrate that the size is affected by the concentration of the polymer. A higher concentration gives a larger particle size. The encapsulation obtained was high, although the release only lasted approximately 30 hours, with a similar initial burst as in the other studies.

These two drugs have a high molecular weight compared to MCHB ($C_{16}H_9ClN_2O_2$, M=296.7g/mol). A more resembling drug is 9-Nitrocamptothecin ($C_{20}H_{19}N_3O_6$, M=393.4 g/mol) and was studied by Derakhshandeh et al.[18] Besides having smaller molecular weight, the two cancer drugs are both lipophilic. The encapsulation efficiency for 9-Nitrocamptothecin obtained was 33% using PLGA 50:50 and in the following study, it was a maximum of 45% using PLGA-PEG. The drug was released during more than 5 days. Acetone was used to dissolve the polymer and the drug, and polyvinyl alcohol (PVA) was used as a stabilizer. In contrast to the study made on the risperidone, a stabilizer in the receiver solution had a strong effect on this encapsulation.

Throughout these studies, the nanoprecipitation method has shown the possibility to form particles with a good size and a good dispersity. Hydrophobic drugs can be encapsulated and released with a constant rate. Finally in another study, it has been shown that the particle size can be controlled by the choice of solvent, as well as the ratio of organic solvent to aqueous solution. This study was made with a drug called docetaxel ($C_{43}H_{53}NO_{14}$, M=807.9 g/mol). The stronger miscibility of the solvent in water, as well as a smaller solvent to water ratio, were shown to decrease the particle size. The effect of the ratio was less important. Acetone and acetonitrile are the two most common solvents for the nanoprecipitation method and were therefore used in this project. Dimethylformamide (DMF) was tested in the docetaxel study and gave a smaller particle size than for the two solvents. Unfortunately, this solvent has a boiling

point higher than water and to keep the preparation method easy, it was not tried. Finally, it was also revealed that a higher concentration of polymer increases the size.[19]

3.3. Solution Enhanced Dispersion of Supercritical fluids

3.3.1. Description

Polymeric nanoparticles, capable of delivering drug, can also be produced using supercritical fluids (SCF), which are substances at a temperature and a pressure above their critical point (Figure 6). SCF have good physical properties, which can easily be controlled by the pressure and the temperature of the fluid due to its high compressibility. Nanostructures can be formed due to the combination of SCF's low surface tension and the high mass transfer rate. Therefore, a solute can be brought over its saturation point, stimulating the nucleation of the polymer dissolved in it.

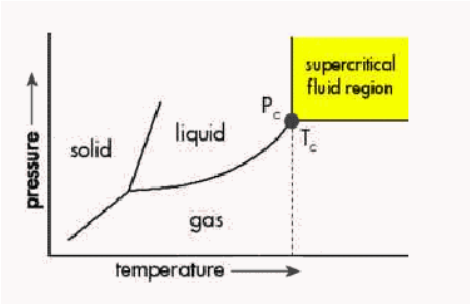

Figure 6. Graph showing the supercritical fluid region.

Carbon dioxide is the most commonly used supercritical fluid as it reaches the supercritical fluid region at a practical temperature and pressure (T_c=31.58°C and P_c=75.8bar). At these conditions, it is possible to work with biologically active compounds such as proteins and nucleic acids. It is also important to work below the glass transition temperature (T_g) of the polymer to keep its properties and avoid plasticization. Finally, CO_2 is a good option because it is nonflammable, inexpensive, is considered safe by the US Food and Drug Administration and it doesn't leave any toxic residue of organic solvents.

In this technique, the polymer and drug are dissolved in a co-solvent. The mixing of the two components in the same co-solvent is important in order to avoid any initial burst effect and get a good drug encapsulation. This solution is then sprayed into a high flow of a supercritical fluid. The polymer and drug precipitate into nanoparticles at the mixing point because of the transition from a two-phase system (co-solvent and SC-CO_2) to a one-phase homogeneous supercritical fluid system. As the solvents become miscible, precipitation and formation of particles occur. This is called the anti-solvent effect. The solvents, now miscible in a SCF, will pass through a filter and dry nanoparticles are collected from this apparatus filter.[20]

Instruments using SCF exist in different set-ups, depending on how the contact between the co-solvent and the SCF is made. Supercritical Anti-Solvent (SAS) is one process in which the co-

solvent solution comes into contact with the supercritical fluid in a particle vessel. In another configuration called the Solution Enhanced Dispersion by Supercritical Fluids (SEDS) method, based on the principle of SAS, the co-solvent and the SC-CO_2 are mixed before entering the particle vessel. In fact, the solution is sprayed into a tee, from above, and a flow of SC-CO_2 comes from each side of the mixing point. These two different mixing structures are shown in Figure 7. In the Zarelab, the SEDS method is used and the instrument is based on a modified SAS instrument (SAS50, Thar Technologies (Pittsburgh, PA)).

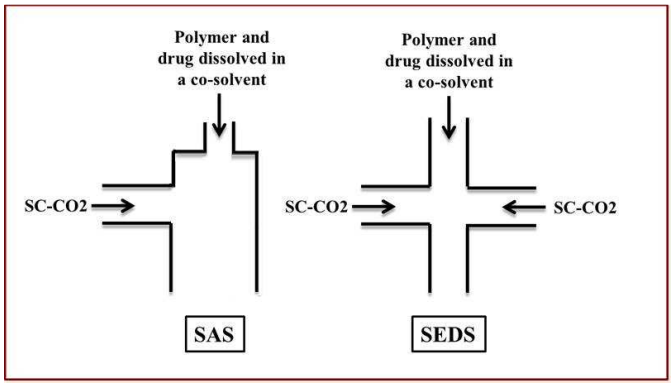

Figure 7. SAS and SEDS mixing point.

A more homogeneous polymer nucleation and therefore a more controlled particle size can be obtained with the SEDS process, in comparison with the SAS method. A frit is located at the entrance of the polymer-drug-co-solvent solution to get small droplets at the mixing point. In a SEDS-configuration, these small droplets are directly subjected to the high flow of SC-CO, which diffuses into them. The rate of this diffusion is very high as SCF have a low surface tension. The droplet expands thereby and gives rapidly a high contact surface between the polymer/drug and the SCF, resulting in the precipitation of particles. At the same time, the solvent diffuses out of the droplet because of its miscibility with the SCF and this increases also the mass transfer. Figure 8 is a schematic image of this mechanism.[21]

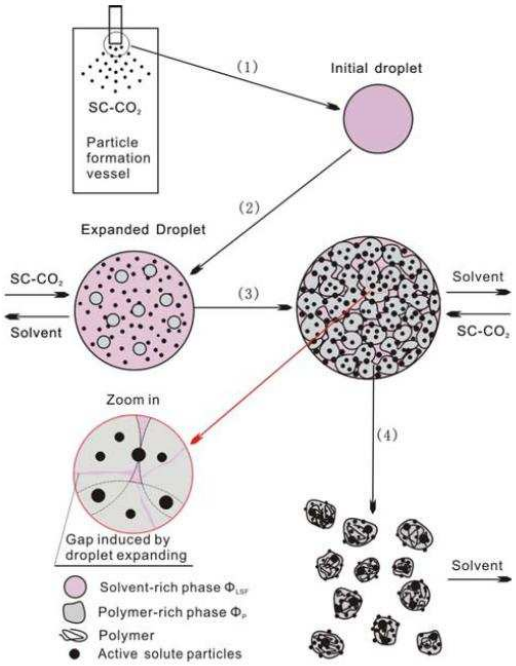

Figure 8. Mechanism diagram for encapsulating an active component into a polymeric carrier by the SEDS process.[21]

The procedure consists of setting a flow of CO_2 at the desired temperature, pressure and flow rate in order to condition the system. The co-solvent containing the polymer and the drug is prepared and is then injected through a nozzle into the tee described previously. The nozzle is used to form droplets and therefore increase the contact surface area. After the co-solvent solution has been injected, the precipitated particles are dried by simply continuing to inject CO_2 into the system for a duration of 20-30 minutes. Dry particles are obtained in the apparatus filter and no post-treatment is needed. Indeed, the organic solvent is completely removed with the SCF by pressure reduction and the dry particles are ready for use or can easily be stored in a freezer. The process yield is determined at this point. Figure 9 shows the different parts of the SEDS instrument.

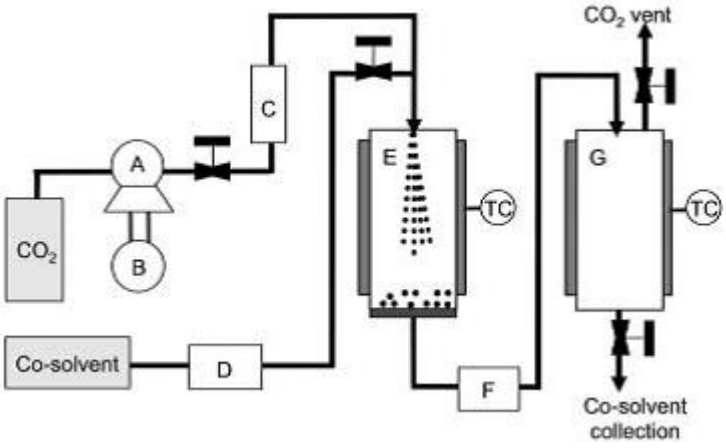

Figure 9. Schematic of the SEDS setup: A, CO2 pump; B, cooling bath; C, heat exchanger; D, cosolvent pump; E, particle vessel; F, automatic back-pressure regulator; G, coalescer; TC, temperature controller.[22]

3.3.2. Previous studies for SEDS

In previous studies performed in the Zarelab, the biodegradable polymer L-Poly(lactic acid) (L-PLA) has been used to form nanoparticles for drug delivery. This polymer is FDA approved and is known to release drugs in a controlled manner. It is also compatible with SC-CO_2, which is a prerequisite when using the SEDS method.

Hydrophilic compounds such as siRNA have successfully been encapsulated. The polymer used was L-PLA, which is soluble in dichloromethane (DCM). As siRNA is not soluble in DCM and because the drug and the polymer have to be dissolved in the same co-solvent (in order to prevent from an initial burst effect), siRNA was coupled with the cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP). The prepared polymeric nanoparticles had a size of 100-300 nm and showed a drug release during 40 days (Figure 10). Nanoparticles were also made by using L-PLA-PEG. This was done in order to reduce the particle agglomeration and it showed a reduced release time of about half.

Sustained drug release from L-PLA nanoparticles (with a size of 250-300nm) was also shown in a study using luciferin, as it can be seen in the *in vivo* results (Figure 10). The release was observed for up to 40 days with up to 90% drug recovery.

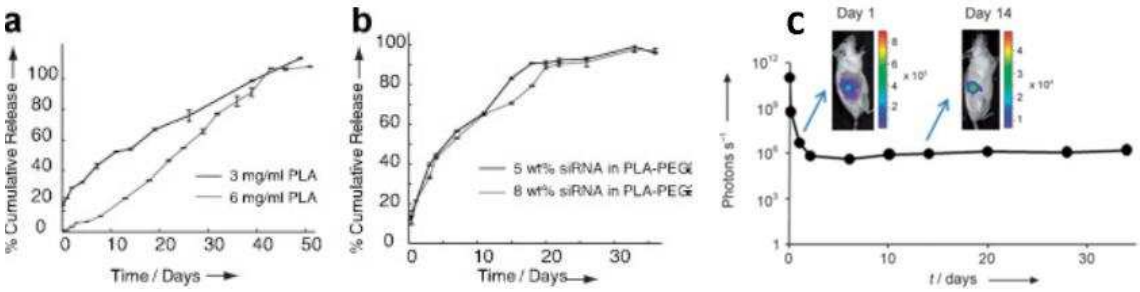

Figure 10. Percent cumulative release of siRNA in PBS from NP containing (a) 5 wt% siRNA in L-PLA (50 kDa), comparing SEDS runs using 3 or 6 mg/mL polymer concentrations in dichloromethane, and (b) 5 and 8 wt% siRNA in 6 mg/mL L-PLA (70 kDa)-PEG (5 kDa).[23] (c) Sustained release of luciferin after subcutaneous injection of PLA particles containing 2 wt% luciferin. Scale bar units are photons per second. [23]

SEDS permits a rapid nucleation and a particle growth into nanostructures of about 100-300 nm. The control of the particle size has been studied by making quercetin particles. The flow rates, solvent selection, polymer and drug concentration, temperature and pressure were all shown to be parameters that can be varied in order to decrease the particle size. For example, increasing the CO_2 flow rate and decreasing the injection flow rate has been shown to result in a smaller particle size as the ratio of SC-CO_2 to solvent increases.[22]

In the experiments performed in this project, the study was emphasized on the effect of the weight percentage of drug, the solvent and the effect of a covalently bonded PEG on the polymer. L-PLA (Mw = 50 000 Dalton) and L-PLA-PEG (Mw = 75 000 Dalton) were used. The temperature was set to 40°C, the pressure in the particle vessel to 100 bars, the CO_2-

flow to 120-140 g/min and finally the co-solvent flow to 0.5 ml/min (see Appendix for the complete formulations)

3.4. Lipid and PEG coating

After having obtained nanoparticles containing the drug, the next step was to coat the particles with a lipid and polyethylene layer. As it has been described, the lipid layer enhances the circulation time in the body and has many advantages, but another important role for the lipid layer is the possibility to attach a N-Hydroxysuccinimide-group (NHS). This chemical group is important for the following part of the project as it gives the possibility of attaching the peptide for specific targeting.

The lipid coating was simply done by sonication, which is the process of applying ultrasound energy to a solution. This action results in the agitation of the different components in the solution, which gives the chance for the lipid and the DSPE-PEG to bond to the nanoparticles surface. Figure 11 shows the schematic image of the desired nanoparticle structure.[24]

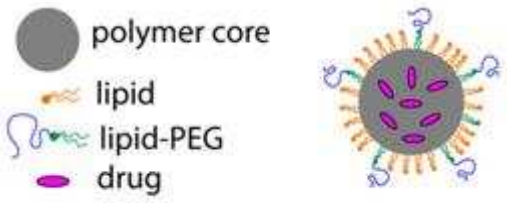

Figure 11. Schematic image of a polymeric nanoparticle containing the drug and surrounded by a lipid-PEG layer.

This procedure consisted of adding lecithin (dissolved in methanol) and DSPE-PEG (also dissolved in methanol) to a water solution. This aqueous solution was heated up to 40°C under stirring in order to get a good dispersion of the components. Depending on the formulation method, mixing with the polymeric nanoparticles was done at two different times. For the nanoprecipitation method, this process was done at the same time as the formulation step. Indeed, the mixture of drug-polymer, dissolved in an organic solvent, was added to the aqueous receiver solution containing the lecithin and the DSPE-PEG. However for the nanoparticles prepared by SEDS, the addition of a lipid-PEG layer was done separately. This post-coating consisted of adding the dispersed lipid-PEG in water solution to the aqueous solution containing the SEDS-nanoparticles, which had previously been sonicated in order to break as much aggregates as possible (see Appendix for the complete protocol).

The amount of lipid was set to 30wt% of the total amount of polymer, as this proportion has shown to give a homogeneous layer around the nanoparticles.[24] 90% of that amount was added as lecithin and the remaining 10% was fluorescein (FITC)-DSPE labeled PEG. In fact, this fluorescent component was purchased (from NanoCS) in order to analyze the coating, as will be described in the next section.

4. NANOPARTICLE ANALYSIS

In order to see if the prepared nanoparticles met all the required objectives, several types of equipment were used. The measurements of the size, zeta potential and the drug release are described in this section.

4.1. Size

The particle size is an important parameter as the drug has to be directly delivered into the cells and it was shown that a size inferior to 150 nm is needed. Two techniques were thereby employed to measure both particle size and particle size distribution. Indeed, the polydispersity index (PDI) is also an important factor as uniform particles are wanted with a narrow size range. A value of 0.3 is considered to be good for the characterization method used. In fact, the closest the PDI value is to zero the more similar the particles are in size.

4.1.1. Dynamic light scattering (DLS)

Theory

Also called Photon Correlation Spectroscopy, dynamic light scattering is a characterization method used to determine the size, usually in the submicron region, of particles, emulsions or molecules dispersed in a liquid.

This determination method uses the fact that all suspensions have a random movement in the liquid, called the Brownian motion. This random path is due to the contact with solvent molecules, which are themselves in movement due to their thermal energy. A laser is directed on the sample and when it hits the particles in the solution, the light is scattered in all directions, which is called the Rayleigh scattering. The scattering intensity varies time-dependently due to the distance variation of the particles in the solution, which is itself due to the Brownian motion. These intensity fluctuations are analyzed and are dependent to the particle size. Using the Stokes-Einstein equation, the hydrodynamic size can be determined:

$$D = \frac{k_B T}{6\pi\eta r}$$

Where: D is the diffusion constant, k_B is Boltzmann's constant, T is the temperature, η is the viscosity and r is the radius of the spherical particle.

DLS is a commonly used technique to determine the particle size as it has several advantages: it is an accurate and fast method (1-2 minutes); the preparation for the analysis is not complicated and the measurement is done in the native environment of the material; the only knowledge necessary is the liquid viscosity.[25][26]

Procedure

The prepared particles were dispersed in a water solution, added to a cuvette and analyzed by DLS (Malvern Zetasized Nano ZS90), in order to obtain the size and its dispersity. For the nanoprecipitation particles, the solution was diluted with water to get a count rate of 100-200 kcps. Due to a high aggregation of the SEDS nanoparticles, these were added to a of 0.5 wt%

Pluronic® F68/H_2O solution, sonicated and diluted in order be break the aggregates as much as possible. However, it was very difficult to get a good size distribution for SEDS nanoparticles. The aqueous solution containing SEDS particles was also filtered (1 µm filter) to remove the biggest particles, but as the polymeric particles are hydrophobic, most of them were trapped in the filter.

4.1.2. Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) analysis was performed to get a better understanding of the size, as well as to verify the results obtained with the DLS. In fact, DLS does not always provide the actual physical size, especially when the shape of the particles is not uniformly spherical and the polydispersity is high. SEM is a technique commonly used to study the surface topography of a sample and gives a 2D-image of the particles.

Theory

A beam of high-energy electrons is focused on the sample and when it penetrates the surface, it causes an interaction between the electron and the surface atoms. Secondary, back-scattered and Auger electrons are formed and these produced signals can be detected using Energy-Dispersive X-ray Analysis (EDX). The secondary electrons, formed during the scanning of the beam on the surface, are detected and reveal information such as the texture, the chemical composition and the crystalline structure of the surface of the sample. It is important to notice that the depth to which the electrons penetrate the sample can be controlled and therefore the image seen on the screen of the SEM is not only showing the surface.

SEM is widely used as it is a good method to see the topography of nanometer scale objects with a high resolution. The analysis is relatively fast and easy to operate, however a sample preparation must be done. The sample must be clean and needs to be attached to a SEM sample holder. A vacuum environment and an electrical conductivity are essential in SEM-analysis. Therefore, if the sample is non-conductive, it must be coated with a very thin layer (200-300 Å) of a conductor.[27][28]

Procedure

Some droplets of the diluted nanoprecipitation prepared solution (the diluted solution, prepared for the DLS measurements, was used) were poured on a SEM holder and left to dry. For the SEDS-particles, carbon sticky tape was used to attach them onto the SEM sample holder. Both samples were sputter coated with Pd/Au, with an intensity of 30 mA during 30 seconds, in order to make the samples conductive. A FEI XL30 Sirion SEM with FEG source, EDX detector was used. When the magnification was increased, the SEDS particles visibly melted together and it was therefore difficult to get images with a good quality. This problem didn't occur for the nanoprecipitation particles as they were highly dispersed in the water solution and therefore on the sample holder.

4.2. Zeta potential

This term gives information about the electrostatic potential of the particles in solution, as well as their colloidal stability. A low zeta potential value is wanted in order for the particles to be as neutrally charged as possible and therefore less interference will occur *in vivo*. That will allow a better circulation all the way to the target site. At the same time a value near zero means instability and rapid coagulation. Colloidal stability is obtained at a value higher than +30mV or lower than -30mV.[29] In this project, the first criterion is the most important and the pegylated brushed layer has been thought to prevent particle aggregation. Finally, a positive value is not desired because it is often a sign of toxicity.

Theory

The zeta potential is determined by measuring the electrophoretic mobility, which is the velocity of the particles when an electric field is applied to them. Indeed, if a particle is charged, it will move with a constant velocity (after equilibrium) to the electrode of opposite charge, in the presence of an electrolyte. This velocity depends of the zeta potential as it is seen in Henry's equation:

$$U_E = \frac{2\varepsilon\zeta f(Ka)}{3\eta}$$

Where: ε is the dielectric constant, ζ is the zeta potential, f(Ka) is Henry's function (equal to 1.5 or 1.0) and η is the viscosity.

The solution containing the particles is put into a zeta capillary cell (Figure 12). A laser beam is directed toward the sample and the fluctuation intensity of the scattered light is detected. The electrophoretic mobility is proportional to this variation and can therefore be determined. This technique is called Laser Doppler Velocimetry.[29]

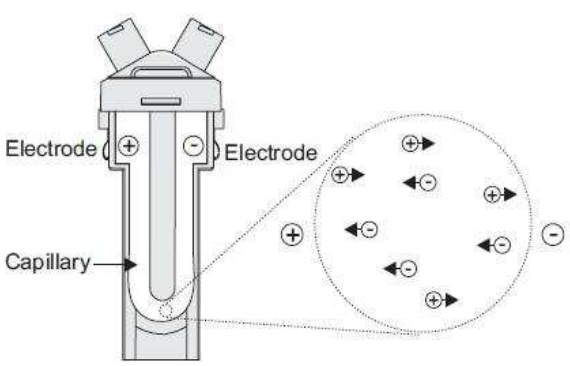

Figure 12. Schematic image of a Zeta capillary cell.[29]

Procedure

The prepared nanoparticle solution previously prepared for the DLS measurement is used in this analysis. A small amount of phosphate buffered saline (PBS) is added in order to get charges in the solution and mimic the body. Indeed, this buffer solution has a ionic concentration similar to

the one in the human body. A total H_2O: PBS ratio equal to 20:1 is used. This solution is then added to the Zeta capillary cell and the measurement is done by a Zetasizer instrument (Zetasizer, Malvern).

4.3. Release study

4.3.1. Fluroscence spectroscopy

MCHB is fluorescent (excitation at 575nm and emission at 625nm) and this property is an advantage when doing release studies. The concentration of the drug in a solution can be easily determined at any time using fluorescence spectroscopy. Without this property, the use of another technique such as high performance liquid chromatography (HPLC) coupled with a UV detector would have been necessary.

Theory

Fluorescence is the phenomena due to the absorption and thereafter the emission of light by a molecule. Photons of light are sometimes absorbed by molecules and this will lead to the passage of an electron from the ground state to the excited state. When the molecule then collides with other ones, it releases some of the absorbed energy and the electron drops to a lower excited state. The surrounding molecules can't always receive all that energy and the remaining energy is emitted. This absorption and emission phenomena is illustrated in the Jablonski diagram, in which S_0 represents the ground state and S_1 and S_2 are the excited states.[30]

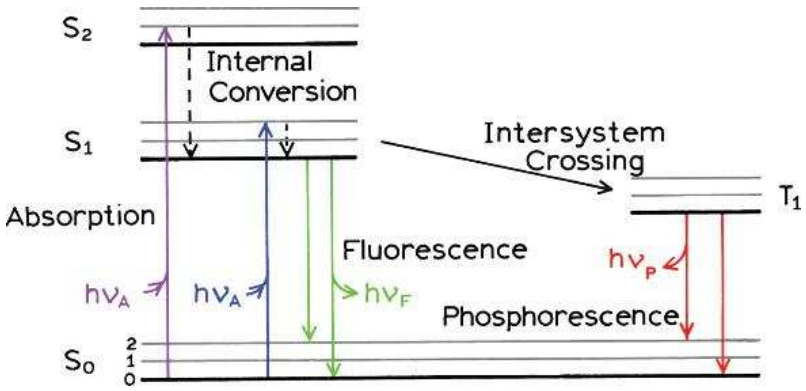

Figure 13. Jablonski diagram.[30]

The fluorescence spectroscopy technique uses a light beam, which is usually an ultraviolet light, in order to excite the molecules in the sample. The excitation wavelength is held constant and using a monochromator, the intensity of the fluorescent signal is measured through a certain emission interval.

Procedure

A fluorescence spectroscopy (SPECTRAmax Gemini XS) was used to get the fluorescence intensity of the samples. 3x100 µl were taken from the desired solution and pipetted into a 96-

Well plate (96 Well Costar black/clear bottom). Parameters such as the wavelength for the excitation and emission and the type of plate were selected in the setup before the reading.
In order to determine the amount of free or released drug, a standard concentration curve had to be done. MCHB was dissolved in octanol at a specific concentration. By diluting this stock solution to different extent, several solutions were made and analyzed with the fluorescence spectroscopy. The concentration curve of MCHB in octanol was thereby obtained (Figure 14)

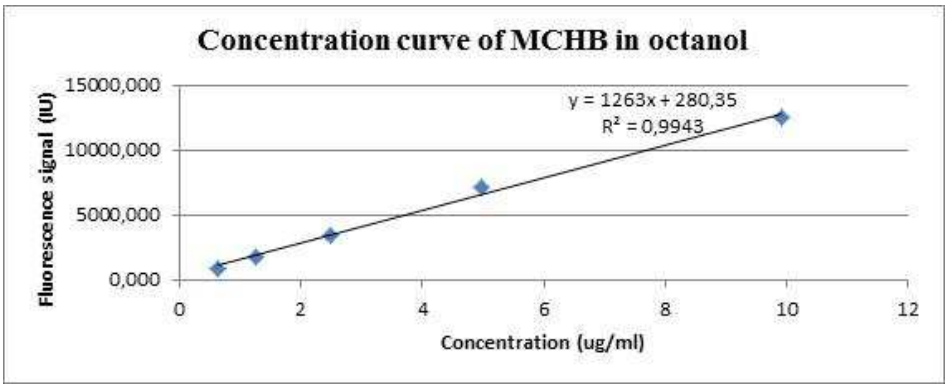

Figure 14. Standard concentration curve of MCHB in octanol.

4.3.2. Description of the release study

The method to determine the release of MCHB had to be rethought. The first trials consisted of dispersing the nanoparticles in a buffer medium (PBS) and to take out a small volume of this solution at predetermined time intervals. The samples were put into an incubator shaking them horizontally and set to 37ºC, in order to mimic the human body. In order to calculate the free MCHB, the drug had to be dissolved in an organic solvent which would not dissolve the polymer (the drug would otherwise be totally released). 1-octanol was therefore used and as this solvent is not miscible in water, two phases were obtained. After centrifugation, the organic solvent was analyzed by fluorescence spectroscopy. The aqueous phase was analyzed with DLS in order to see if the remaining nanoparticles were still there, which they were.

After 2-3 weeks, no MCHB release was detected. The fluorescence signal was too low to give a positive value and it did not increase during the release period. In fact, as the drug is highly hydrophobic, the release in water seemed to occur to a very small extent. Released MCHB was either absorbed to the polymeric surface or precipitated instantaneously to form particles, which sedimented. When using this procedure for the nanoparticles prepared by SEDS, a problem of homogeneity was also visible. Aggregates of particles were present in the solution and therefore it is not truly representative when a sample is taken from the solution.

A new procedure for the release study was necessary. The goal was set to have the released MCHB directly dissolved in the solution without affecting the polymeric nanoparticles. Octanol was therefore added to the prepared nanoparticle solution during the entire release. By letting the solution always be in contact with this organic solvent, the free drug could be dissolved and not interfere with the remaining release. In fact, this two-phase system gives the possibility of easily determining the amount of MCHB released after specific time intervals.

The procedure for the release study consisted thereafter of adding PBS to the prepared nanoparticle-solution (by nanoprecipitation)/ a small amount (4-5mg) of dried nanoparticles (by SEDS) and to add octanol. The volume of organic solvent added was set in order to get a concentration in the interval of the standard MCHB concentration curve (Figure 14). The vial was shaken and vortexed to assure contact between the free MCHB and octanol. Centrifugation was then used to get a distinct phase separation and the octanol-phase could finally be analyzed with fluorescence spectroscopy. The amount of drug not encapsulated or absorbed on the surface of the particles could therefore be determined. During the release time, the solutions were in an incubator at 37ºC and shaking horizontally.

The cumulative release could thereby be plotted against time for each formulation and a comparison between them could be made. As the nanoparticles get directly in contact with the octanol, the amount of drug at time zero is the amount that is not encapsulated or absorbed on the surface of the nanoparticles. The cumulative release is equal to the amount of drug divided by the total amount of drug in the solution. For every release point, the standard error was calculated and integrated in the graph. This value is equal to the standard deviation divided by the square root of the sample size.

There is a drawback of using this procedure with octanol. Due to the strong aggregation of the SEDS nanoparticles, a surfactant was used to disperse them. PF-68 was used with a concentration of 0.5 wt% in PBS (this concentration is below the critical micelle concentration). The surfactant formed a layer between the octanol and the aqueous phases and as it can be seen in the following graph (Figure 15), a small amount of MCHB was trapped in it. These curves are normalized by subtracting the amount free MCHB at time zero. The release rate is not affected as it can be seen, but the encapsulation efficiency is. The following release studies were therefore made without any surfactant. Aggregations were visible but the release was obtained.

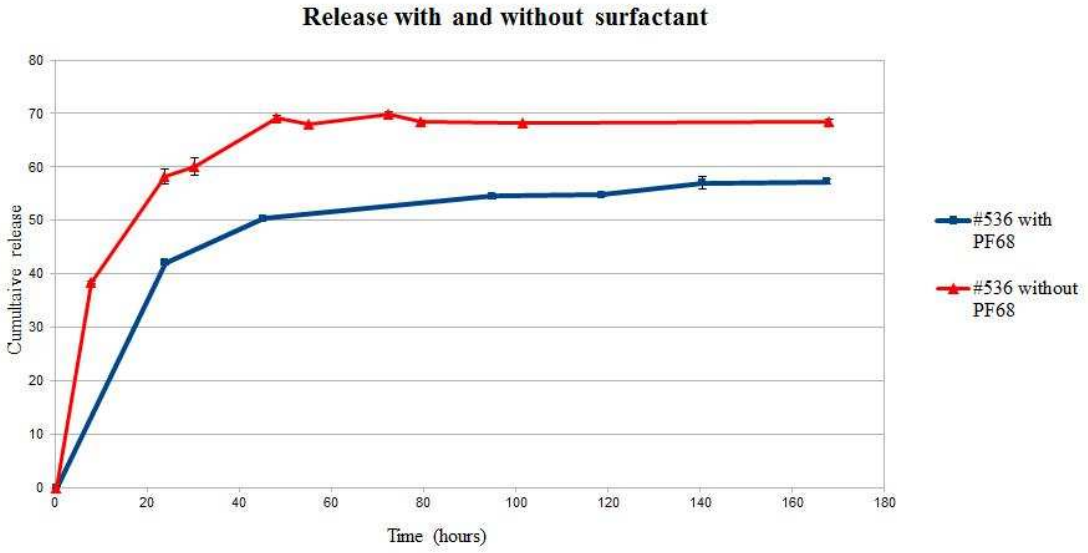

Figure 15. Effect of using a surfactant during the release study.

In order to determine if the release is completed or not, dichloromethane, in which the polymer and the drug are both soluble, was added to the remaining aqueous solution to purposely dissolve the nanoparticles. DCM is not water-miscible and all the remaining MCHB will therefore be in this organic phase. By taking a sample of that phase, rotary evaporating the dichloromethane and dissolving the dried MCHB with octanol, the amount of drug could be determined by fluorescence spectroscopy. The polymer seemed to hinder the flurorescence signal of the drug or to entrap some of it. A better option was therefore to add DCM directly to the two-phase system (octanol and aqueous phase), shake the solution vigorously and rotavap the DCM. In fact, this organic solvent has a lower boiling point (40°C) that both water (100°C) and octanol (196°C).[31]

4.3.2. Encapsulation efficiency

In order to compare the different formulations, the amount of drug incorporated in the nanoparticles was determined for each one. The expression for the encapsulation efficiency if the following:

$$Encapsulation\ efficiency(\%) = \frac{Amount\ of\ drug\ in\ the\ nanoparticles}{Initial\ amount\ of\ drug} \times 100$$

For the nanoparticles prepared with the nanoprecipitation method, the initial amount of drug is the total amount detected, including the loss in the preparation beakers and the free MCHB in the solution.

For the SEDS particles, the encapsulation efficiency was determined differently. For this technique, dry particles are collected in the apparatus filter. However, these particles do not contain all the MCHB injected into the instrument and this preparation loss has to be determined. The encapsulation efficiency is however calculated only from the amount of drug totally present in the particles collected (inside and on the surface of the particles). This value is therefore equal to:

$$Encapsulation\ efficiency(\%) = \frac{Amount\ of\ drug\ inside\ the\ nanoparticles}{Total\ amount\ of\ drug\ present\ in\ the\ collected\ nanoparticles} \times 100$$
$$= \frac{Total\ amount\ of\ drug\ present\ in\ the\ collected\ nanoparticles - Free\ amount\ of\ drug\ at\ t = 0}{Total\ amount\ of\ drug\ present\ in\ the\ collected\ nanoparticles} \times 100$$

Finally, the yield of the process is determined by comparing the amount of polymer/drug collected by the amount injected in the SEDS instrument.

4.4. Use of the confocal microscopy to see the efficiency of the lipid coating.

This type of microscope gives a sharp 2D image of a sample and can easily be applied to fluorescence studies. It has been used in this project in order to determine if the lipid coating was successful or not. MCHB fluoresces and to determine if a lipid layer successfully surrounded the polymeric particles, a fluorescent lipid-polyethylene (DSPE-PEG-FITC) was

used. It was important to choose a lipid with a different emission wavelength interval, in order to minimize any interference.

Theory

Similarly to the fluorescence spectroscopy, the confocal microscope uses a laser to excite the molecules in the specimen, but it uses the technique of point illumination in order to eliminate out-of-focus signal and therefore increase the optical resolution. In fact, the light is focused on one point on the sample by using a pair of lenses and the detector can measured the emitted light, like it is seen in Figure 16.

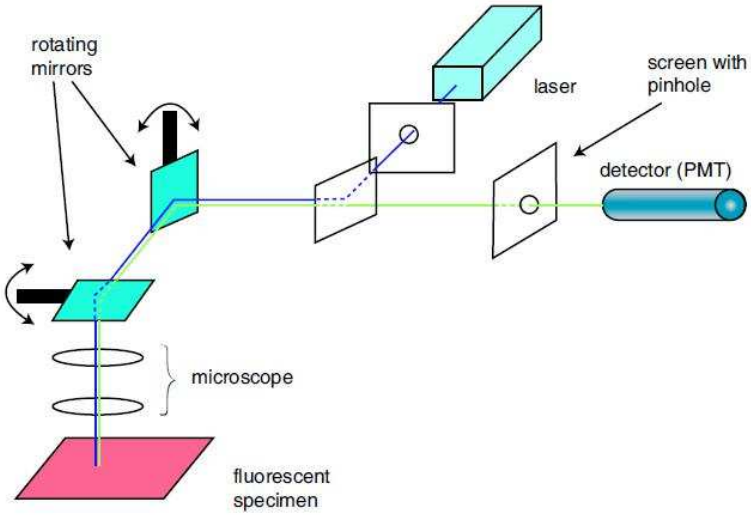

Figure 16. Schematic picture of the confocal miscroscope setup.[32]

The optical resolution is increase from a normal fluorescence miscrope, but the signal intensity is although decreased as fewer emitted photons are collected with the presence of pinholes.

Procedure

It was first essential to insure that the fluorescence of the MCHB could be seen in the microscope, as well as to distinguish its emission from the fluorescein's (FITC). MCHB is excited at 575nm, but as the confocal microscope can only excite at certain wavelength (458, 476, 488, 496, 514, 543 and 633nm), the nearest wavelength was taken, 543nm.

Three droplets of MCHB dissolved in acetone were put on a slide and the solvent was let to evaporate. Water was added to the dried MCHB and put under the objective. An extremely low fluorescence signal could be seen from the MCHB as Figure 17 shows and it seems that the drug is forming a crystalline structure. The fluorescence signal is most probably quenched by the water. When looking at SEDS nanoparticles containing MCHB, a much stronger signal was seen, but it seemed like the particles were repulsing the water so that air droplets were formed. In order to assure complete contact between the nanoparticles and the water molecules, the samples were observed with the objective (Objective 20X/0.5W) immerged into the aqueous solution and the fluorescence of MCHB could be seen (Figure 17). It is important to notice that

the focus is not the same for all the particles seen on the screen and that the particles not fluorescing in the pictures most certainly do at another focus.

The image in the top left corner refers to the fluorescence detected in MCHB's emission interval; in the top right corner it is the microscope image and finally the bottom image is the superimposed image.

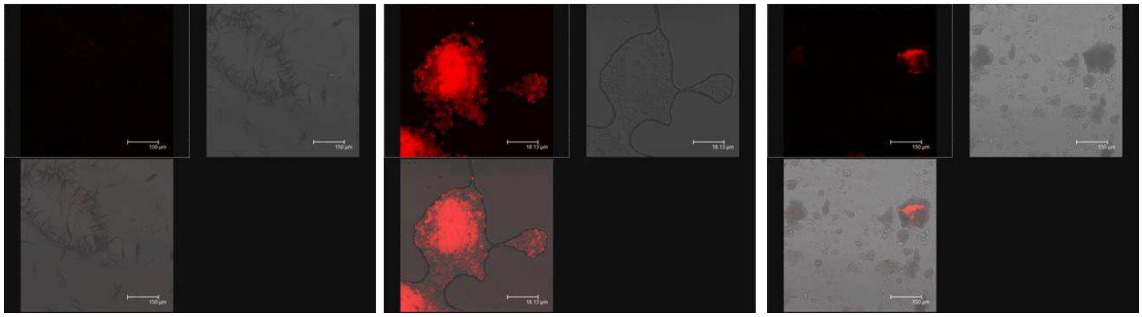

Figure 17. Images taken with confocal miscroscopy showing MCHB in water (left image); MCHB in polymeric nanoparticles, on a slide (middle image); and MCHB in polymeric nanoparticles immerging the objective into the solution (right image).

The next step was to determine if the lipid coating was successful or not. FITC can be excited at 490 nm with an emission at 510 nm and the laser beam at 488nm was therefore chosen. The emission interval was set to 500-535 nm for FITC and 610-625 nm for the MCHB. For the images showing the fluorescence of FITC and MCHB, the top right corner is representing the fluorescence from the emission between 500-535 nm and the top left corner from 610-625 nm. After trials, it appeared that the MCHB could also be excited at that wavelength and in fact with a higher intensity (the signal intensity was also higher when using 488nm as the excitation wavelength on the fluorescence spectroscopy), but its emission was seen at different emission intervals. Parameters for the detectors were preset using polymeric samples with only MCHB and ones with only FITC, in order to see only the respective fluorescence (see Figure 31 in Appendix). These parameters (Gain of 500V and an offset of 0% for both detectors) were kept through all the analysis, to insure the corresponding fluorescence and avoid any crosstalk phenomena (when a fluorophore is detected in another one's emission interval).

In order to decrease the fluorescent background, the particles were washed with water. The procedure consisted of centrifuging the samples at 10 krpm for 3 minutes, taking out as much upper phase as possible and finally pure water was added. If the background remained too strong, this was re-done. Finally it is important to notice that only particles in the micrometer scale can be seen in the confocal microscope and therefore the efficiency of the lipid coating is analyzed on aggregates.

5. EXPERIMENTS AND RESULTS

5.1. Nanoprecipitation method

Due to the fact that the release study method did not work in an aqueous buffer solution and it had to be rethought, several solutions were made without having the possibility to accurately measure the encapsulation efficiency, as well as the drug release behavior. Instead these solutions were used to set up the described analysis procedure, as well as understanding some phenomenon like the partial MCHB encapsulation in only lipid vesicles. Only the size and the zeta potential could successfully be measured. However the most promising solutions were re-done and analyzed with the right methods. Parameters, such as the effect of the organic solvent, the PLGA ratio of lactid to glycolic acid and the composition in the receiver solution, were investigated.

Effect of the concentration of the polymer in the solution

This effect was investigated by adding two different amounts of PLGA (85:15), dissolved in acetone, into a water solution. No drug was added for these two experiments. The size increases and the zeta potential has a more negative value when a higher polymeric concentration is used. The following table shows the results, obtained with DLS and Zetasizer.

Table 1. The effect of the concentration of the polymer (PLGA 85:15) on the size, PDI and zeta potential of the nanoparticles.

Total PLGA concentration	Size	PDI	Zeta potential
0,56 mg/ml	113.6 ± 1.5 nm	0.17 ± 0.01	-14.1 ± 0.6 mV
1,0 mg/ml	185.6 ± 0.7 nm	0,13 ± 0.03	-29.4 ± 1.4 mV

This result matches with previous studies and is due to a higher viscosity of the organic solvent when a higher amount of polymer is in the solution. Bigger nanodroplets are obtained when the solvent diffuses through the aqueous solution and therefore the size is bigger.[17]

Effect of the solvent

MCHB and PLGA were first dissolved in acetone and acetonitrile because these two organic solvents are the most commonly used for the nanoprecipitation method, as well as they give a smaller size than 150 nm. Methanol, in which the drug is soluble but not the polymer, was added to the receiver solution to different extents in order to see its effect. This was done in order to increase the solubility of the drug in the solution and therefore increase its precipitation time.

The release of MCHB was very fast and everything had already released at the first release point, taken after approximately 20 hours, respectively 7 hours for acetone and acetonitrile (see Figure 32 and Figure 33 in the Appendix). Some trends concerning the encapsulation efficiency could although be seen and are visible in the following table (Table 2). It seems that adding

methanol to the receiver solution decreases the percentage of drug incorporated in the polymeric nanoparticles. Slightly higher encapsulation efficiencies were obtained when using acetone as the solvent. The size and the zeta potential were also measured and revealed a smaller size, as well as a value nearer to zero, when using acetone.

Table 2. Results from the experiments using acetone and acetonitrile, with different amounts of methanol in the receiver solution.

Solvent	Receiver solution	Amount MCHB	Encapsulation efficiency	Size	PDI	Zeta potential
Acetone	Water	1.25wt%	27%	105.9 ± 0.5 nm	0.13 ± 0.04	-16.5 ± 1.0 mV
Acetone	Water + 5% Methanol	1.25wt%	22%	98.7 ± 0.5 nm	0.13 ± 0.02	-18.1 ± 0.5mV
Acetone	Water + 10% Methanol	1.25wt%	22%	114.3 ± 0.7 nm	0.19 ± 0.04	N/A
Acetone	Water + 15% Methanol	1.25wt%	21%	107.2 ± 1.8 nm	0.13 ± 0.03	N/A
Acetonitrile	Water	1.25wt%	26%	134.5 ± 1.2 nm	0.04 ± 0.04	-23.0 ± 2.1 mV
Acetonitrile	Water + 5% Methanol	1.25wt%	16%	126.2 ± 1.3 nm	0.07± 0.02	-22.2 ± 3.3 mV

The release points were taken at shorter time intervals for all the releases that followed. Acetone was used as the principal solvent as the percentage of drug encapsulated was slightly higher, but mostly because a smaller size for the nanoparticles was obtained. Acetone is also preferred over acetonitrile concerning its toxicity.

Effect of the ratio lactic: glycolic acid

PLGA 50:50 and PLGA 85:15 were used to determine the effect of the ratio lactic: glycolic acid on the polymer. Exactly the same preparation method was used for the two polymers and the results showed that PLGA with a higher lactic amount gives a smaller size, as well as a zeta potential value closer to zero.

Table 3. The effect of the polymer ratio lactic: glycolic acid on the size, PDI and zeta potential of the nanoparticles.

Sample	Size	PDI	Zeta potential
PLGA 50:50	105.3 ± 1.7 nm	0,11 ± 0.02	-15.8 ± 7.2 mV
PLGA 85:15	97.9 ± 0.5 nm	0,15 ± 0.01	-3.7 ± 1.4 mV

It can also be observed that the presence of drug during the preparation of the nanoparticles, gives a smaller and less negative value of the zeta potential (if comparing with Table 1, PLGA 85:15 had here a total concentration of 0.91 mg/ml).

Thereafter, two different approaches were used: one where the polymer-drug mixture dissolved in acetone was added to water and another one where CHF was added to the acetone mixture before the addition into the receiver solution. In this last case, methanol had to be added to the aqueous solution (1:2 Water/Methanol) in order to obtain a complete miscibility (see complete protocol in the Appendix). Due to the fast release immediately after incubation at 37°C, the encapsulation efficiency was determined from the fluorescence signal at t=0, as well as t=5min,. The second method (using CHF) encapsulated a bigger amount of MCHB, but after only five minutes the amount of the drug remaining was approximately the same (Table 4).

Table 4. Encapsulation efficiency (E.E.) for the two different methods.

Polymer	Solvent	Receiver solution	Amount MCHB	E.E. (t=0 / t=5min)
PLGA 50:50	Acetone	Water	1.25wt%	11% / 7%
PLGA 85:15	Acetone	Water	1.25wt%	10% / 7%
PLGA 50:50	Acetone/CHF (2:1)	Water/Methanol (1:2)	1.25wt%	16% / 6%
PLGA 85:15	Acetone/CHF (2:1)	Water/Methanol (1:2)	1.25wt%	24% / 9%

The effect of the ratio lactic: glycolic acid seemed to have different effect for the two methods. For the first method, the encapsulation efficiency is the same for PLGA 50:50 and PLGA 85:15. However, for the second method, a higher encapsulation efficiency is obtained when using PLGA 85:15, but this difference is not as strong after the first release point (after 5 minutes in the incubator. The ratio lactic: glycolic acid did not seem to have strong influence on the release in our two-phase system.

Increasing MCHB's solubility

During a preparation, it was seen that the drug was not completely soluble in acetone. In fact, when looking at the sample, the dissolution seemed complete, but looking extremely thoroughly in the light, clouds of very small particles could be seen. These particles were most certainly in the micrometer scale. As the polymeric particles are in the nanoscale, it would be impossible for them to encapsulate a drug with a scale above the nanometer scale.

A 200 nm filter was used to eliminate these bigger drug particles which were not dissolved in acetone. The new stock solution of drug in acetone was thereafter analyzed with fluorescence spectroscopy in order to determine the new concentration. Indeed, it had decreased by almost nearly half (122,5µg/ml to 62,5µg/ml).

The two methods used previously were thereby re-done and the results showed better encapsulation efficiencies, especially for the second method. Figure 18 and Figure 19 show the release curves of the new preparations compared to the old ones. The release rate was not affected by this increase in solubility and the release rate was still very fast.

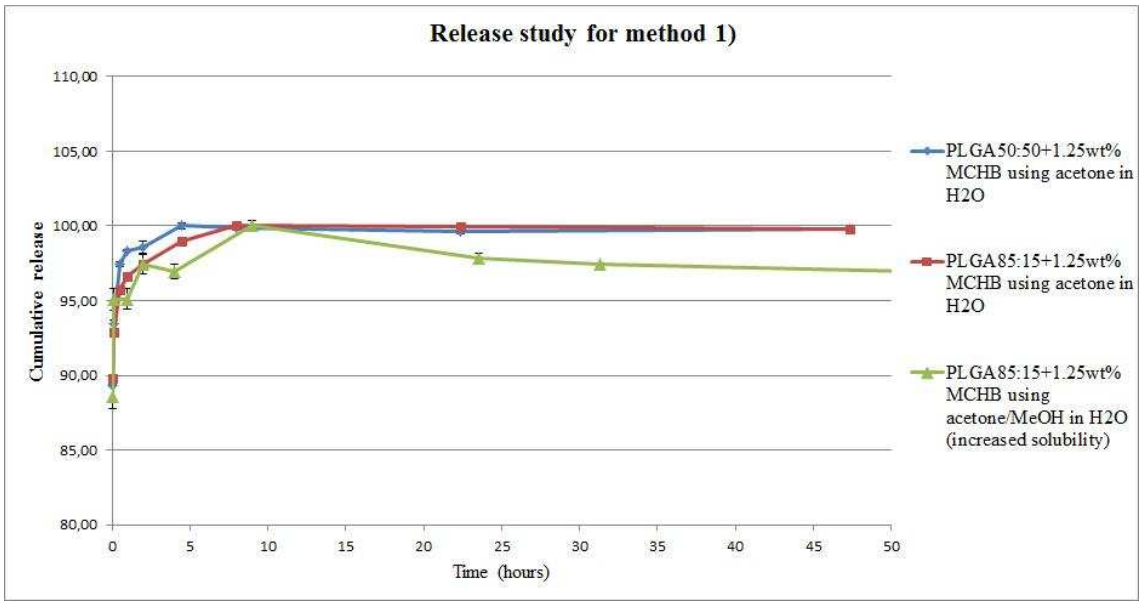

Figure 18. Release study for the first method.

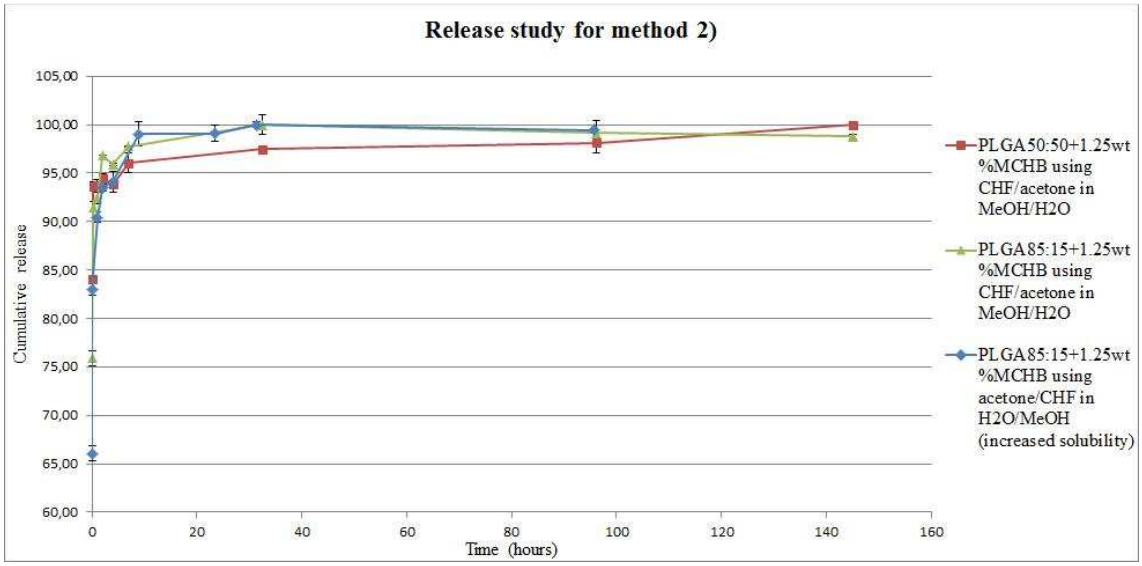

Figure 19. Release study for the second method.

In order to summarize, the following table shows the encapsulation efficiency obtained for these two described methods. As the release is very fast, this efficiency was also calculated by using the amount of free MCHB after five minutes.

Table 5. Encapsulation efficiency (E.E.) for the solutions prepared with the two different methods

Sample	Solvent	Receiver solution	Amount MCHB	MCHB E.E	MCHB E.E. after 5min
PLGA 50:50	Acetone	Water (1:1 with acetone)	1.25wt%	11%	7%
PLGA 85:15	Acetone	Water (1:1 with acetone)	1.25wt%	10%	7%
PLGA 85:15 (increased MCHB solubility)	Acetone	Water (1:1 with acetone)	1.25wt%	13%	5%
PLGA 50:50	Acetone/CHF (2:1)	Water/Methanol (1:2)	1.25wt%	16%	6%
PLGA 85:15	Acetone/CHF (2:1)	Water/Methanol (1:2)	1.25wt%	24%	9%
PLGA 85:15 (increased MCHB solubility)	Acetone/CHF (2:1)	Water/Methanol (1:2)	1.25wt%	**34%**	17%

Finally, it was seen that the size was decreased and a zeta potential value around -20 mV was obtained by solving this insolubility problem, (Table 6).

Table 6. Size, PDI and zeta potential of the two prepared solution with an increased solubility of MCHB in the solvent.

Polymer	Solvent	Receiver solution	Size	PDI	Zeta potential
PLGA 85:15	Acetone	Water (1:1 with acetone)	81.1 ± 0.3 nm	0.08 ± 0.02	-21.1 ± 1.9 mV
PLGA 85:15	Acetone/CHF (2:1)	Water/Methanol (1:2)	77.1 ± 0.7 nm	0.14 ± 0.04	-22.1 ± 7.3 mV

SEM images

As the DLS doesn't always give the actual physical particle size, it was confirmed by analyzing the particles with the SEM. For the different formulations made in this project, the particles prepared had all the same spherical structure (Figure 20). The size visible in the SEM-images corresponded to the DLS results.

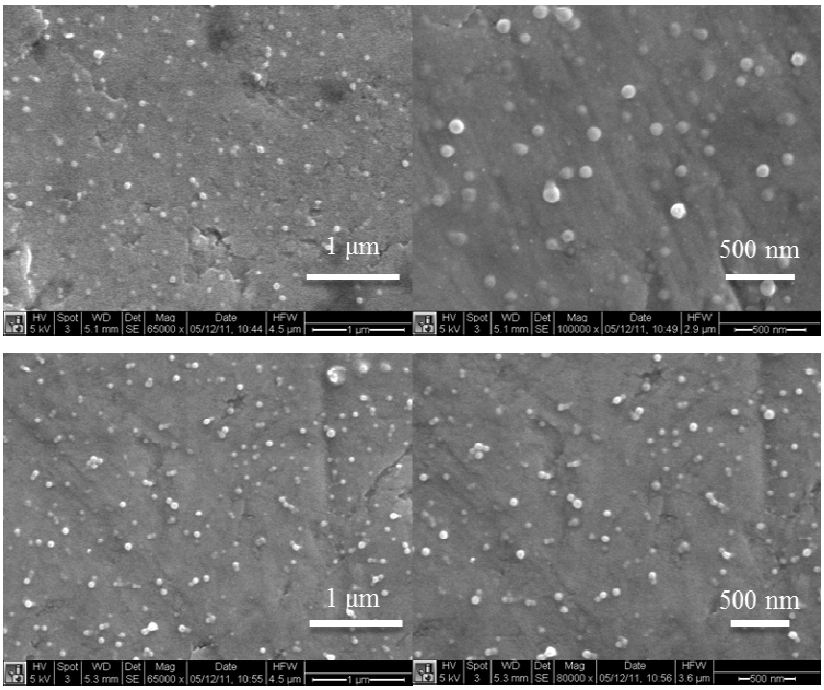

Figure 20. SEM images for the prepared nanoprecipitation particles. Top images: Using method 1. Bottom images: Using method 2.

5.2. SEDS method

Effect of the co-solvent

As it was described previously, the amount of drug lost during the process is not taken into account in the release graphs. The encapsulation efficiency is here the amount of drug inside the particles divided by the total amount of drug inside and outside the particles. The polymer and MCHB were dissolved in two different co-solvents. In one, these components were dissolved in dichloromethane (DCM) and methanol (ratio 3:1) and in the other mixture hexafluoro-isopropanol (HFIP) was the solvent alone. The following release curves were obtained (Figure 21).

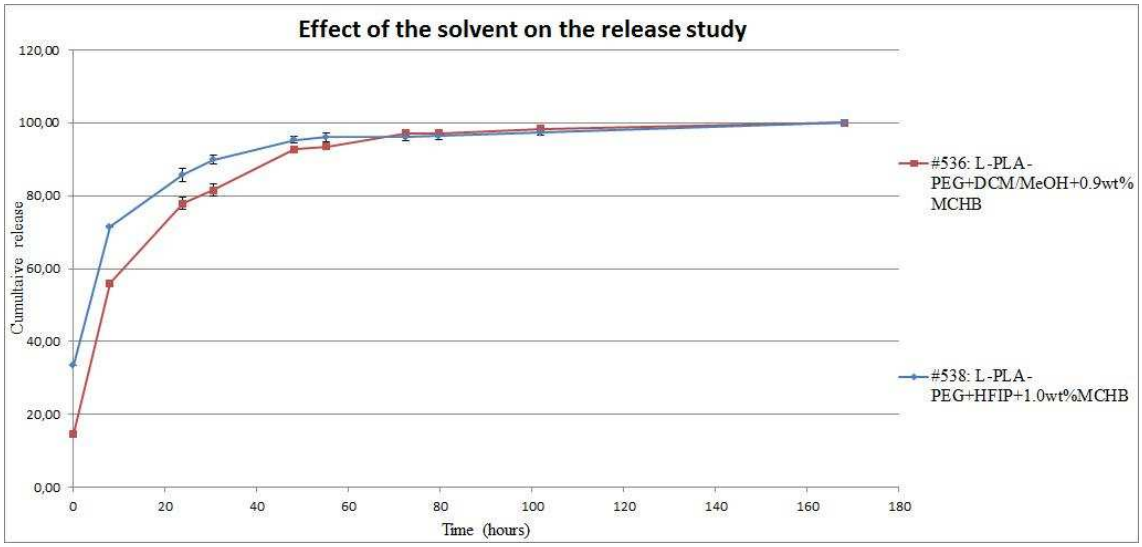

Figure 21. Release curves for SEDS nanoparticles prepared by using DCM/methanol or HFIP.

The encapsulation percentage is higher for the nanoparticles prepared with DCM/methanol (85% compared to 67%). However, the difference between the two co-solvents is much bigger if the drug lost during the process is taken into account. The total encapsulation efficiency is thereby 80% when using DCM/methanol against 27% when using HFIP.

The prepared nanoparticles were observed by SEM and the images (Figure 22) show a difference in the particle structure. When DCM/methanol was used, all the particles had a spherical structure. However, for the particles prepared with HFIP, some spherical particles can be seen but a large number of particles have a more elongated and smaller structure.

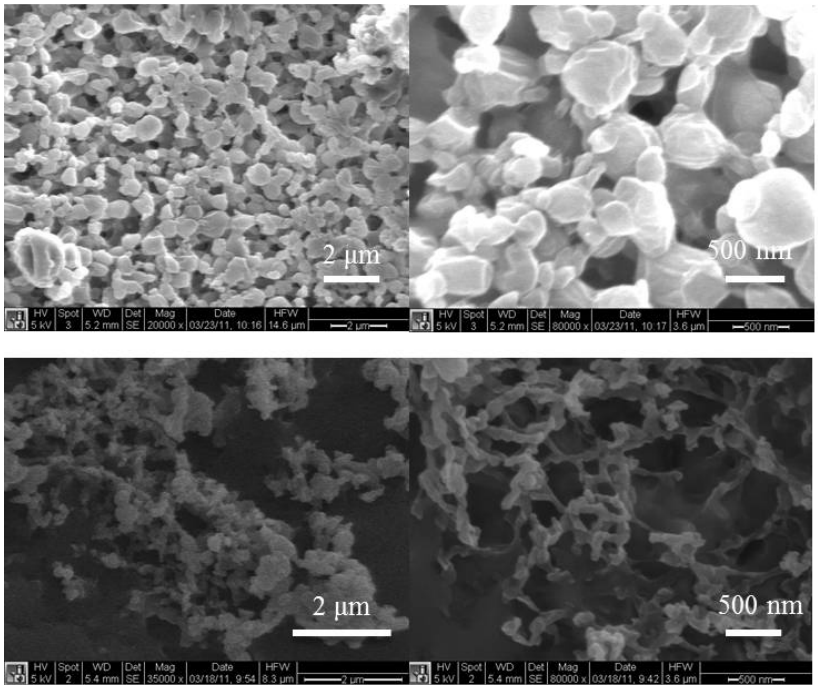

Figure 22. SEM-images from SEDS nanoparticles. Top images: using DCM/MeOH (#536) and bottom images: using HFIP (#538).

Effect of the weight percentage drug to polymer

Experiments with different amount of MCHB to polymer were done in order to see its effect on the release and the encapsulation efficiency. It appeared that when decreasing the amount of drug in the formulation, the encapsulation efficiency increases. The release curves have the same shape for the different formulations (Figure 23). However, the rate is slower for the first experiment made (3 days for #536 compared to 1 day for #543 and #545) and the cause of this difference will be discussed later. It is also important to notice that the ratio of DCM to methanol was decrease from 3:1 to 2:1 for the sample having a higher drug weight percent. The bigger volume of methanol was necessary to solubilize the bigger amount of MCHB and may also have an effect on these results.

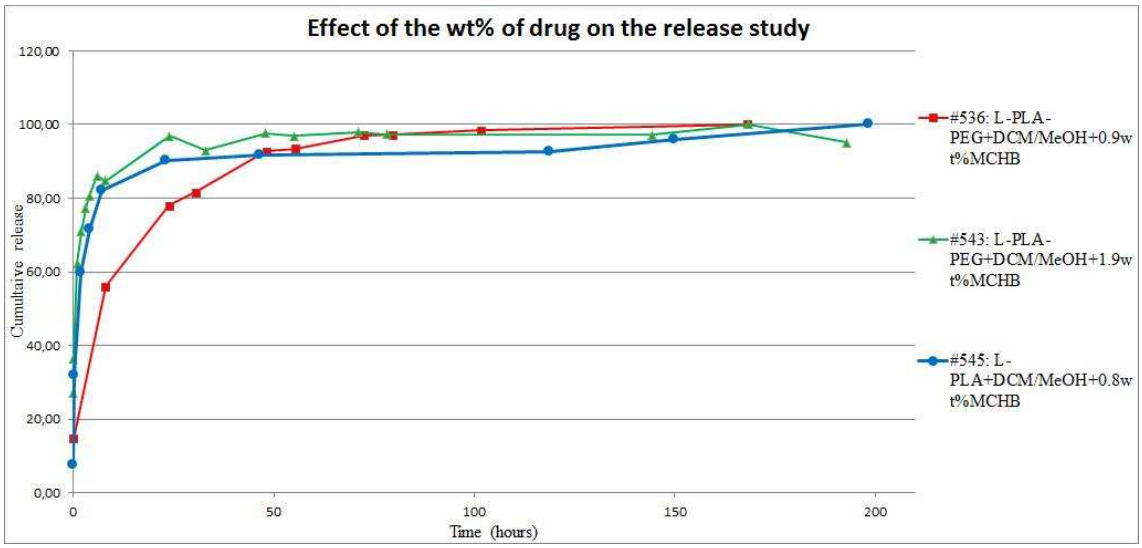

Figure 23. Effect of the drug weight percentage on the release rate and the encorporation efficiency.

Effect of the PEG label on the PLA

Finally, the presence of PEG on the polymer was studied by preparing nanoparticles with L-PLA-PEG and L-PLA. Drug was released only during the first hour for the PLA nanoparticles and after eight days, they were dissolved by using dichloromethane. MCHB was still encapsulated and had not been released yet. The drug release with PLA is much slower than for PLA-PEG and that is most certainly due to the solubility of PEG in octanol.

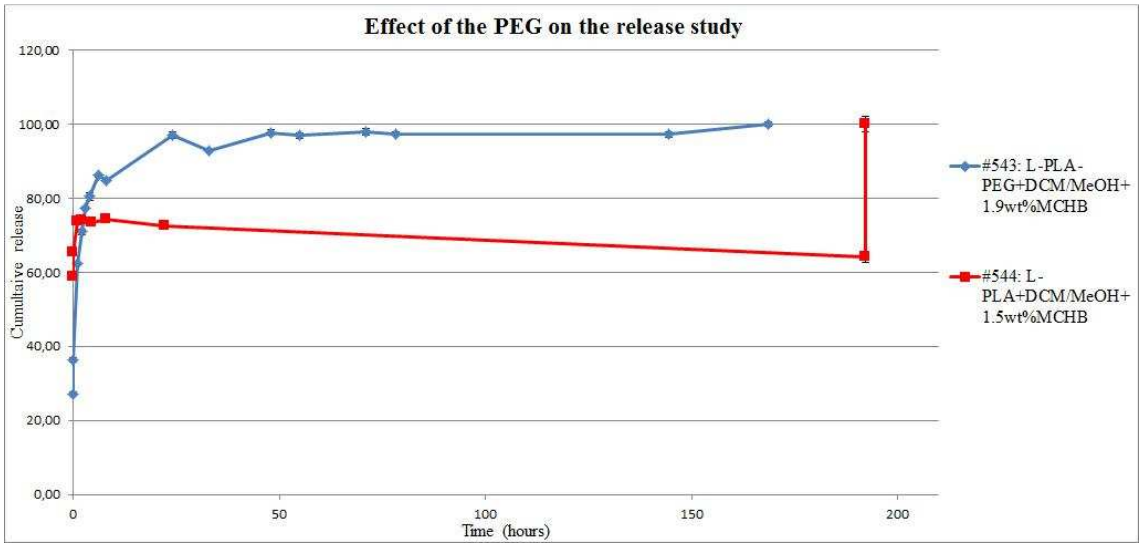

Figure 24. Effect of the PEG on the release study.

SEM was used to see the effect of the PEG-label on the particles. Figure 25 shows the two samples have the same spherical shape and approximately the same size for the two samples. The size is in the nanometer scale, but unfortunately a precise size cannot be determined as the particles are melting together when increasing the magnification.

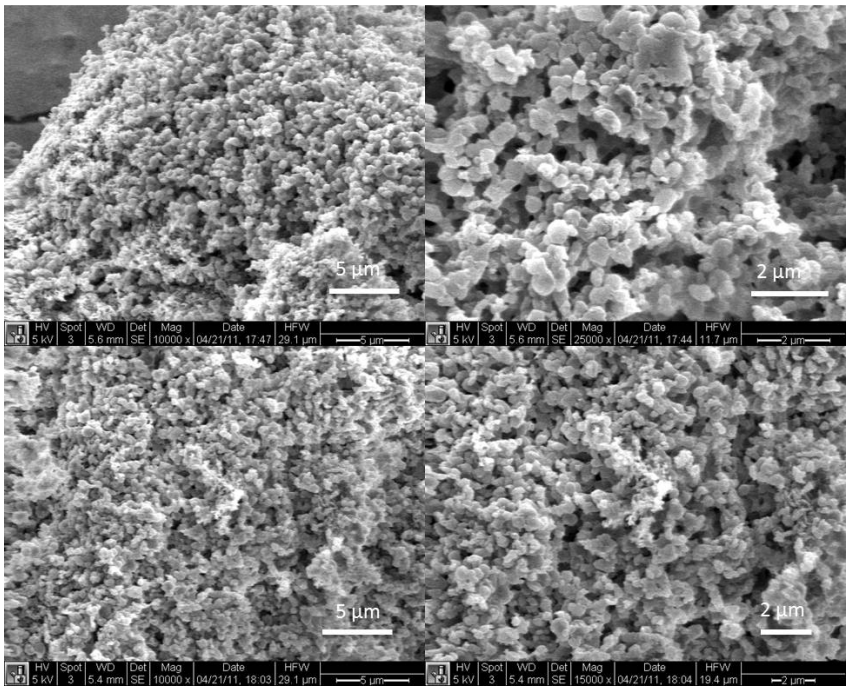

Figure 25. SEM-images from SEDS nanoparticles. Top images: PLA-PEG (#543) and bottom images: PLA (#544).

Summary of the SEDS experiments

The first two experiments showed that when using HFIP instead of DCM/methanol, a big amount of MCHB was lost during the process step and the less it was encapsulated. The drug seemed highly soluble in HFIP, more than in DCM/methanol. Therefore, an explanation of this big drug loss in the SEDS is that the MCHB follows the co-solvent instead of precipitating together with the polymer. The co-solvent should therefore be chosen to solubilize the drug and the polymer only to a certain extent.

Table 7. Summary of the results gotten for the SEDS nanoparticles

Sample	Components	wt% MCHB	Solvent	MCHB loss during SEDS process	MCHB encapsulated in collected particles	Total MCHB encapsulation
#538	L-PLA-PEG	1.0	HFIP	60%	67%	27%
#545	L-PLA-PEG	0.8	DCM/MeOH (ratio 3:1)	77%	92%	21%
#536	L-PLA-PEG	0.9	DCM/MeOH (ratio 3:1)	6%	85%	80%
#543	L-PLA-PEG	1.9	DCM/MeOH (ratio 2:1)	64%	73%	26%
#546	L-PLA	0.8	DCM/MeOH (ratio 3:1)	~100%	N/A	N/A
#544	L-PLA	1.5	DCM/MeOH (ratio 2:1)	74%	41%	11%

In this table, it is important to fully notice that the percentage of MCHB encapsulated does not take into account the amount lost during the process. In fact, this loss can be seen in the column to its left. As it has described a higher encapsulation efficiency is obtained when using a smaller

weight percentage of drug to polymer and finally more drug is incorporated in the nanoparticles when using PLA-PEG compared to PLA.

When analyzing the drug loss during the process, no trend is visible. However this strong difference between the experiments was due to a problem of the SEDS instrument. While preparing the two first samples (#536 and #538), the pressure drop was 9 bars, respectively 40 bars. For all the other formulations this pressure drop was much higher, 130 to 170 bars and the CO_2 had to be decreased manually from 140 to 100-120 g/min. This was done in order to assure a pressure drop under the maximum value (200 bars). Due to this strong difference in the pressure drop, the pressure in the particle vessel was not 100 bars as it was set, but 230 to 270 bars. This means that the supercritical environment at the precipitation point was not the same between the samples and that the components had different solubility.

So what could have caused this difference in pressure drop? For the first two samples, a frit of 2 µm was located in the mixing tee (Figure 7), but this piece was lost between the sample #538 and #543. It was replaced by a new frit of 0.5 µm, but this one was not integrated in the tubing and mechanical strength was automatically applied onto it. It seemed that the new frit got blocked in the tubing and was the reason for the increase in pressure in the particle vessel.

Even if this difference in pressure drop was present, the results discussed are likely to be valid. The two first experiments comparing the effect of the solvent were made before the problem and this comparison is therefore valid. The evaluation of the effect of PEG covalently bonded to PLA is correct as the two samples were made one after each other. Finally for the effect of the weight percentage, which compares both the sample made before and after this problem, it would be necessary to make new experiments. The increase of the encapsulation efficiency with a lower weight percent drug is also very credible.

5.3. Lipid coating

The first formulations of nanoprecipitation particles were including the lipid addition, but after some experiments, it was discovered that the lipid encapsulates by itself the MCHB. The drug is highly lipophilic and instead of forming polymeric nanoparticles surrounded by a lipid layer, both lipid vesicles and polymeric nanoparticles were formed. Therefore, it was thought to first find a good formulation for polymeric drug carriers and thereafter include the lipid to it.
The highest encapsulation efficiency was obtained using the method 2, previously described, and the lipid-PEG was therefore coated for this formulation, in a one-step process. As the nanoparticles are very light and dispersed, only some aggregates of polymeric particles were observed.

Figure 26 shows some images from this prepared solution, taken with confocal microscopy. The bottom right image shows one aggregate which is most probably constituted of polymeric nanoparticles. The fluorescence of the lipid and the drug comes from the same position. Some crystalline needle structures are visible around the aggregate and are a sign of some free MCHB on its surface. Other types of particles (both images on the left) were also visible in the sample. MCHB particles by itself or with a very small amount of polymer were

observed and the lipid seemed to be present at the same position. The top right image shows a lipid vesicle, recognizable by it smooth shape. The intensity of the drug and the lipid is very strong and is a proof of the lipophilicity of the drug.

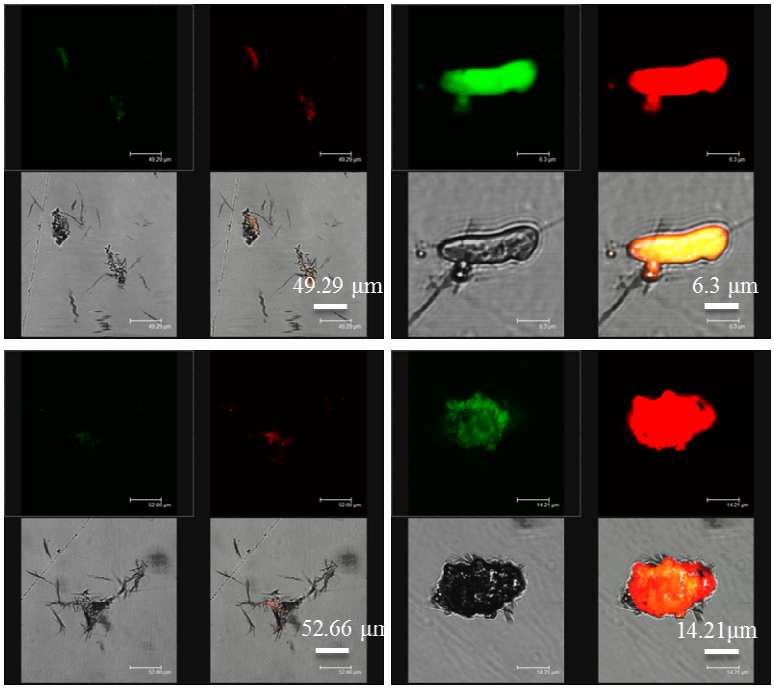

Figure 26. Particles containing MCHB and lipid made by the nanoprecipitation method. Top left: Lipid/MCHB. Top right: Lipid vesicle containing MCHB. Bottom left: Lipid/MCHB. Bottom right: Polymeric particles with some free drug.

SEDS-particles made with PLA-PEG were lipid coated and observed using confocal microscopy. Figure 27 shows two different aggregates of polymeric particles. The location of the fluorescence from the lipid and the drug correspond and therefore it can be conclude that lipid coated particles are obtained. However, the analysis showed that some PLA-PEG particles were not coated.

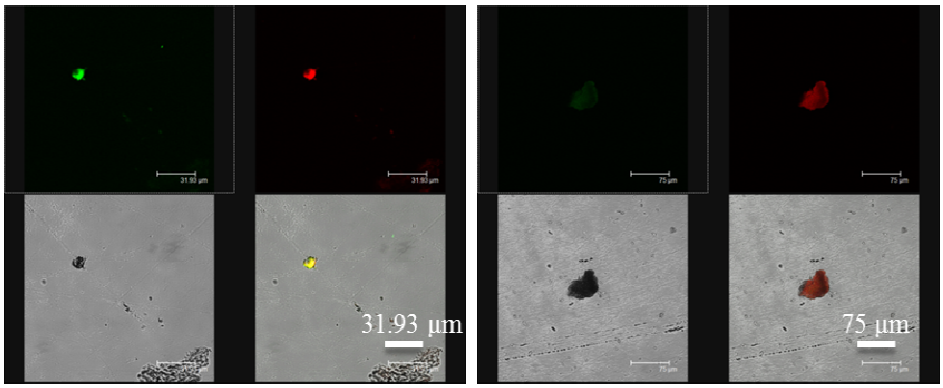

Figure 27. PLA-PEG particles containing MCHB and coated with lipid.

Some PLA particles containing MCHB were also coated and analyzed with the microscope. For this sample, more particles seemed to be coated. Like for the PLA-PEG particles, the location of the lipid and the drug was the same.

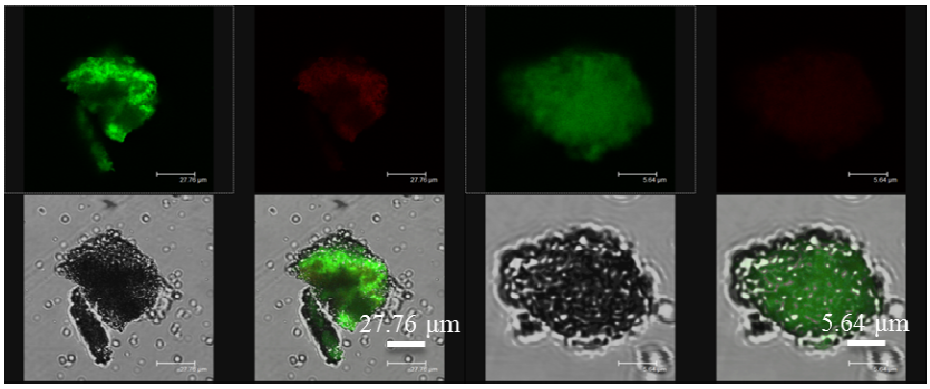

Figure 28. PLA particles containing MCHB and coated with lipid.

Nevertheless, the intensity of the MCHB is not strong. This can be due to quenching of the lipid and/or the polymer. It can be concluded that a lipid coating is possible on the polymeric nanoparticles, which was the objective of this study. The coating works better on L-PLA particles than on L-PLA-PEG. The presence of the polyethylene glycol has most certainly a repulsion effect on the lipid.

As no fluorescent signal came from the smallest particles, the lipid-coating was re-done using only fluorescent lipid (100% DSPE-PEG-FITC and no lecithin). For this experiment, only the big aggregates of L-PLA-PEG particles were coated, but for the L-PLA particles even the smallest particles gave a fluorescent signal (Figure 29). This confirms the better coating on particles without any PEG.

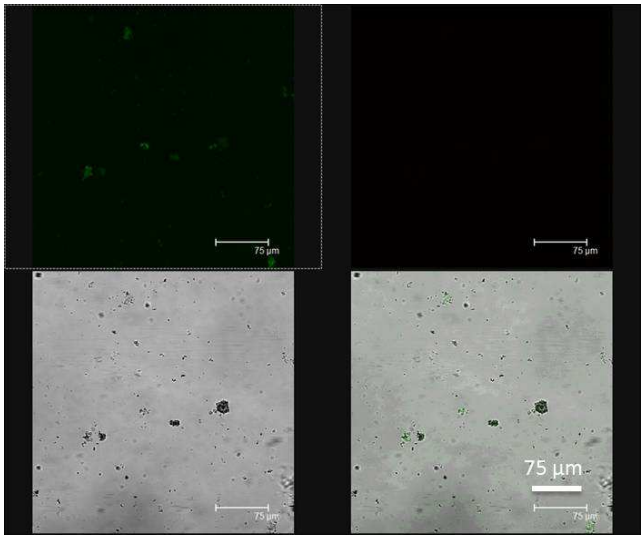

Figure 29. PLA particles coated with only DSPE-PEG-FITC.

The excitation wavelength for MCHB was determined to 575 nm, by measuring the absorbance spectra of MCHB in dimethyl sulfoxide. As it was described in the section 4.4., 488 nm was used for this analysis because the intensity of the signal seemed visibly stronger than at 543 nm. This big difference was studied by running some tests. In fact, the absorbance spectra for MCHB encapsulate in polymeric nanoparticles (prepared by SEDS), as well as particles with FITC and particles with both FITC and MCHB were obtained. This spectra (Figure 30) shows

that FITC has an absorbance peak at 490 nm as it should, but that MCHB encapsulated inside polymeric particles fluoresce but does not present any peak.

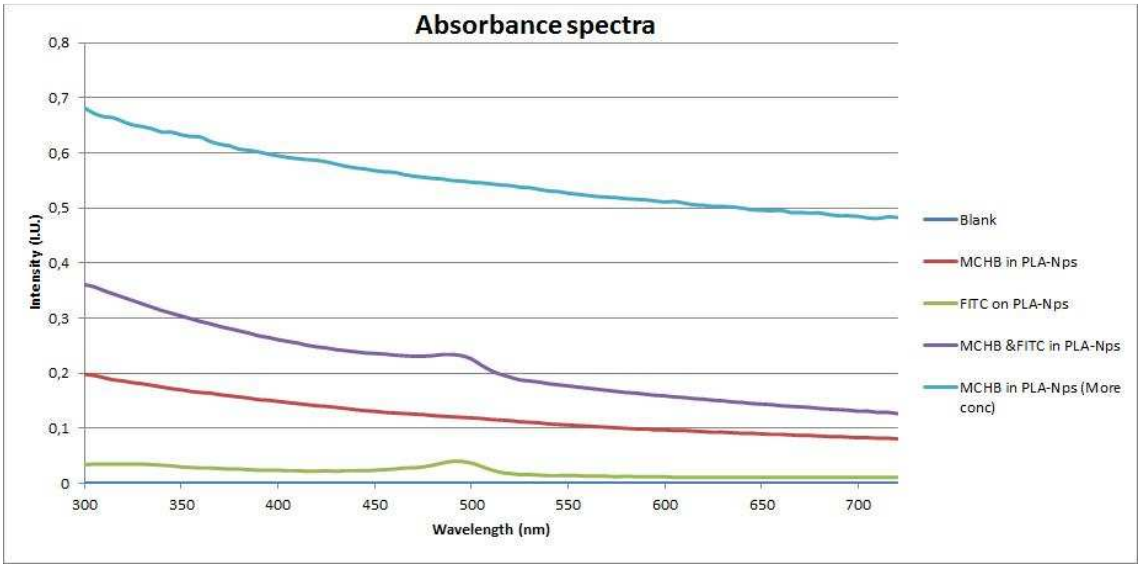

Figure 30. Absorbance spectra for L-PLA nanoparticles (dispersed in water) with: only MCHB (red curve), only MCHB but a more concentrated solution (light blue curve), only FITC (green curve), both MCHB and FITC (purple curve). The blank consisted of water (dark blue curve).

The emission of these components was also quantitatively measured when exciting at 479 nm and 543 nm (using an atomic absorption spectrophotometer) and showed no emission of FITC when exciting at 543 nm, but an emission peak for MCHB at approximatively 610 nm. This result seems normal as FITC is not excited at 543 nm. At 479 nm, the same emission peak for MCHB around 610 nm was visible, but with a higher intensity (which matches with the observations in the confocal microscope). The other component, FITC, emitted with a very high intensity at the interval chosen (500 to 535 nm) and the signal decreased at higher wavelength up to 660 nm, where emission was not visible anymore. The fluorescein was therefore also probably emitting in the interval set for MCHB.

6. DISCUSSION

6.1. Nanoprecipitation nanoparticles

PLGA nanoparticles were synthesized with a good size and polydispersity. The objective of forming particles with a size inferior to 150 nm was therefore accomplished. The zeta potential was also found to be in the correct range.

Unfortunately, the encapsulation efficiency was not high and the maximum value obtained was 34%. A big amount of drug was not incorporated in the polymeric particles, but was instead precipitating alone. Indeed, the MCHB seemed to precipitate into a crystalline needle structure, according to the images taken with the confocal microsope. The use of surfactant could have helped the encapsulation process as Derakhshandeh et al. showed in their study. Unfortunately, when using the octanol to study the release, the presence of this component is impossible.[18]

However, the encapsulation efficiency is not the biggest problem with the prepared nanoparticles. In fact, the amount incorporated drug is in the same order as the amount of 9-Nitrocamptothecin Derakhshandeh managed to encapsulate in PLGA nanoparticles [18]. 9-Nitrocamptothecin and MCHB have the similar nitrosubstituted benzene rings and both are lipophilic drug with a similar molecular mass.

The biggest concern for the prepared nanoparticles containing MCHB is the fast drug release. Only five minutes in an incubator at 37°C gives an important release and after less than 10 hours, almost everything is out of the polymeric particles. In comparison with previous studies using the same polymer, this release time is very fast. It is although important to notice that those release studies were done in water/buffer solutions.

Two mechanisms are combined for the drug release in a nanoparticle: the diffusion of the drug out of the particle and the erosion process. Smaller particle sizes, as well as a higher drug loading affect these release mechanisms by accelerating them [8]. The nanoparticles are always in contact with the octanol during the release and as this organic solvent has different properties than water, such as a higher viscosity, the release may have been accelerated. Possible reasons for the fast release are the fast erosion process of the nanosphere by the octanol (due to its high viscosity) or the entry of octanol into the matrix system giving a faster diffusion of the drug out of the nanoparticles. These reasons also depend on the structure of the nanoparticle. It is important to highlight that the environment in the body is different and therefore that the release *in vivo* or *in vitro* in cells would most probably be different than these results obtained *in vitro* in a vial.

However, this fast release can also be due to the poor entrapment of the drug in the nanoparticles, due to a difference in the precipitation time. This is highly probable as no difference in the release rate is seen between PLGA 50:50 and PLGA 85:15. Indeed, the release should be slower with a higher lactic to glycolic acid ratio.

Concerning the lipid coating, the one-step process was not successful and a two-step process seems to be a better option. In fact, MCHB seems highly lipophilic. Therefore,

when the lipid coating is done at the same time as the polymeric nanoparticle formation, lipid vesicles encapsulating MCHB are also formed. One aggregate of polymeric nanoparticles were observed and showed a good lipid coating. However, some free drug was on the surface of this aggregate.

6.2. SEDS nanoparticles

For the nanoparticles prepared by the SEDS method, higher encapsulation efficiencies were obtained. For the best experiment made, only 6% MCHB was lost during the process and 85% of the drug in the collected particles was encapsulated. Particles with a higher encapsulation efficiency was also obtained, but more drug was lost during the preparation. Nevertheless, this difference was due to a problem in the SEDS-instrument.

The release was also slower compared to the release for the nanoprecipitation particles. For the nanoparticles made before the "pressure-drop" problem occurred, the release of drug took place during 3-4 days for L-PLA-PEG particles. This rate is still fast, but as it was described earlier, the two-phase system is a method to observe if the drug is released or not. This differs most certainly from *in vivo* and *in vitro* analysis in cells. It is also important to notice that PEG is soluble in octanol and as it was dissolved, this most certainly created space for the drug to diffuse out of the particles. PLA particles showed however no significant release after 8 days, at which point they were dissolved and showed the presence of drug encapsulated.
The solvent used to dissolve the drug and the polymer was found to have an effect on the encapsulation efficiency and especially on the loss of MCHB during the process. It seemed that the drug was highly soluble in HFIP and as the solution was mixed with supercritical carbon dioxide, some drug did not precipitate but went through the filter, now dissolved in the SCF phase. The solvent did not however have an effect on the release rate.

A problem for these prepared nanoparticles was instead the bigger size and the strong aggregation. The size obtained was higher than 150 nm. Nevertheless, a sustained release of siRNA was seen in a study made by Jacobson et al., in which SEDS-particles, with a size of 100 to 300 nm, were capable to enter cells.[20]

6.3. Best option and future plans

This two-phase water-octanol system used for release studies is different from the environment in which cancerous cells are. The next step in this project is therefore to try the best nanoparticle formulations *in vitro*, using cancerous cells. The laboratory of Professor Matin is responsible for this analysis.

As the polymeric particles made by nanoprecipitation are releasing too fast and because some MCHB particles are hard to separate from the solution, these particles are not chosen for this next step. Instead, as lipids have seemed to be highly capable of encapsulating the drug, solid lipid nanoparticles (SLN) are going to be prepared by nanoprecipitation and injected into cells. Lipid-based drug delivery systems have been shown to be good carrier with a flexible release and a pegylation is possible.[10] The formulation of these particles consists of

the same steps but lecithin is used instead of polymer. This was tried and a homogeneous pink solution was obtained. Unfortunately, no release study was done as lipids are soluble in octanol. However, when organic solvents (such as acetone or octanol) were added to the lipid/MCHB prepared nanoparticle solution, the color changed to a more intense pink, which could be a sign of drug encapsulation.

After having solved the pressure problem in the SEDS instrument, the best experiment (#536) will be re-done and these particles will be brought to this next *in vitro* analysis. Both L-PLA and L-PLA-PEG particles will be tried. From the results obtained in this master thesis, L-PLA-PEG particles have shown better encapsulation efficiency than L-PLA, but a faster release (most certainly due to the solubility of PEG in octanol and the formation of "pores" in the nanostructure). L-PLA nanoparticles did not show any release after 8 days but did encapsulate some drug. The lipid coating was much more effective for the non-pegylated nanoparticles as the presence of PEG on the surface of the polymeric particle interfere with it.

For this *in vitro* analysis, these nanoparticles will be incubated in presence of noncancerous cells in order to see if the nanoparticles can enter the cells and deliver the MCHB. At this very moment, the peptide is soon ready to be tested and attached to the nanoparticles. Therefore, after having attached the NHS-group to the lipid, the peptide will be easily linked to the drug carriers and the efficiency of targeting will be determined. This ligand will hopefully help the incorporation of the nanoparticles in the cancerous cells.

CONCLUSION

This master thesis has shown the possibility of formulating nanoparticles encapsulating this new cancer drug called MCHB. Two different anti-solvent techniques have been used to accomplish this and they have showed different advantages and inconveniences. Nanoprecipitation was a better method to obtain smaller and more dispersed nanoparticles. However, SEDS was found to be the best option as a higher encapsulation efficiency was obtained, as well as a more sustained release of the drug. The release study was not possible in water and needed the presence of an organic solvent to which the free drug could diffuse. This two-phase system can be used for other extremely hydrophobic drug such as MCHB. The post-lipid coating was possible by sonication and was the most efficient for the non-pegylated particles. *In vitro* experiments will now be done in which the best formulations of nanoparticles are going to be incubated with cancerous cells.

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APPENDIX

Formulation protocols

Nanoprecipitation

Method 1:

1. Have the "MCHB in acetone" solution prepared (C_{MCHB}=69.51 µg/ml)
2. Prepare a 5 mg/ml PLGA 85:15 (respectively PLGA 50:50) in acetone.
3. Mix 0.7 ml (=3.5 mg) of this solution with 0.629 ml (43.75 µg => 1.25wt% MCHB to PLGA)
4. Add 2.658 ml of H_2O to a beaker (in order to get a ratio of 2:1 H_2O/Acetone).
5. Add drop-wise the "MCHB/PLGA in acetone" solution to the aqueous receiver solution, under stirring.
6. Keep under stirring for 2h at room temperature.
7. Rotavap until the remaining organic solvent has evaporated.

Method 2:

1. Have the "MCHB in acetone" solution prepared (C_{MCHB}=69.51 µg/ml)
2. Prepare a 5 mg/ml PLGA 85:15 (respectively PLGA 50:50) in acetone.
3. Mix 0.7 ml (=3.5 mg) of this solution with 0.629 ml of "MCHB in acetone"(43.75 µg => 1.25wt% MCHB to PLGA)
4. Add 0.443 ml CHF (in order to get a ratio of 3:1 Acetone/CHF)
5. Prepare a 3.544 ml H_2O / 7.088 ml Methanol (ratio of 1:2 H_2O/Methanol and ratio of 2:1 H_2O/Acetone) in a beaker.
6. Add drop-wise the "MCHB/PLGA in acetone/CHF" solution to the aqueous receiver solution, under stirring.
7. Keep under stirring for 2h at room temperature.
8. Rotavap until the remaining organic solvents have evaporated.

Lipid coating the method 2:

1. Prepare a DSPE-PEG-FITC in methanol solution ($C_{DSPE\text{-}PEG\text{-}FITC}$=1.9 µg/µl) and a lecithin in methanol solution ($C_{lecithin}$=10 µg/µl).
2. 30wt% of lipid=1.05 mg: Mix 55.26 µl (=105 µg) of "DSPE-PEG-FITC in methanol" with 94.5 µl (=945 µg) of "lecithin in methanol".
3. Prepare a 3.544 ml H_2O / 7.088 ml Methanol (ratio of 1:2 H_2O/Methanol and ratio of 2:1 H_2O/Acetone) in a beaker.
4. Heat up this solution to 40°C and add the prepared organic solvent containing the lipids.
5. Have the "MCHB in acetone" solution prepared (C_{MCHB}=69.51 µg/ml)
6. Prepare a 5 mg/ml PLGA 85:15 in acetone.
7. Mix 0.7 ml (=3.5 mg) of this solution with 0.629 ml of "MCHB in acetone"(43.75 µg => 1.25wt% MCHB to PLGA) and add 0.443 ml CHF (in order to get a ratio of 3:1 Acetone/CHF)
8. Add drop-wise the "MCHB/PLGA in acetone/CHF" solution to the aqueous receiver solution, under stirring.
9. Keep under stirring for 5 min. Sonicate for 5 min.
10. Keep under stirring for 2h at room temperature.
11. Rotavap until the remaining organic solvents have evaporated.

SEDS

- #536
  - 0.45 mg MCHB
  - 50 mg PLLA-PEG (0.9 wt% MCHB) *(mtot=50.45 mg)*
  - 13.5 ml DCM + 4.5 ml MeOH (ratio 3:1) *(Vtot=18 ml)*

  Collected: 18.3 mg SO A YIELD OF 36.3%
- #538
  - 0.6 mg MCHB
  - 61.5 mg PLLA-PEG (1.0 wt% MCHB) *(mtot=62.1 mg)*
  - 5 ml HFIP *(Vtot=5 ml)*

  Collected: 18.6 mg SO A YIELD OF 30.0%
- #542-543
  - 1.4 mg MCHB
  - 73.9 mg PLLA-PEG (1.9 wt% MCHB) *(mtot=75.3 mg)*
  - 28 ml DCM + 14 ml MeOH *(Vtot=42 ml)*

  542 => 12 ml of the solution => 21.5 mg

  Collected: 6.5 mg SO A YIELD OF 30.2%

  543 => 17 ml of the solution => 30.5 mg

  Collected: 19.4 mg SO A YIELD OF 63.6%
- #544
  - 1.1 mg MCHB
  - 72.0 mg PLLA (1.5 wt% MCHB) *(mtot=73.1 mg)*
  - 22 ml DCM + 11 ml MeOH *(Vtot=33 ml)*

  544 => 12 ml of the solution => 26.6 mg

  Collected: 10.7 mg SO A YIELD OF 40.2%
- #545
  - 0.45 mg MCHB
  - 55.5 mg PLLA-PEG (0.8 wt% MCHB) *(mtot=55.95 mg)*
  - 13.5 ml DCM + 4.5 ml MeOH *(Vtot=18 ml)*

  Collected: 32.7 mg SO A YIELD OF 58.2%
- #546
  - 0.45 mg MCHB
  - 54.2 mg PLLA (1.5 wt% MCHB) *(mtot=54.65 mg)*
  - 13.5 ml DCM + 4.5 ml MeOH *(Vtot=18 ml)*

  544 => 14.5 ml of the solution => 44.02 mg

  Collected: 11.7 mg SO A YIELD OF 26.6%

Lipid coating:

1. Weigh some mg of SEDS-particles: $m_{\#544}$=1.9 mg
2. Prepare a DSPE-PEG-FITC in methanol solution ($C_{DSPE\text{-}PEG\text{-}FITC}$=1.9 µg/µl) and a lecithin in methanol solution ($C_{lecithin}$=10 µg/µl).

3. 30 wt% of lipid=0.57 mg: Mix 30 µl (=57 µg) of "DSPE-PEG-FITC in methanol" with 51.3 µl (=513 µg) of "lecithin in methanol". Add 38.7 µl of methanol in order to have 4% of methanol to water.
4. Heat up 3 ml of H_2O to 40°C and add the prepared organic solvent containing the lipids. Keep under stirring for 20 min.
5. During this time, put the weighed SEDS-nanoparticles to 5 ml H_2O. Sonicate in order to disperse the particles in the aqueous solution.
6. Add the DSPE-PEG-FITC/lecithin solution in this aqueous solution.
7. Keep under stirring for 5 min. Sonicate for 5 min.
8. Keep under stirring for 30 min at room temperature.

Confocal microscopy

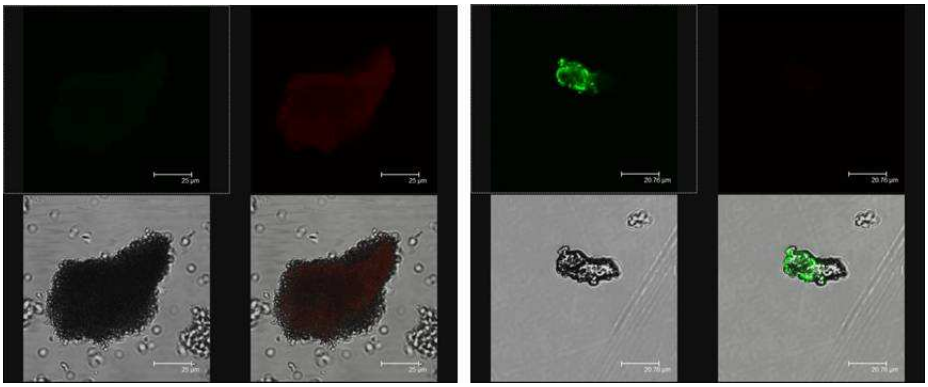

Figure 31. PLA-PEG particles containing MCHB but with no lipid (left image) and PLA-PEG particles coated with lipid but no MCHB (right image).

Release curves

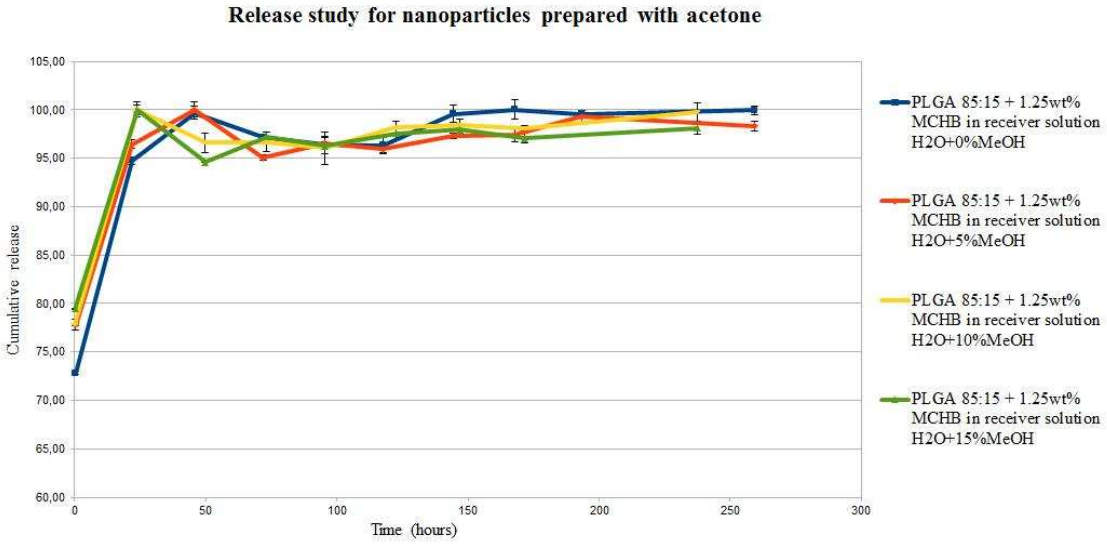

Figure 32. Release curve when using acetone as the drug and polymer solvent. The receiver solution is composed of different amounts of methanol (0%; +5%; +10% and +15%).

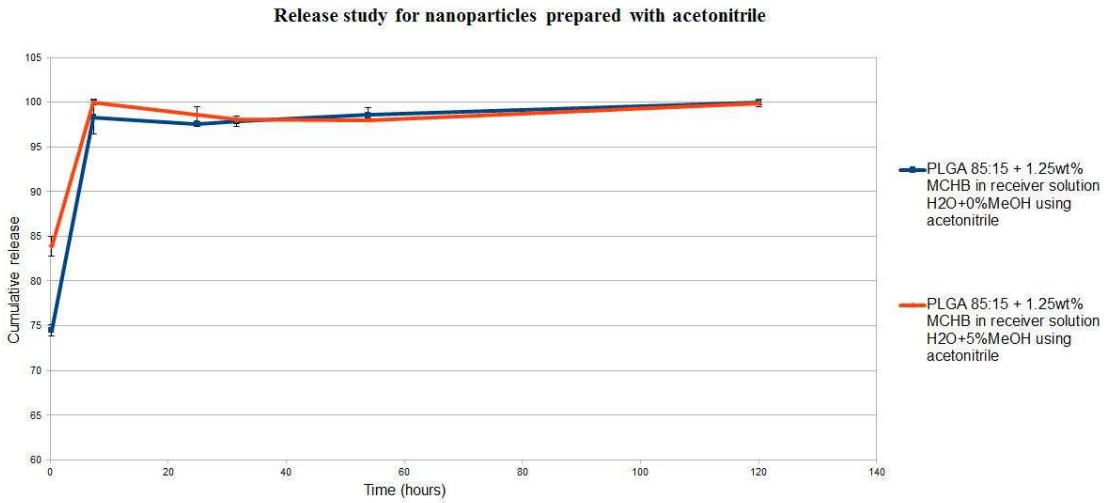

Figure 33. Release curve when using acetonitrile as the drug and polymer solvent. The receiver solution is composed of different amounts of methanol (0%; +5%).

Emission spectra

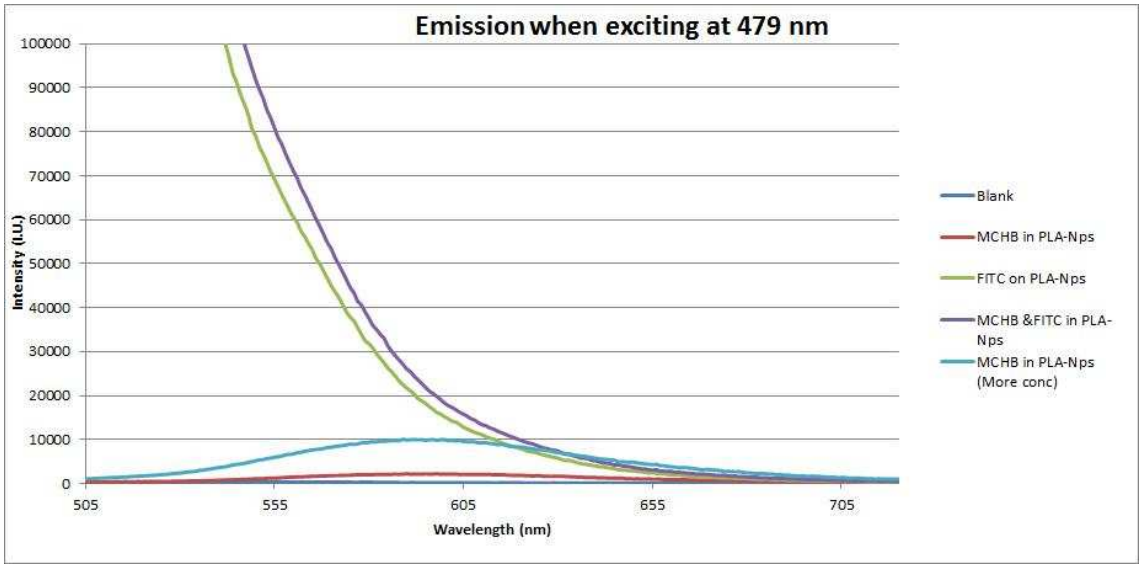

Figure 34. Emission spectra with an excitation at 479 nm for L-PLA nanoparticles (dispersed in water) with: only MCHB (red curve), only MCHB but a more concentrated solution (light blue cruve), only FITC (green curve), both MCHB and FITC (purple curve). The blank consisted of water (dark blue curve).

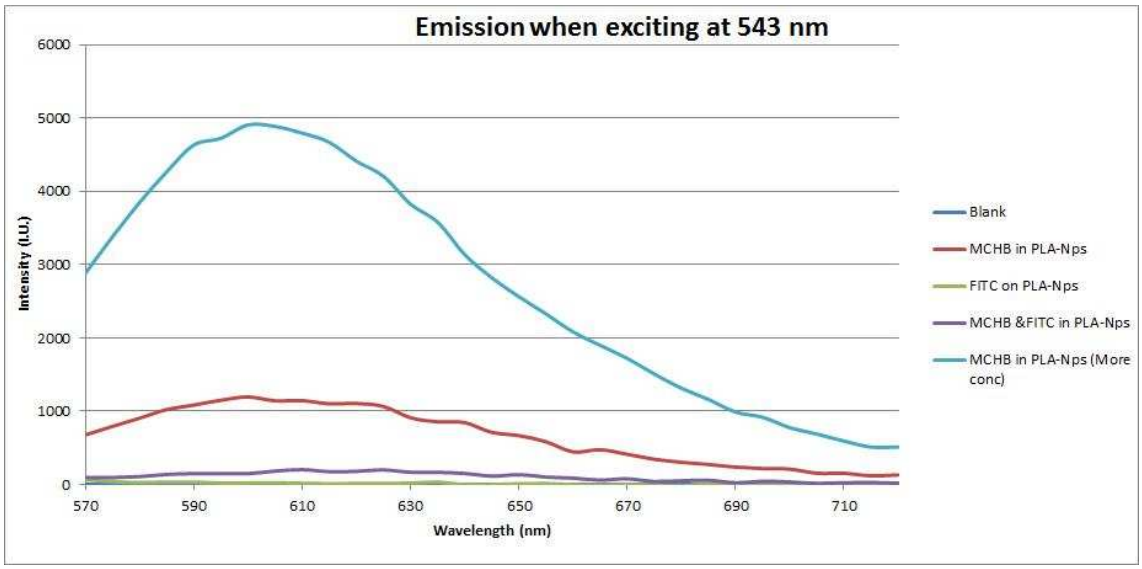

Figure 35. Emission spectra with an excitation at 543 nm for L-PLA nanoparticles (dispersed in water) with: only MCHB (red curve), only MCHB but a more concentrated solution (light blue cruve), only FITC (green curve), both MCHB and FITC (purple curve). The blank consisted of water (dark blue curve).