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Master Thesis Report:

Development of thermoresponsive liposomes as a delivery system of HRP

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ABSTRACT

Significant research in tissue engineering has been devoted to the replication of cartilage by developing suitable scaffolds. The approach of Ren et al was to develop an injectable Hyaluronic acid-tyramine hydrogel. HA-tyr hydrogels can be crosslinked by adding hydrogen-peroxide (H_2O_2) and the enzyme horse-radish peroxidase (HRP). However, the reaction is almost instantaneous and temporal control is difficult. By separating HRP from HA-Tyr and H_2O_2, the mixture is liquid. Hence, the main goal of this project was to develop a thermoresponsive system for HRP delivery aiming to trigger and control HA-Tyr crosslinking reaction.

Most of this study was inspired from the Ren et al (2015) research. However, two types of themoresponsive liposomes were formulated within this study, having a different composition as well as different methods of preparation. Both types of liposomes were formulated and their release properties were investigated.

Ren et al. (2015), developed a binary mixture of thermolabile synthetic phospholipids. In their research, they have successfully encapsulated the enzyme HRP but the process was difficult to reproduce. Multiple steps were shown within this project for ameliorating the sensitive processing. The second type of liposome was formulated with artificial synthetic phospholipids, synthesized by Professor Zumbühl and his team at the University of Fribourg. These formulations were shown to be more user-friendly and could be manipulated easily.

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CONTENT

INTRODUCTION

1.1 Cartilage

Currently, articular deficiencies and injuries (age-related, sport…) are increasingly observed. Due to the limited healing capacity of cartilage, this can lead to functional limitation or, if left untreated, to complete cartilage loss.[1] Thus, the patient's life quality can be greatly affected. Hence, it is important to develop new cartilage repair strategies. Currently, new techniques such as tissue engineering investigate long term articular cartilage repair that can withstand high mechanical loading.[2]

Tissue engineering or regenerative medicine tries to reproduce the biomechanical and biochemical characteristic of the native tissue. Even though cartilage tissue is avascular, aneural and alymphatic, it has a complex structure.[3] Articular cartilage is mainly composed of water, accounting for 70-80% of its weight, and it contains structural proteins. The cells, commonly called chondrocytes, are embedded within the dense extracellular matrix (ECM) mainly made of collagen and proteoglycans. It is the collagen and the proteoglycans that provide the porous structure of cartilage and they also ensure most of the mechanical properties of the joint.

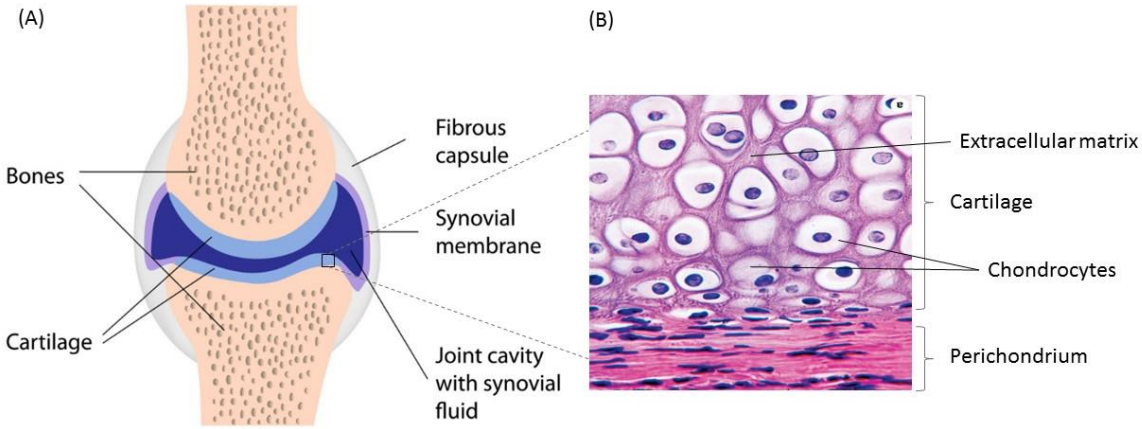


Figure 1: (A) Knee joint anatomy[4] (B) A section of cartilage's structure showing chondrocytes embedded within the extracellular matrix (ECM). The fibrous structure of ECM is given by the collagen and the proteoglycans.[5]

Since several years, significant research in tissue engineering has been devoted to the replication of cartilage by developing suitable scaffolds[6,7]; a support that must act as a temporary ECM by providing mechanical support and bioactivity. Several properties including biocompatibility, bioresorbability, promoting cell migration and tissue growth are required[8]. Currently, several limitations remain in the development of an appropriate scaffold for tissue engineering. For example, invasive surgery is often required which involves risk of infection or failure to heal. Secondly, it is difficult to perfectly mold a material into a defect site[1].

In order to address these issues above, a possible approach is to develop injectable scaffolds[9]. The method proposed by Ren et al. was based on the injection of a liquid hydrogel called hyaluronic acid-tyramine, that can crosslink in the defect site in response to a stimulus[10].

1.2 Hyaluronic acid-tyramine hydrogel

Hyaluronic acid-tyramine (HA-Tyr) hydrogel can be synthetized by crosslinking conjugated tyramine hyaluronan (HA-Tyr) in the presence of hydrogen-peroxide (H_2O_2) and the enzyme horse-radish peroxidase (HRP).

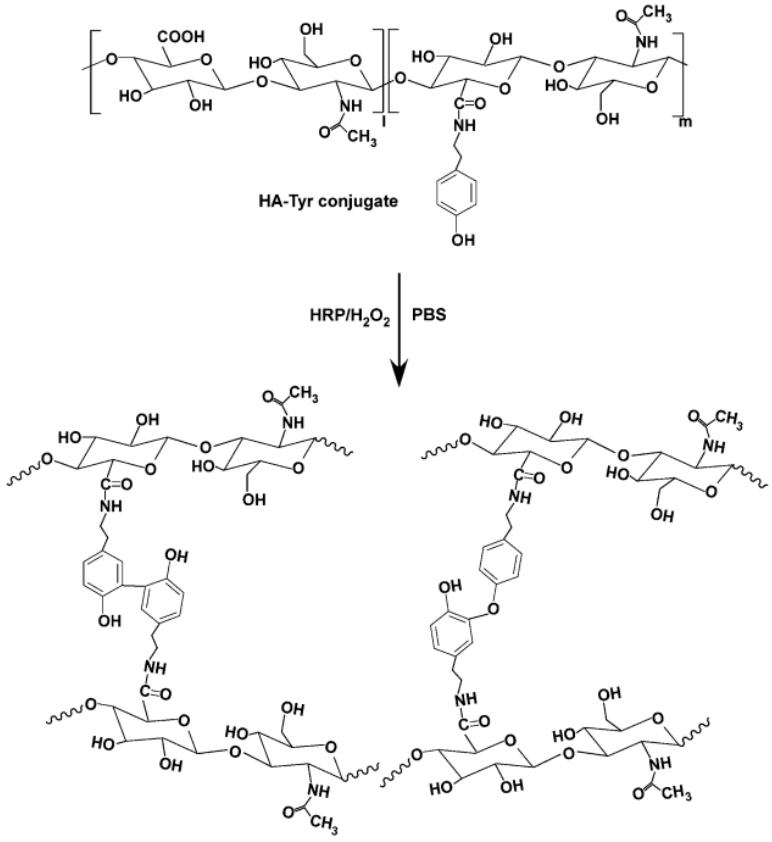


Figure 2: Crosslinking reactionof biodegradable HA-Tyr hydrogel[11].

HA-Tyr is a hydrogel with a covalently bound network. The crosslinking reaction occurs by an oxidative coupling of the phenol using hydrogen peroxide (H_2O_2) and a peroxide. The enzyme HRP is mainly used as an intermediate to oxidate the phenol groups, which will be described in more detail in this section[11]. The phenol hyaluronan tyramine derivative is a biocompatible and biodegradable polysaccharide and it is a major constituent of the extracellular matrix of the joint [12]. It was also shown that this hydrogel is adhesive to cartilage, hence it favors implantation[4]. In sum, HA-Tyr hydrogel seems very promising for the development of a cartilage tissue engineering scaffold.

The complete schematic of the reaction is illustrated below. The crosslinking of phenols by HRP involves two successive steps; first, HRP is oxidized by H_2O_2 to form an intermediate [HRP(I)], which then oxidizes the phenol (S). Finally two oxidized phenols ($R^{\cdot}$) crosslink to form a covalent network[12].

$$(1)\ HRP + H_2O_2 \overset{k_a}{\rightarrow} HRP(I) + H_2O$$

$$(2)\ HRP(I) + S \overset{k_b}{\rightarrow} HRP(II) + R^{-}$$

$$(3)\ HRP(II) + S \overset{k_c}{\rightarrow} HRP + R^{-} + H_2O$$

$$(4)\ 2R^{-} \overset{K_d}{\rightarrow} R - R$$

HA-Tyr hydrogel can provide a wide range of applications of injectable hydrogels, such as tissue regeneration or drug delivery. In order to produce a suitable scaffold, it needs to have structural similarities such as porosity, shape or surface morphology to reproduce as close as possible the mechanical and biological properties of the native tissue[13].

The crosslinking density (mechanical strength) of this hydrogel is solely affected by the concentration of H_2O_2[11]. It was shown that the crosslinking density directly affects the biodegradability of the gel which is essential since a scaffold should preferably be absorbed by the surrounding tissues. A second important factor for the development of an injectable scaffold is the gelation rate. Indeed, for drug delivery systems or micro-fissure tissue regeneration, hydrogels should rapidly form after injection, otherwise, uncontrolled diffusion of the gel, or contamination of surrounding tissue with the drug molecules might occur. The gelation rate of HA-Tyr hydrogel is influenced by the amount of HRP and the gelation time ranges from 1 second to 20 minutes[11]. In fact, the criteria for an injectable hydrogel would be that during manipulation the hydrogel remains in a liquid form and when it is injected into the defect site, the hydrogel should form and consolidate as rapidly as possible.

The solution proposed by Ren et al. to control the gelation rate is to encapsulate HRP into thermoresponsive liposomes: by separating HRP from HA-Tyr and H_2O_2 at RT, the mixture would remain liquid[10]. Upon injection into the defect site and exposure to body temperature, the microcapsules would release HRP, resulting in the subsequent reaction (fig. 3) and the formation of a hydrogel scaffold.

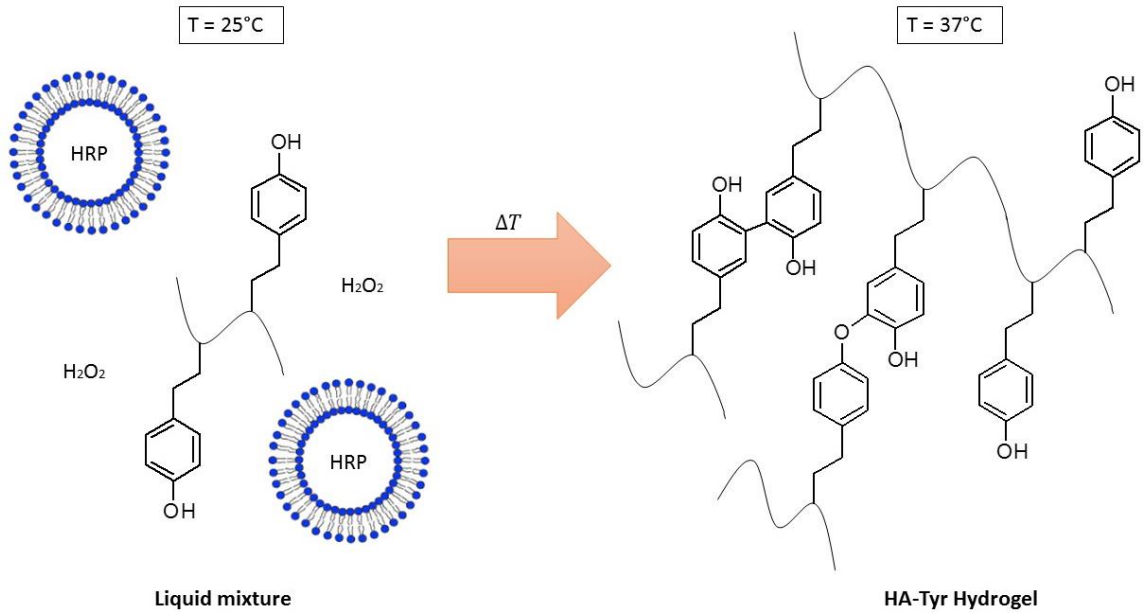


Figure 3: Model of an injectable HA-Tyr hydrogel by encapsulation HRP into thermoresponsive liposomes

Thermoresponsive liposomes are vesicles that are sensitive to temperature. Choosing a specific liposomal composition enables the release of a cargo at a specific temperature. The difference between room temperature (25°C) and body temperature (37°C) is not negligible, hence very interesting to discharge drugs or substances into a specific body site by means of thermoresponsive liposomes. The next chapters describe in detail how this phenomenon can occur.

1.3 Liposomes

Liposomes are closed spherical vesicles composed of one or more phospholipid bilayers[14]. The major components of liposomes are phospholipids and cholesterol. It is the chemical properties of these lipids that control the behavior of the liposome. The amphiphilic character of phospholipids is due to their hydrophilic polar head and hydrophobic apolar tail. Thermodynamically, the combination of these lipids into a membrane is favored in the presence of water[15]. The polar end is associated with water and the apolar tails are in the middle of the membrane (Fig. 4). The spherical structure is spontaneously formed as it has been thermodynamically proven that the free energy of the system is lower in a spherical geometry than in planar bilayer[16].

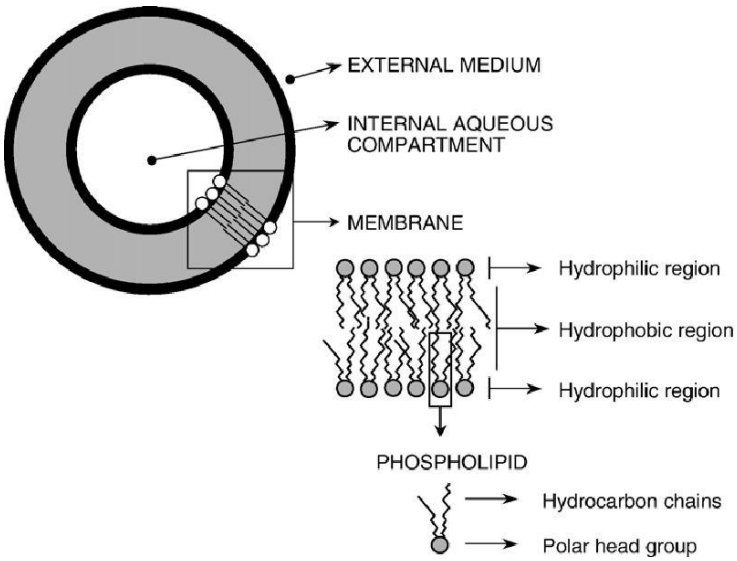


Figure 4: Structure and composition of liposomes[14]

The structure of liposomes, forming an inner and an outer aqueous space, enable the encapsulation of both hydrophobic and hydrophilic material. The center of the membrane provides a place for non-polar substances and the lumen of the liposome provides a place for polar substances. Presently, liposomes are extensively used as drug carriers for numerous molecules in cosmetic and pharmaceutical industries[17]. This type of delivery system has the advantage to entrap unstable compounds, shield their functionality and deliver the drug at a targeted place. Several other advantages using liposomes are their biocompatibility, biodegradability and non-toxicity[18].

The lipid bilayer offers a basic barrier for the diffusion of entrapped material. Permeability of the membrane is affected by (1) lipid composition (2) surface charge (3) physical properties of the medium such as pH and temperature, and (4) shear stress[18]. For instance, unsaturated phospholipids (found in natural sources such as egg or soybeans) give much more permeable and less stable bilayers then saturated phospholipids with long acyl chains (e.g. dipalmitoylphosphatidylcholine, DMPC)[18].

1.4 Composition of liposomes

As mentioned above liposomes are mainly composed of phospholipids.

The structure of a phospholipid is composed of two major portions: a hydrophilic head and a hydrophobic tail. All phospholipids are composed of a glycerol backbone, a phosphate group as well as two hydrocarbon chains as shown in the figure below.

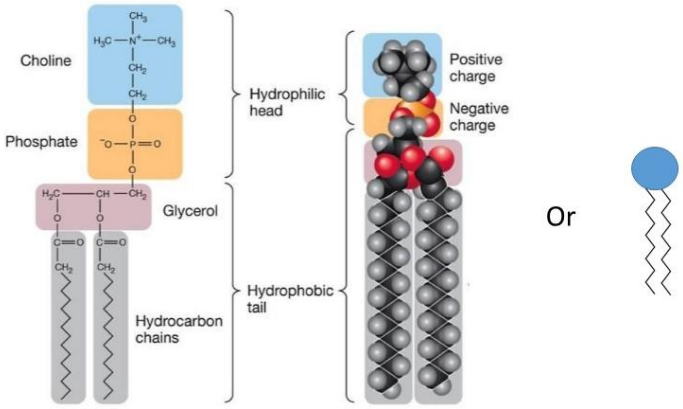
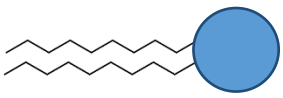


Figure 5: General structure of a phospholipid[19]

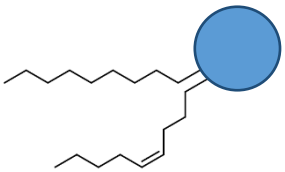
The hydrophilic head can either be neutral, negatively or positively charged. To further modify the properties of liposomes it is common to add a sterol or an ionic substance. The addition of an ionic substance gives a charge to the membrane that can, for instance, prevent vesicle aggregation due to repulsion forces or provide a higher hydrophilic loading efficiency[20].

The length and the degree of saturation of the two hydrocarbon chains affect the permeability of the membrane. In brief, the higher the number of carbon atoms, the longer is the chain and the longer the chain, the smaller is the lateral expansion between the two chains (due to greater hydrophobic interactions)[21]. Hence, permeability of the membrane is increased with the length of the chain. Another parameter that can affect the chain length is the configuration. There are two possible configurations, *cis* or *trans*. In *trans* configuration, the chain is fully extended, whereas in *cis configuration* the chain is bended, and thus reduces its size. The figures below show the difference between *trans* and *cis* conformations.

Saturated phospholipid in trans conformation



Unsaturated phospholipid in cis conformation



Usually, an unsaturated chain has a *cis* conformation that induces lower possibility for hydrophobic interactions and lateral expansion between the two chains. This phenomenon implies greater degree of freedom.

To summarize, saturated phospholipids with long chains (such as dipalmitoylphosphatidylcholine, DPPC) form a rigid and rather impermeable bilayer, whereas membranes composed of unsaturated lipids with short chains are much more permeable and less stable.

Temperature is also a factor that can affect the permeability of the membrane. Indeed, each phospholipid has a specific phase transition temperature (Tc)[22] (see annex A1). It is defined to be the temperature at which the lipid physical state converts from an ordered gel phase to a disordered liquid crystalline phase (see figure below).

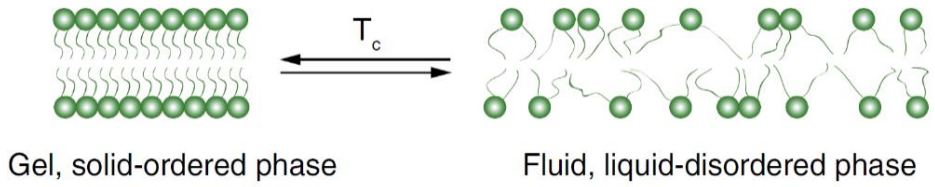


Figure 6: Phase transition temperature (Tc) of phospholipids going from gel to fluid physical state[23]

In the gel phase, lipid molecules are ordered and packed together forming a two-dimensional plane membrane. Upon heating, mobility of the lipids increases gradually. The energy given by the increase of temperature (in the form of heat) enables the carbon-carbon bonds to rapidly rotate around their axes forming different conformers[21] (see annex A2). This phenomenon decreases the hydrophobic interaction between the chains. Therefore, it enables the lipids to move more freely within the plane. As a result at Tc the membrane becomes fully fluidized and is permeable.

Tc depends on the hydrocarbon chain length, degree of saturation, charge and head group species (see Table in Annex A1). The use of phospholipids with higher phase transition temperatures generates bilayers, which are more stable and rigid. This decreases the possibility for premature leakage of encapsulated components. On the other hand, if the phase transition temperature of the selected phospholipid is too high, high temperature might denaturate the encapsulated cargo during the process of liposome formulation. Indeed, as will be explained later in this report, in order to formulate liposomes the medium temperature has to be higher than the phase transition temperature of the lipids within the mixture.

In all, the molar percentage of phospholipids can vary between 55 and 100%[22]. The remaining percentage is composed of components that can be added to the formulation in order to impart specific property to the final vesicles. For instance, to stabilize the bilayer from leaky properties additional sterol can be employed. As shown on Figure 7, cholesterol is one sterol that is widely used for this purpose.

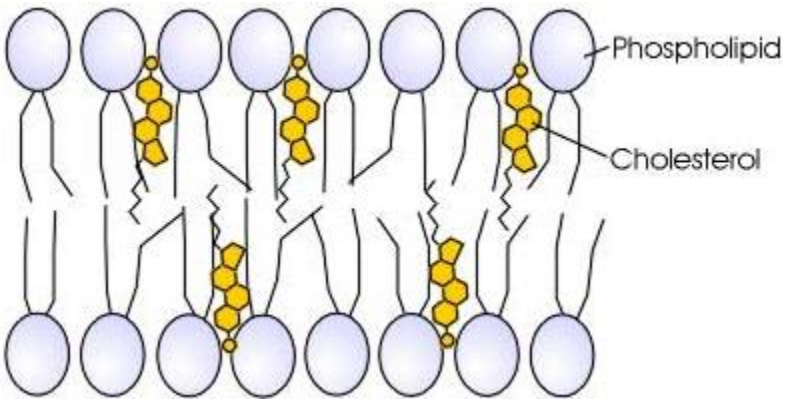


Figure 7: Cholesterol molecules embedded into a phospholipidic bilayer[24]

The small and rigid structure enables it to lodge between the phospholipid's apolar chains, which in turn limits their degree of freedom. Overall, cholesterol increases the rigidity of the phospholipid bilayer. Most studies have shown that in order to prepare stable and controlled drug release vehicles, a molar composition of 70% of lipids and 30% of cholesterol is required[25].

1.5 Classification of liposomes

Generally, liposomes are classified by two factors: their size and their number of bilayers[21]. Both of these parameters influence the encapsulation efficiency of the liposomes.

Effective and stable encapsulation depends on the physico-chemical properties of the substance that should be encapsulated (size, charge, hydrophobicity), but also on liposome features (size, type, composition and concentration of lipids)[22]. As mentioned before, both hydrophilic and lipophilic molecules can be encapsulated in the lumen of the liposome or within the bilayer, respectively. There are two ways to encapsulate a substance: one called the passive way, where the encapsulation occurs during liposome preparation, and the second the active way, where the cargo is introduced into preformed liposomes[22]. Depending on the liposome formulation method and the substance that should been capsulated, the active or passive way can be used and for a better understanding, liposomes have to be classified.

Liposome size can vary from very small being several nanometers in size, to large vesicles with a few micrometers in diameter. Furthermore, they can have one or more bilayer membranes. They are typically classified into four main categories as can be seen in figure 8: 1) Small Unilamellar Vesicles (SUV), 2) Large Unilamellar Vesicles (LUV), 3) Multilamellar Vesicle (MLV) and 4) Multivesicular Vesicles (MVV).

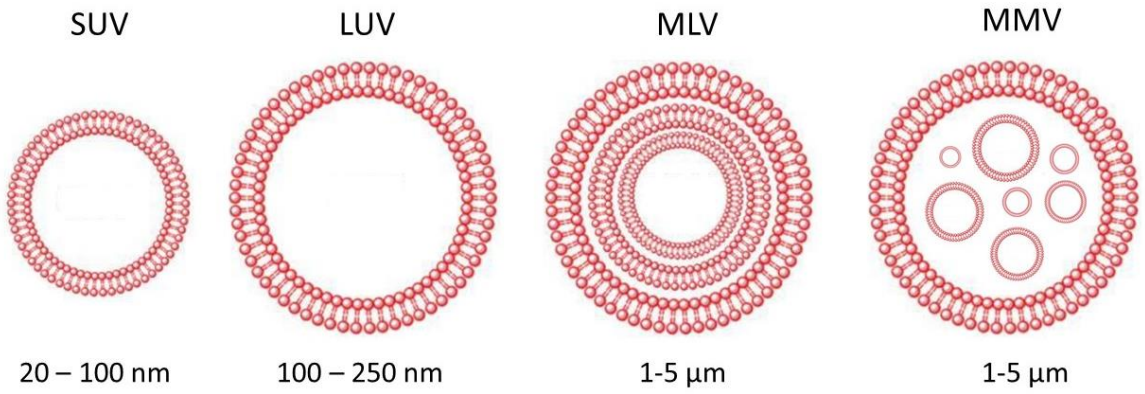


Figure 8: Classification of liposomes based on size and lamellarity[18]

In unilamellar structure, the vesicle has a single phospholipid bilayer, whereas in multilamellar structure, the vesicles have an onion-like structure. In the case of Multivesicular Vesicles several unilamellar vesicles are formed inside of larger liposomes, forming a multilamellar structure of concentric phospholipid spheres.

The encapsulation efficiency of liposomes increases with the size of the liposome and decreases with number of bilayers (for hydrophilic compounds only). Likewise, the larger the radius curvature, the weaker is the tension surface. Hence, optimum liposome encapsulation efficiency and stability is reached when the vesicles have a size between 80 and 100 nm[22].

1.6 Liposome preparation methods

Although liposome formation can occur spontaneously when the lipids are transferred into an aqueous medium (thermodynamic equilibrium), it often requires supplementary steps. The preparation method has a great influence on the properties of the liposomes: size, shape, stability and drug loading efficiency.

Figure 9 depicts the four basics stages for the preparation of liposomes: 1) Dispersing the lipids into an organic solvent, 2) Forming a thin lipid film by evaporating the organic solvent, 3) Hydrating the lipid film and 4) Processing additional steps to get a homogeneous liposome formulation.

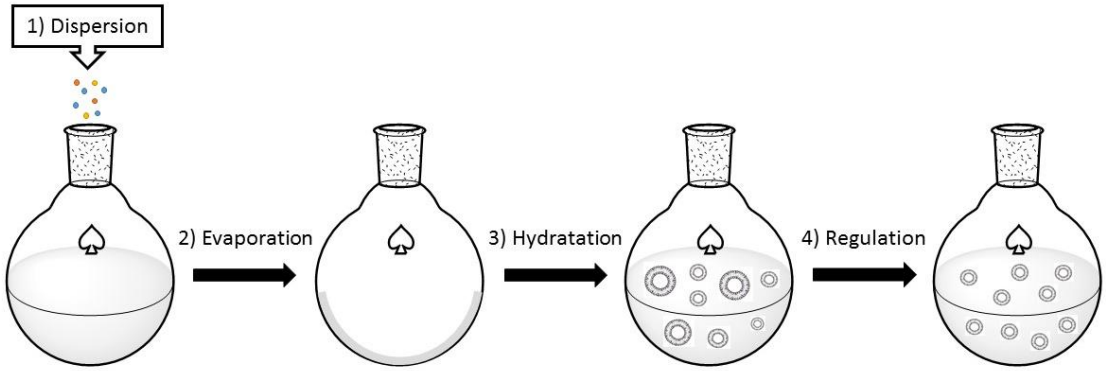


Figure 9: General steps for liposome's formulation

Regardless of the mixture of lipids (neutral or ionic), the dispersion step is always the same. The different lipids are first dissolved into an organic solvent, usually chloroform and/or methanol, to ensure a homogeneous mixture of lipids. The organic solvent is then removed to obtain a thin lipid film on the wall of the round bottom flask. Usually, evaporation is performed using a rotary evaporator connected to a vacuum pump. The round bottom flask can then be placed under vacuum overnight to ensure complete removal of residual solvent. The third hydration step, is performed by adding an aqueous solution. It is important to work at a temperature that is always higher than the phase transition temperature of the lipids in order to permit liposome formation. Encapsulation efficiency as well as the size of the liposomes (MLV) varies depending on the processing time and agitation. MLV, obtained by this method, can be homogenized by sonication or extrusion.

In the sonication process, ultrasound is used to break down the MLV into SUV (generally 20 to 50 nm)[22]. A sonication bath or a probe tip sonicator can be used. Generally, probe tip sonication is mostly applied since the power is far higher than bath sonication. The only drawback is that the probe can contaminate the suspension with traces of metal which have to be eliminated by subsequent centrifugation. Sonication has to last until the formulation becomes clear and slightly opalescent at a temperature higher than the Tc. The cloudy effect is due to light dispersion on the remaining MLV. The encapsulation efficiency by the sonication process is very low. Furthermore, it is challenging to reproduce exactly the same sonication condition between each batch and the liposome's size dispersion will differ batch dependently.

A second method for the regulation of liposome suspension is by extrusion. This technique consists of forcing the liposomal suspension through a filter with a given pore diameter, in order to recover liposomes with a size near the pore diameter of the filter. The extrusion process also has to be performed at a temperature higher than the Tc. If the liposomal suspension passes through the filter more than 10 times it is possible to have LUV with an average diameter of 120 to 140 nm[22]. There are three advantages of extrusion: first the size of the liposomes is reproducible from one batch to another, second this method is very fast and third encapsulation efficiency can be higher than 30%[22].

Modern novel methods for liposome preparation are continuously developed[26]. There are several possibilities to formulate liposomes[27], but the two methods that were used in this work are described below:

1. Freeze-drying method

Pre-formed SUVs are frozen and then dried during a lyophilization process. The dried lipids, in the form of lyophilized powder, are then directly hydrated with the aqueous phase. During hydration, SUV membranes merge to form MLV with a high encapsulation volume. This method is generally used when a component is thermolabile and would be destroyed during the hydration or regulation step.

2. Freeze-thaw method

This method applies several freeze and thaw cycles on pre-formed SUVs. The fusion of SUVs with each other results in the formation of LUVs with high encapsulation efficiency (between 30-80%). This technique can also be used during the hydration of lipid films receiving LUVs directly instead of MLVs.

1.7 Liposome size's characterization

In order to assess the quality of liposomes and to determine whether batches are reproducible from one another, the size of the vesicles can be measured. In the present study, the size determination of the liposome was performed using dynamic light scattering (DLS).

Dynamic light scattering (DLS) is a well know technique to measure the size and the size distribution, more particularly in the sub-micron region of suspended particles. A laser emits through the solution and the Brownian motion caused by the vesicles in suspension causes laser light to be scattered at different intensities. As the Brownian motion and the intensities are related, using Stokes-Einstein relation, the size of the particles can be then determined.

EXPERIMENTAL DESCRIPTION

Thermoresponsive liposomes are vesicles which release their cargo in response to heating. Lipids undergo a specific phase transition temperature that changes the conformation of phospholipids going from a gel to a fluid phase, allowing the diffusion of material through the membrane. Hence, the liposome composition is determined in function of the target release temperature.

2.1 Ren et al. experiment

In summaryl, Ren et al. wanted to develop an injectable HA-Tyr hydrogel[10]. By encapsulating HRP into thermolabile liposomes, the HA-Tyr system would remain liquid at room temperature. Through exposure to body temperature, the components of the liposomes would undergo a gel to liquid phase transition resulting in the release of the cargo. Once HRP, HA-Tyr and H_2O_2 are brought together, the crosslinking reaction occurs almost spontaneously forming a biodegradable and biocompatible hydrogel. This injectable hydrogel seems very promising as it can perfectly mold defect sites.

The composition of thermolabile liposomes has to be especially defined to trigger release at body temperature (37°C). Until today, there are no natural or synthetic phospholipids on the market that have a phase transition temperature precisely at 37°C (see table 1, annex A1). Thus, a mixture of different phospholipids has to be chosen. Ren et al. decided to take a combination of two different phospholipids: dipalmitoyl pholsphatidylcholine (DPPC) and dimyristoyl pholsphatidylcholine (DMPC). Both of them are neutral saturated phospholipids having 16 and 14 carbon atoms in the hydrocarbon chains, respectively. Their phase transition temperatures are: Tc (DPPC) = 41°C and Tc (DMPC) = 23°C.

Mabrey and Sturtevant have investigated the phase transition temperature of a binary mixture DPPC/DMPC, by high sensitive differential scanning calorimetry[28]. The phase diagram was constructed by specifying (on the bottom line) the onset temperature and (on the upper line) the completion temperature. The straight line is an extrapolation of six DSC measurements, hence is the empirical phase transition temperature of DPPC/DMPC. In this case, figure 10 depicts, that the phase transition temperature of the binary mixture does not behave ideally (where the dashed line is the ideal phase diagram). As first approximation, in order to have a system with a phase transition temperature of 37°C, the molar amount of DPPC has to be four times higher than the molar amount of DMPC according to Marbrey and Sturtevant binary phase diagram.

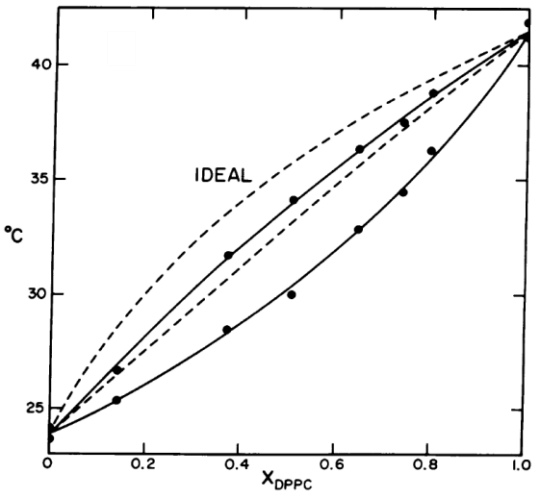


Figure 10: Phase diagram constructed from initiation and completion temperatures of a binary mixture DPPC/DMPC. (- -) represent the ideal phase diagram whereas (--) is an extrapolation of six measurements[28].

Initially, the liposome preparation method proposed by Ren et al. was the freeze-dried method which is also called Dehydration-Rehydration Vesicle technique (DRV)[10]. As mentioned in the "liposome preparation methods" chapter, this method is based on controlled rehydration of preformed dehydrated vesicles.

2.2 Tanasescu et al. experimental approach

Another batch of completely different thermoresponsive liposomes was formulated in the chemical laboratory of Professor Zumbühl at the University of Fribourg. Indeed Prof. Zumbühl and his team have developed artificial phospholipids 1,3-diaminophospholipid, named Pad-PC-Pad[29]. They have noted that Pad-PC-Pad forms faceted large and giant unilamellar vesicles (Fig. 11 A). The particularity of these faceted liposomes originates from the fact that hydrocarbon chains interdigitate (Fig. 11 B) with each other as a main factor for the stabilization of facets.

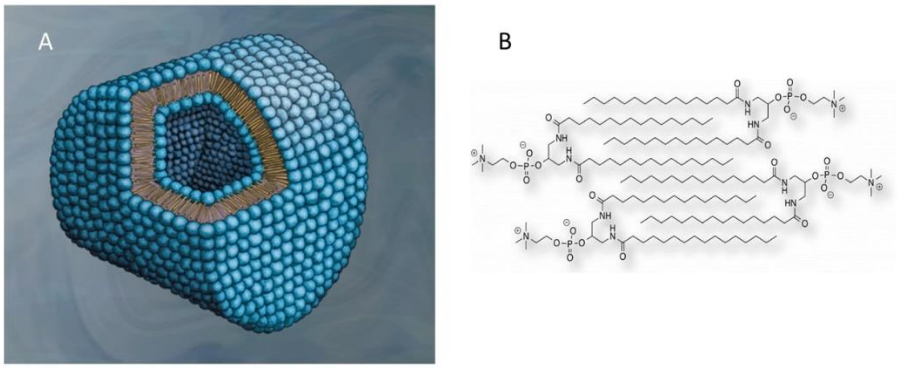


Figure 11: A) Faceted geometry of Pad-PC-Pad[30]. B) Pad-PC-Pa bilayer[29].

The faceted Pad-PC-Pad liposome has the specificity to be mechanosensitive and thermosensitive at the same time. According to the research conducted by Tanasescu et al. [30], the phase transition temperature of Pad-PC-Pad is around 37°C (as depicted in the figure below). This property makes it interesting for the development of thermoresponive liposomes that can release a cargo at body temperature. The figure below shows the differential scanning calorimetry (DSC) of Pad-PC-Pad liposomes containing different amounts of cholesterol.

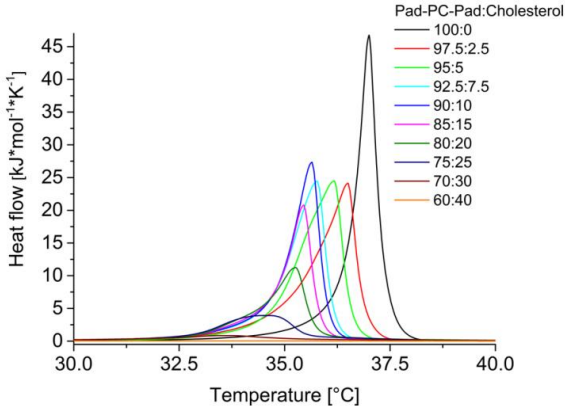


Figure 12: Differential Scanning Calorimetry scans of a mixture of Pad-PC-Pad and cholesterol[30].

As the amount of cholesterol increases, the phase transition temperature of the liposomes decreases. Hence, pure Pad-PC-Pad liposomes can lead to a discharge of material at a temperature of 37°C.

The preparation method of Pad-PC-Pad liposomes is produced by going through the four basics steps of liposome formulation explained in the ‘’liposome’s preparation methods’’. Freeze-thaw cycles were performed to obtain LUVs during the hydration step. In the next chapter (or in Tanascescu et al., 2016) the detailed method for faceted Pad-PC-Pad liposome formulation is elucidated.

MATERIALS AND METHODS

3.1 Materials

- Phosphate Buffered Saline, PBS, pH 7.4, 1X (Gibco, Life Technologies, USA)
- 1,2-dimyristoyl-syn-glycero-3-phosphocholine, DMPC (Avanti Polar Lipids INC., USA)
- 1,2-dipalmitoyl-sin-glycero-3-phosphocholine, DPPC (Avanti Polar Lipids INC., USA)
- Cholesterol, grade $\geq$ 99% (Sigma Aldrich, USA)
- 1,3-diaminophospholipid, Pad-PC-Pad (Fribourg university, CH)
- Peroxidase from horseradish, type I, RZ: 146 units /mg Solid (Sigma Aldrich, USA)
- Carboxyfluoroscein, (Sigma Aldrich, CH)
- Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Labeling & Detection, Life Technologies, USA)

3.2 Liposome Formulation

Method 1

Given ratio of DPPC, DMPC and cholesterol (unless other mentioned: 80.9 mg, 25.2 mg and 29.4 mg respectively) were dissolved in 5 mL chloroform in a 50 mL round-bottom flask. The organic solvent was evaporated with a rotary evaporator (Büchi rotavapor R-205) at 50°C (Büchi heating bath B-490) under reduced pressure (Büchi vacuum Controller V-800). The lipid film was hydrated with 5 mL phosphate-buffered saline (PBS 1X) during 30 minutes in a 50°C water bath with intermittent vortexing. The formulation was sonicated with a probe-tip sonicator (Bandelin sonopuls HD 2070) at a power of 50 watt within a water bath at 55°C during 30 minutes. Then after, the suspension was cooled at RT, solution of HRP (unless other mentioned: 2 mg/mL in PBS 1x, 5 mL) was added into the formulation. The whole was frozen at a temperature of -80°C during at least 3 hours then freeze dried overnight (Kühler Freeze dryer ALPHA). Dried liposomes were rehydrated with phosphate-buffered saline solution (PBS 1x, 0.5 mL) and vortexed until homogenization.

Method 2

Same method as *Method 1,* but instead of adding the enzyme after the sonication process, HRP was added into the freeze-dried powdered formulation. Dried liposomes as well as HRP were mixed manually with a spatula until a homogenous solution was obtained. Finally, the lyophilized product was rehydrated with PBS 1x (0.5 mL).

Method 3[30]

One milliliter of chloroform solution of ready to use synthetic lipids (4mg/mL), C16 1,3-diamidophospholipid, named Pad-PC-Pad, was dried under reduced pressure, and left under high vacuum overnight. One milliliter of 5(6)- carboxyfluorescein-containing buffer [10 mM HEPES buffer, 50 mM 5(6)-carboxyfluorescein dissolved in ultrapure water, pH 7.4 (NaOH), 200 mOsm (NaCl/L)] was added, and the film was hydrated within a water bath (65°C) for 30 minutes. Afterwards, five freeze/thaw cycles were preformed using liquid nitrogen and a 65 °C water bath. The suspension was extruded 11 times at 65°C using a miniextruder (Avanti Polar Lipids) and a track-etched filter membrane (pore size: 100 nm, Whatman). Finally, the liposomes were purified using a size exclusion chromatography (PD-10 desalting columns, GE Healthcare) and diluted to 50 mL with additional outer buffer (HEPES). The final suspension was left to rest at room temperature during 24 hours, in order to allow the formulation to reach equilibrium.

Another batch was formulated with exactly the same method but different liposome composition. Instead of Pad-PC-Pad, DPPC, DMPC and cholesterol (16.2 mg, 5.0 mg, 1.3 mg respectively in 1 mL HEPES buffer) was used.

Method 4

Given amounts of DPPC, DMPC and cholesterol (16.2 mg, 5.0 mg, 1.3 mg respectively) were dissolved in 1 ml chloroform. The solvent was removed under reduced pressure with a rotary evaporator at 41°C. Then the given dried film was left under vacuum overnight. The thin lipid film was then hydrated with one milliliter PBS and left within a water bath at 45°C for more or less 24 hours until complete hydration of the thin film. The suspension was then extruded 11 times at 45°C using a miniextruder (Avanti Polar Lipids) and a track-etched filter membrane (pore size:100 nm, Whatman). Finally, liposomes were purified using size exclusion chromatography (PD-10 desalting column, GE-Healthcare) and diluted to 50 mL with PBS 1x.

3.3 Liposome's size characterization

Liposome size was determine by Dynamic light scattering (DLS) using a Malvern Instrument (Zen 3600) connected to the Zetazizer software. The liposome's formulation (from above) was diluted 10^3-folds in PBS and 1 mL solution was analysed into a universal dip cell. The temperature of analysis was 25°C and feature setting of 1.450 as for refractive index and 0.001 as for absorbance.

3.4 Release measurement

Measurement of HRP release

For the measurement of HRP release, the formal formulation of liposome (from above) was first diluted 10-fold. Then two lots of seven aliquots of 100 µL diluted suspension were incubated for one hour at either room temperature or 37°C. In order to record a release trend, HRP quantification was measured at different time steps. Before HRP quantification the formulation was centrifuged (8000 g, 4 min) and the HRP concentration in the supernatant was measured using Amplex Red Kit. HRP was quantified in terms of units of enzymatic activity using a microplate reader (Wallac 1420 Instrument) at 544 nm (excitation) and 590 nm (emission). The control 0% HRP delivery was determined at time zero whereas 100% delivery was set to an hour.

Measurement of 5(6)-carboxyfluoroscein release

Nine 1.5 mL aliquots of 5(6)-carboxyfluoroscein liposomal suspension were incubated at 37°C during a set amount of time. The release trend was recorded at different time steps (0, 5, 10, 15, 20, 30, 40, 60 min), where 5(6)-carboxyfluorescein was quantified in triplicate using a fluorimeter (HIDEY Sense Microplate Reader) at 485 nm (excitation) and 535 nm (emission). The control 0% dye delivery was determine at time zero whereas 100 % dye delivery was determined by adding 2 vol % of a 10 vol % Triton-X100 to a sample.

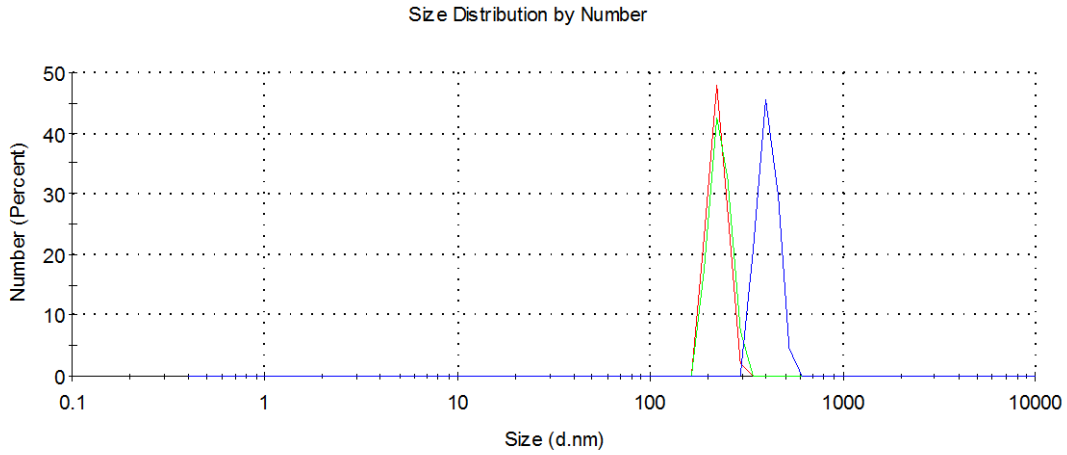
RESULTS & DISCUSSION

As a recall, the main aim of this study was to first develop thermoresponsive liposomes in order to develop an injectable HA-Tyr hydrogel. Indeed, such hydrogel would be a tremendous progress in regenerative medicine and in the tissue engineering domain. Ren et al. developed a process to trigger a crosslinking reaction with temperature sensitive vesicles resulting in a possible repair of joint micro-crack. In fact, despite the accurate protocols and explanations given by Ren et al., it was not as obvious as it seems to reproduce their experiment. Hereafter, is the chronological approach that was tried to replicate the Ren et al. research.

At the beginning of the project, the liposome composition was chosen in order to target a phase transition temperature of 37°C. According to the Mabrey and Sturtevant study (Fig. 10, Section 2.1), the binary phase diagram of DPPC and DMPC gives a molar ratio of 3 for such a temperature. Hence, a ratio of 75% DPPC and 25% DMPC was chosen regarding the liposomal lipid composition. As for the addition of cholesterol, in most literature[25] it is recommended to have 30% in mole of cholesterol within the composition. Actually, it helps to stabilize the lipid membrane as well as it prevents unwanted leakage. Several experiments were performed following the Method 1 in Section 3.2, until a first release trend was observed.

4.1 First result

First of all, the class of liposome has to be determined by calculating the vesicles size with dynamic light scattering (DLS). The graph below shows the size distribution expressed by a number (percentage) with regard to the particle diameter (nm). The measurements were taken in triplicate.



Graph 1: Here the size distribution of the formulation prepared with method 1 is shown. This batch has the following composition: 80.9 mg DPPC, 25.2 mg DMPC, 29.4 mg cholesterol in 10 mL PBS, giving a lipid concentration of 10.5 mg/ml.

The first two measurement are transposed on each other (green and red line), whereas the last measurement (blue line) shows larger vesicles. Nevertheless, this phenomenon is very common for liposome DLS measurement. Indeed, the suspension of liposomes is never completely homogeneous; hence variations in the measurement are often seen. The average size of this batch of liposome (mean size calculated with regard to each percentage) is $409 \pm 49\ nm$. This value corresponds to the class of multi-lamellar vesicles MLV.

The picture below allows the global visualization of this population of vesicles.

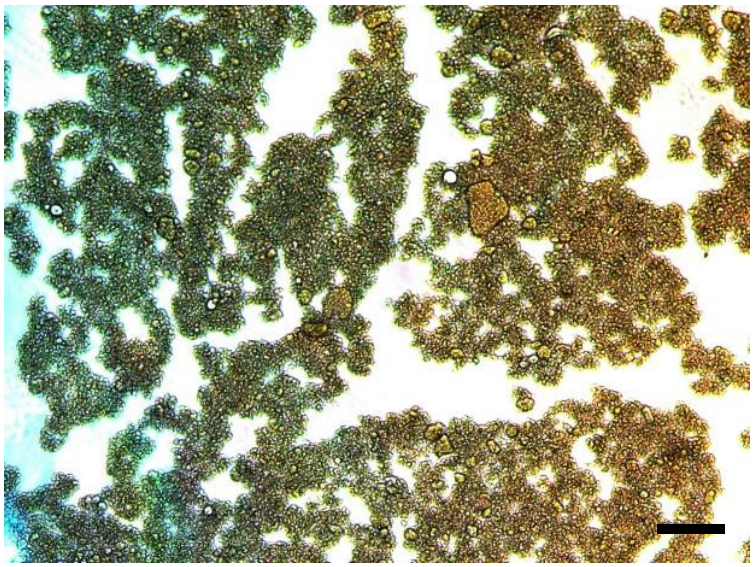
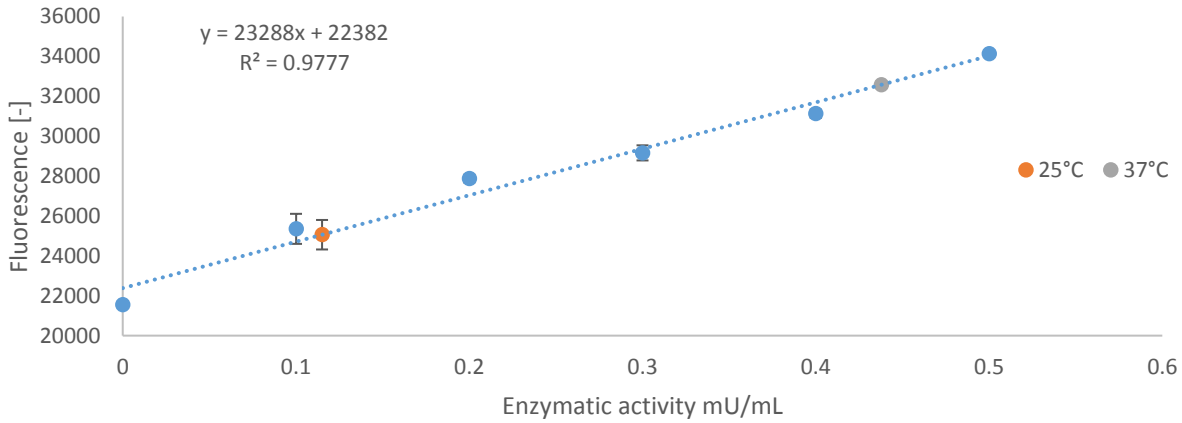


Figure 13: Liposome formulate with Method 1 in Section 3.2. The scale bar represent 100 µm.

Despite that the vesicles are very small and that they are aggregated, Figure 13 shows clearly the size dispersion of MLV. From small vesicles to bigger ones, the population of liposome is dense and not homogeneous. However, it is this batch of liposome that gives the first HRP release. In Graph 2 is depicted the fluorescence with regards to a concentration expressed in terms of enzymatic activity. A sample of the formulation of liposome was left at 25°C and another sample at 37°C and the enzymatic activity of HRP in the supernatant of each sample was measured after one hour. In order to convert the fluorescent signal to a concentration, the HRP standard curve was also plotted.



Graph 2: HRP release of two samples held for 60 min. at 25°C and 37°C. The blue dot line is the standard curve, the concentration in the supernatant is given by the orange point for the sample held at 25°C and the grey point for the sample held at 37°C.

Graph 2 highlights the presence of a release when two samples of a common batch were held at 25°C and 37°C during one hour. In fact, the enzymatic activity of the sample left at 37°C is 4-fold higher than the sample left at room temperature.

This experiment was performed once again in order to validate the model. Unfortunately, after several trials, this experiment was never reproducible. In order to understand the reason of these

discrepancies, every step of the formulation was investigated: such as the composition of the liposome, the release measurement method or the method of preparation.

4.2 Composition optimization

Since no release was observed at any other stage, the optimization of the composition of the liposome was begun. Perhaps the problem was due from the Tc of the bilayer being too loose (all HRP would have been released at 25°C, hence no release would be observed) or was too rigid (nothing could diffuse through the lipid membrane). Thus, formulations of different ratios of DPPC and DMPC with a constant amount of cholesterol (30% in mole) were formulated. Three different molar ratios were investigated: 1:1, 3:2 and 19:1 (DPPC and DMPC respectively). In fact, HRP release was not observed in any of these experiments. As the phase transition temperature of the vesicle is linked to the composition of the liposome, by decreasing the molar ratio DPPC/DMPC, Tc also decreases. Hence, it was expected that with lower amount of DPPC, with a same incubation temperature (37°C), HRP release would be easier. Subsequently, another experiment was performed but this time changing the concentrations of HRP with constant lipid composition: 2, 4 and 8 mg/mL were tried with lipid molar composition of approximately 53% DPPC, 17% DMPC and 30% cholesterol. The results made absolutely no sense either. Hence, HRP release was not observed under any of these conditions so one last experiment was performed using exactly the same liposome composition mentioned in the Ren et al. paper. Liposomes were formulated with 60 mg/mL lipid (DPPC, DMPC with a weight ratio of 2.3), having 5% by weight cholesterol. In terms of molar percentage, it gives a composition of 62% DPPC, 29% DMPC and 9% cholesterol. By significantly increasing the amount of lipids within the formulation (going from 10.5 mg/mL to 60 mg/mL) the encapsulation efficiency should have been increased. In addition, as the amount of cholesterol was lowered the membrane fluidity was increased. Unfortunately, once again no HRP release was observed. Consequently, as nothing worked as expected, the method of quantification of the release of HRP was investigated.

4.3 Release method optimization

As was mentioned before, two lots of liposomal suspension were incubated either at room temperature or at 37°C. At different time steps the concentration of HRP was measured. In order to eliminate the liposomes a step of centrifugation was performed before the quantification. One hypothesis was that the centrifugation step involves the loss of the liposome cargo. If the centrifugation speed is too rapid, liposomes can blow up due to centripetal force resulting in the release of the encapsulated material. This indeed would explain the reason why no release trend was observed. Hence, the speed and the time of the centrifugation step were changed from 8000 g to 2000 g and from 4 minutes to 10 minutes. Unfortunately, the results were not any more encouraging. The second parameter that could be changed in the release method was the temperature of incubation. An additional experiment was run with an incubation temperature of 45°C without any success. Finally, one last investigation was to make a close analysis of the method of formulation of Ren et al.

4.4 Liposome formulation optimization

The method of preparation of Ren et al. is depicted in the figure below. The first step consists of dispersion of the lipids and cholesterol into an organic solvent, in this case chloroform. The second step eliminates the chloroform using a rotary evaporator and proceeding to the third step where PBS is added on top of the thin film. During the hydration step, the temperature has to be higher than the phase transition temperature in order to allow the formation of vesicles. The formulation has to be left in a water bath during 30 minutes with intermittent vortexing. The fourth step is the sonication

step (also 30 minutes) in order to homogenize the population of vesicles and reduce their size. This step has to be at a temperature higher then Tc. Finally when the formulation reaches room temperature, dissolved HRP can be added to the suspension. The whole is frozen and dried and it is during the rehydration step that the HRP is encapsulated into the liposomes.

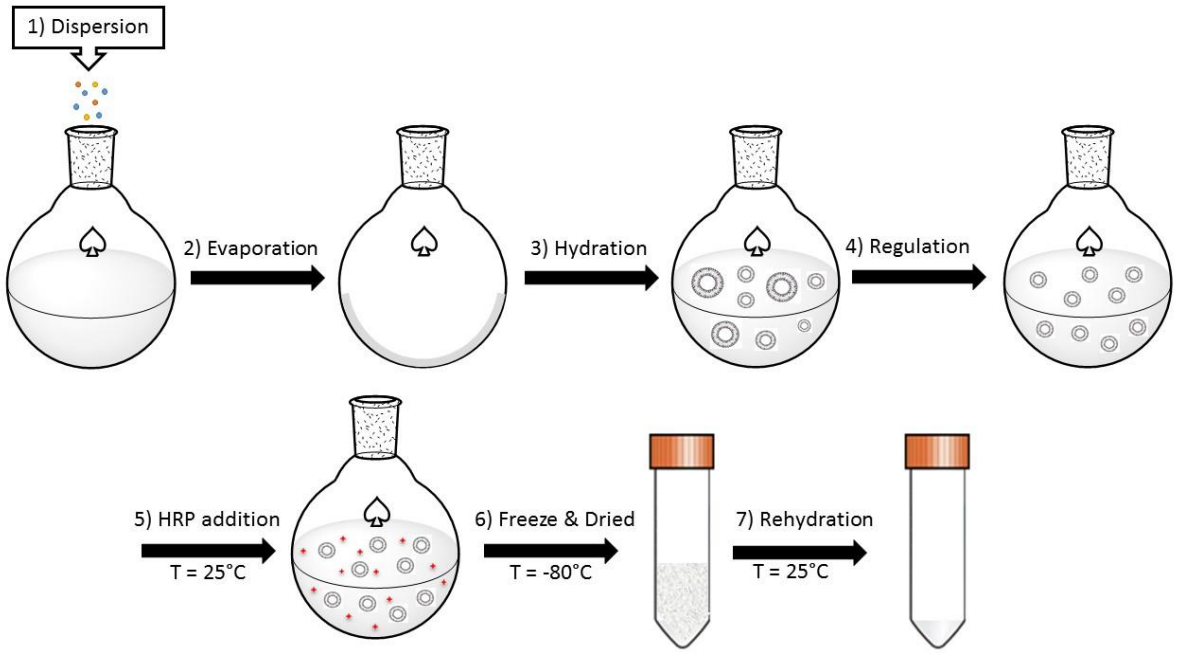
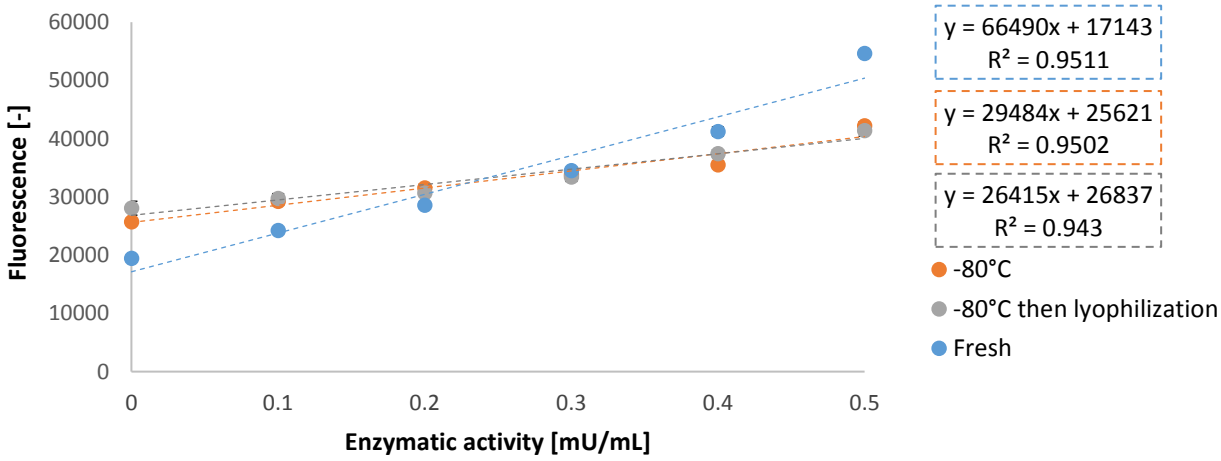


Figure 14 : Ren et al. method for the formulation of liposome.

The sensitive portion of this method is in the last step. HRP is added into the protocol in the fifth step and the encapsulation happens during rehydration. The first thing that was checked was whether HRP was denatured or not during the freeze and dry steps. It is well known that HRP is a very sensitive enzyme and can be easily deactivated. Two samples of a common solution were put at -80°C and one of them was freeze-dried. The enzymatic activity was measured the day of the preparation of the solution and after being at -80°C and freeze-dried. The figure below shows the result of this experiment.



Graph 3: Measurement of the enzymatic activity of HRP the day of preparation (Fresh) and the next day after being one night at -80°C and freeze-dried.

The enzymatic activity is related to the slope of the trend curves. The fresh preparation gives an enzymatic activity of 100% and has a slope of 66490 mL/mU. The enzymatic activity of the freeze (1) and freeze-dried (2) sample can be calculated as follow:

$$(1)\ \frac{29484}{66490} \cdot 100 = 44.3\%$$

$$(2)\ \frac{26415}{66490} \cdot 100 = 39.7\%$$

The activity of the enzyme at -80°C lost 56% of its activity whereas for the freeze-dried process it lost 60%. In conclusion, it seems to be the freeze step of process that deactivated the enzyme explaining the reason why the results are not relevant. Hence the Ren et al. method was changed as follows:

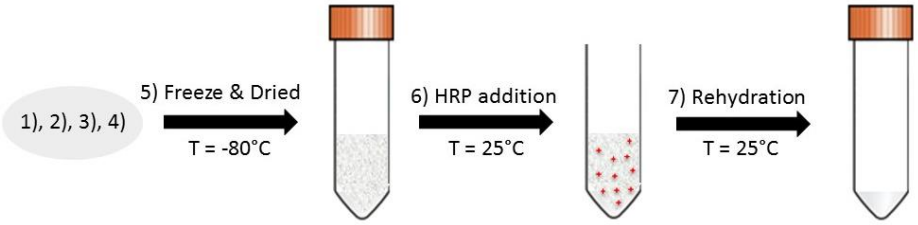
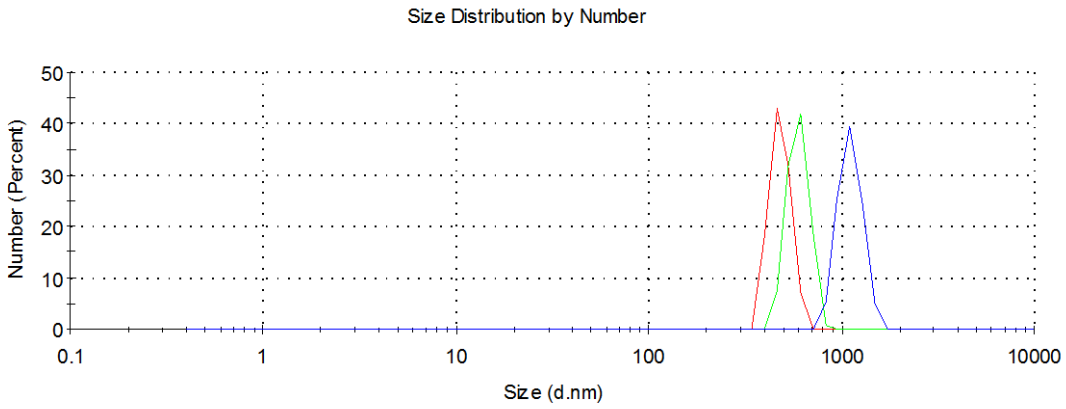


Figure 15: Second method for the formulation of liposome (Method 2 in Section 2.3)

Instead of adding HRP before the freeze-dried process, it was added at the end in its solid form. Dried liposome as well as HRP were mixed manually with a spatula. Then after the normal rehydration step another release experiment was performed but this time instead of keeping the samples at room temperature and 37°C, it was kept at 4°C and 37°C. This time a release was observed.

4.5 Second results

As usual when a release was observed a DLS measurement was performed.



Graph 4: Here the size distribution of the formulation was prepared with the method 2 in Section 3.1. This batch has the following composition: 80.9 mg DPPC, 25.2 mg DMPC, 29.4 mg cholesterol in 10 mL PBS, giving a lipid concentration of 10.5 mg/ml.

The average size of this batch of liposome (mean size calculated in function of each percentage) is $1115 \pm 157\ nm$ that is to say MLV vesicles were obtain once again. In comparison to the first release the liposomes are bigger and the solution is also not very homogeneous (3 different peaks).

The figure below depicts the liposome of this particular batch. Indeed the size of the liposomes are not homogeneous at all. Compared to the first batch prepared with Method 1 in Section 3.2 it seems that they are better dispersed.

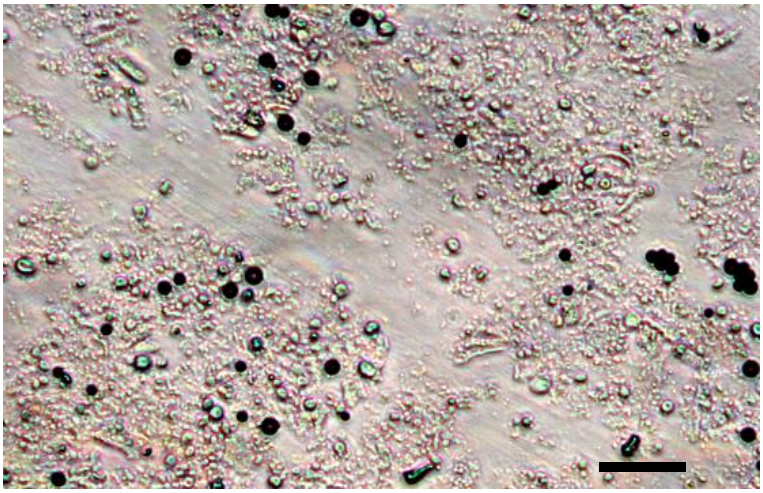
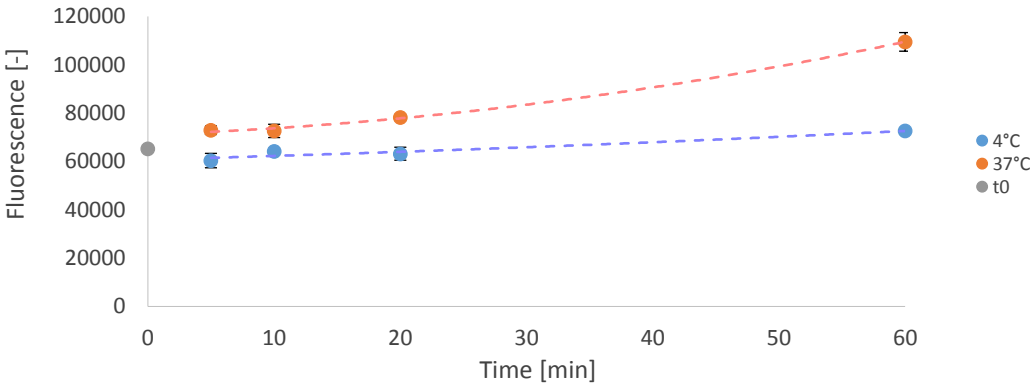


Figure 16 : Liposomes formulated with Method 2 in section 3.2. The scale bar represent 100 µm.

In fact, adding HRP after the freeze-dried process gives larger liposome with high size distribution. This might come from the manual mixing which would induce a destabilization of the preformed liposomes.

The release trend that was observed is showed in the graph below.



Graph 5: Measurement of HRP release (method explain in Section 3.4). Instead of keeping the samples at 25°C and 37°C it was kept at 4°C and 37°C. The fluorescence of HRP in the supernatant (express in the y-axis) was measured at different time step: 0, 5, 20 and 60 minutes, where t0 is HRP activity at time zero.

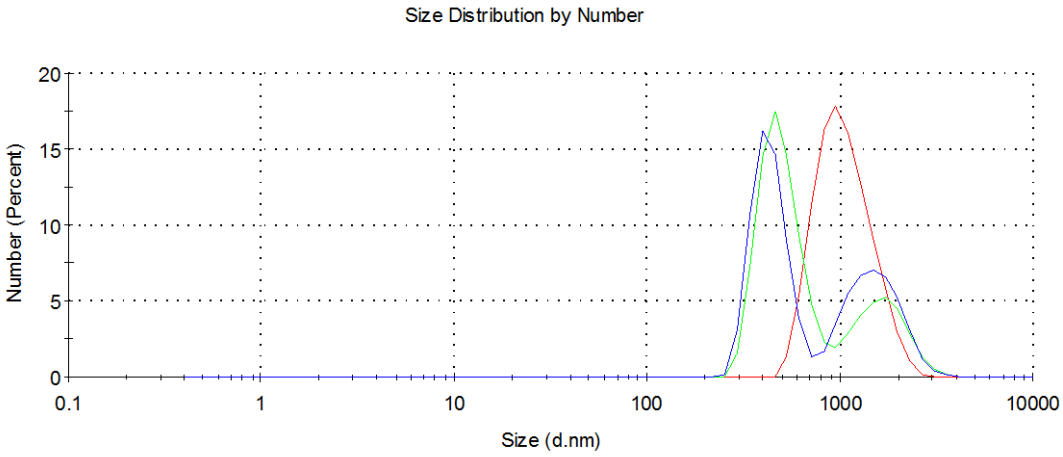
This result is quite surprising. In comparison to Ren et al. release measurement where most of the HRP was out the first 10 minutes, here it is after 60 minutes that the enzyme is out.

Once again, this experiment was performed again without any success.

4.6 Radu et al. encapsulation

In order to understand what could be wrong in the formulation of the liposome, Prof. Zumbühl from the University of Fribourg, was contacted in order to have some practical and technical advices. Luckily, his scientific team has elaborated liposomes that burst at exactly 37°C. Together with one of his assistant (Tanasescu R.), two batches of liposome were formulated using the artificial lipids and the binary mixture DPPC/DMPC. The method of preparation (Method 3, Section 3.2) was completely different from RDV's method. Because Freeze-thaw cycles was used to hydrate the thin lipid it was not possible to encapsulate the enzyme. HRP would be completely deactivated. Carboxyfluoroscein was thus encapsulated. The release was successful with both liposome preparations.

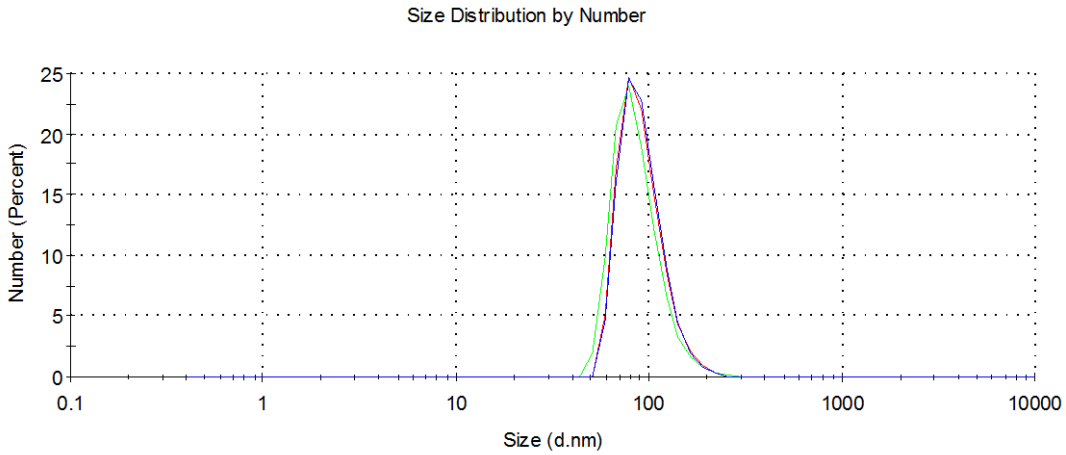
The Graph 6 represent the size distribution of the liposome formulation with Pad-PC-Pad lipids.



Graph 6: DLS of the liposomes formulate with Method 3 in Section 3.2 with Pad-PC-Pad lipids.

The average size of this batch cannot be calculate accurately. There is two peaks, one around 400 nm and another one around 1 micron. As the experiment was performed in the University of Fribourg, the DLS measurement was done only the day after the formulation of liposome. In consequence the liposomes probably form aggregate explaining the result of this DLS measurement.

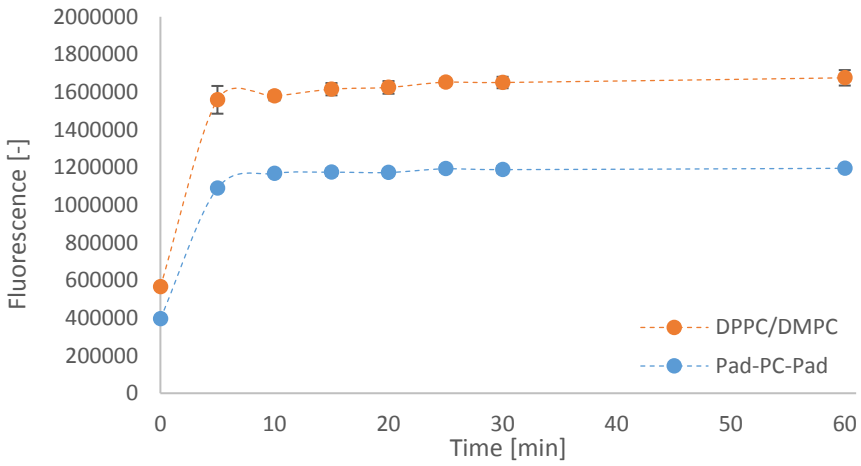
This was not the case for the liposome composed of DPPC and DMPC. Graph 7 depicts, three measurement that are well transpose on each other.



Graph 7: DLS of the liposomes formulate with Method 3 in Section 3.2 with a binary mixture of DPPC and DMPC with 9% mol. cholesterol.

The average size of this batch of liposomes is $92 \pm 25\ nm$ giving a population of Large Unimolecular Vesicles (LUV). Hence the extrusion process is better than sonication to reduce the size of the vesicles and give a homogeneous suspension.

Two, almost, similar release trend were observed. In the graph below is depicted the release trend of carboxyfluoroscein through the lipid bilayer composed of DPPC, DMPC and 9% in mole cholesterol (orange trend) and through a membrane composed of the artificial phospholipids, named Pad-PC-Pad (blue trend).

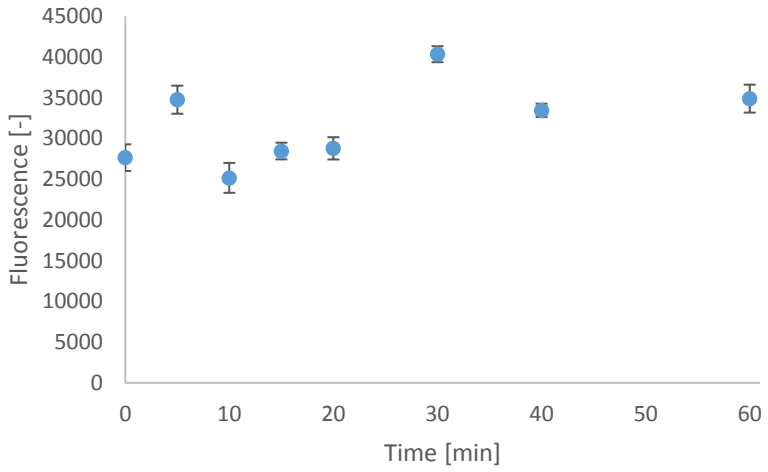


Graph 8: The release trends of carboxyfluoroscein, through a lipid bilayer made of Pad-PC-Pad phospholipids (in orange) and through a bilayer made of a mixture of DPPC, DMPC and cholesterol bilayer (orange).

Both trends tends to release their cargo the first five minute. It seems that the liposome formulation with the binary mixture of DPPC and DMPC can encapsulate a higher amount of carboxyfluoroscein then the liposome formulate with the artificial lipids.

4.7 Last experiment

As the release of carboxyfluoroscein was a success a last experiment was tried. HRP was encapsulated using Tanasescu method and the freeze-thaw step was replace by a mild hydration of the thin film within a water bath of 45°C for 24 hours (Method 4, Section 3.2).



Graph 9: Measurement of the activity of HRP (express in fluorescence) at different time step: 0, 5, 10, 15, 20, 30 and 60 min. Method 4 in Section 3.2 was used to formulate this batch of liposome.

Once again the results are unsatisfactory. The result give no relevant trend.

4.8 Summery

During this master project, it was unfortunately not possible to reproduce the Ren et al. experiment and the "trouble-shooting" experiments took much time and planning. Despite that the approach was well thought using the novel DRVs method to encapsulate a sensitive enzyme, several steps would still have to be improved in order to have reliable and reproducible results.

Below are a few suggestions that were elaborated:

First of all, as performed in Tanasescu's method, after evaporation of the organic solvent with the rotary evaporator, the thin lipid film has to be put under vacuum overnight in order to get rid of any possible traces of solvent. Indeed, remaining solvent during the hydration steps can inhibit the formation of vesicles or deteriorate the formulation equilibrium. It was observed that evaporation of the solvent with rotary evaporator was never reproducible from one batch to another within the same conditions and traces of solvent were almost always perceived before hydration.

Second, as mentioned in the "Liposome's formulation" chapter, the use of a probe tip sonicator is not advised. One the one hand, the solution can be polluted with probable metallic residue and on the other hand the encapsulation efficiency is pretty low. To ensure at least no metallic contamination, the Ren et al. method has to introduce a centrifugation step before the addition of the enzyme. Another point that was observed during the sonication process was that temperature control of the formulation was complicated if not impossible. Due to the high ultrasonic frequency, part of this energy was converted into heat resulting on a temperature increase within the 5 mL batch. Thus, unavoidable evaporation of part of the water, causing non-negligible volume reduction, was occurring. Consequently, most of the time additional PBS was added during the process, implying thermodynamic equilibrium loss. In sum, the probe tip sonicator step is arbitrary and does not ensure reproducible results. A solution to this problem would be to replace the sonication step by an extrusion step. Indeed, during extrusion temperature can be well monitored as well as the size of the particles. It gives a more homogeneous formulation and is more reproducible.

Finally, the last suggestion is to completely change the method of formulation of liposome and take Tanasescu's method. The results demonstrated that encapsulation of carbofluoresceine was a

success and a release occurred at 37°C. This means that the composition of the liposomes as well as the 9 % mol. of cholesterol gives effective liposomes.

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PERSPECTIVE

All along this project, there have been unexpected events as well as pleasant surprises: from many liposome formulation defeats to success by working with grand artificial phospholipids. If the aim of this project was to develop thermoresponsive liposome with a release target temperature of 37°C, then the goal would have been achieved. Two types of thermoresponsive liposomes were developed within this study, the one of Ren et al. and the alternative Tanasescu et al. method. For both of them, the carboxyfluoroscein's release occurs at exactly 37°C. Nonetheless, HRP encapsulation and monitoring the release rate were key factors for the development of an injectable HA-Tyr hydrogel. Therefore, in order to complete this mission, there are still remaining research experiments to be accomplished.

Since two different kinds of liposome were developed, here are already two options to continue this study.

If the desire would be to further develop the encapsulation of HRP within liposome made of DPPC and DMPC, then here are several steps that could be done. First, for the reasons mentioned in the 5.6 Section the preparation method should be revised in order to have a reproducible and stable formulation. Reproducible formulations are key factors for the control of the release rate, especially when it is intended to launch a crosslinking reaction. Multi-lamellar vesicles are hardly stable and to reproduce the encapsulation efficiency of MLV is quasi impossible. Hence, the Method 4 in the "Materials and Methods" chapter should be used. Indeed, this method enables the formation of LUV with low size distribution. The stability of the liposome would, thus be improved compared to MVL. Furthermore, this method uses milder conditions ($T \leq 45°C$), monitors well the temperature and gives opportunity to work with lower volume (0.5 to 1 mL). Having the opportunity to work with lower volume enables one to increase the amount of lipids in a batch. Thus, investigation of encapsulation efficiency with regard to the amount of lipids could be achieved. As the amount of lipids is anyway lower than for a 10 mL batch, more experiments could be done for a same overall cost. It is obvious that before anything, it should be examined that HRP can withstand the shear stress implied by the extrusion step. This can easily be performed by quantifying the activity of HRP before and after extrusion. If the activity remains the same then HRP does not lose any of its activity during the process. If it happens that shear stress denatures the enzyme, hence another method to convert MLV into LUV should be found. Finally, one last point that should be mentioned here, is before starting all the process to record a potential release trend, it should first be proved that HRP is well encapsulated. It is well known that detergent such as Triton X-100 or SDS can solubilize lipid membranes. However, these surfactants are so strong that they might denature the enzyme, resulting on an enzymatic activity loss. A suggestion given by Tanasescu's colleague was to burst liposome by inducing an osmotic gradient between the inner and outer side of the vesicles. Indeed PBS has an osmolarity of 214 mOs, whereas ultrapure water has an osmolarity of zero. The liposome formulation should be done using PBS as usual and in order to check whether HRP has been encapsulated or not, a dilution should be done using on one side PBS and on the other side ultrapure water. A quantification of both solutions will answer the question whether HRP was encapsulated or not.

On the second hand, if the desire would be to further work with artificial phospholipids (1,3-diaminophospholipid, named Pad-PC-Pad) developed by Prof. Zumbühl and his team, then this is another issue. Indeed, these artificial phospholipids seem very promising, giving a novel kind of liposome being thermo- as well as mechano-sensitive. This feature has great potential for the development of any kind of inducible system. Further studies should be performed in order to

encapsulate HRP within their artificial membranes, not forgetting that a collaboration/partnership is necessary as their phospholipids are not on the market yet.

The last point to highlight to assure the project continuation is to perhaps change the HRP quantification technique. Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit is a very sensitive method to detect peroxide. Nonetheless, it might be too sensitive. A high dilution is required (in the order of 10^6-folds) which may create additional potential errors as well as very small impurities which could in turn alter the signal.

In conclusion, to develop a thermoresponsive system for HRP delivery aiming to trigger and control HA-Tyr crosslinking reaction, further research is indeed of merit. The overall goal of this project was quite challenging. However, to improve joint healing by finding a way to formulate an injectable thermolabile hydrogel would be a major step forward.

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ANNEX

A: Supplementary figures

❖ *A1 - Properties of mainly used phospholipids*[22]

Lipids	Abbreviation	Carbons : Saturations	Charge at pH 7.4	Tc (°C)
Neutral natural lipids				
Egg phosphatidylcholine	EPC		0	-15 to -7
Soy phosphatidylcholine	SPC		0	-15 to -7
Neutral synthetic lipids				
Dilauryloyl pholsphatidylcholine	DLPC	12 :0	0	-1
Dimyristoyl pholsphatidylcholine	DMPC	14 :0	0	23
Dipalmitoyl pholsphatidylcholine	DPPC	16 :0	0	41
Distearoyl pholsphatidylcholine	DSPC	18 :0	0	55
Dioleoyl pholsphatidylcholine	DOPC	18 :1	0	-20
Dimyristoyl pholsphatidylethanolamine	DMPE	14 :0	0	50
Dipalmitoyl pholsphatidylethanolamine	DPPE	16 :0	0	63
Distearoyl pholsphatidylethanolamine	DSPE	18 :0	0	74
Dioleoyl pholsphatidylethanolamine	DOPE	18 :1	0	-16
Negatively charged synthetic lipids				
Dilauryloyl phosphatidylglycerol	DLPG	12 :0	-1	4
Dimyristoyl phosphatidylglycerol	DMPG	14 :0	-1	23
Dipalmitoyl phosphatidylglycerol	DPPG	16 :0	-1	41
Distearoyl phosphatidylglycerol	DSPG	18 :0	-1	55
Dioleoyl phosphatidylglycerol	DOPG	18 :1	-1	-18
Dimyristoyl phosphatidic acid, pH 6	DMPA	14 :0	-1	51
Dimyristoyl phosphatidic acid, pH 9	DMPA	14 :0	-2	45
Dipalmitoyl phosphatidic acid, pH 6	DPPA	16 :0	-1	67
Dipalmitoyl phosphatidic acid, pH 9	DPPA	16 :0	-2	58
Dipalmitoyl phosphatidylserine	DPPS	16 :0	-1	51
Positively charged synthetic lipids				
Stearylamine	SA	18 :0	+1	-
di-Oleoylosytrimethylamoniopropane	DOTAP	18 :1	+1	-

Table 1: Main phospholipids used for the formulation of liposomes.

❖ *A2 - Newman conformations of butane*[31]

Rotation around the central C2-C3 bond in butane involves conformers of different energies:

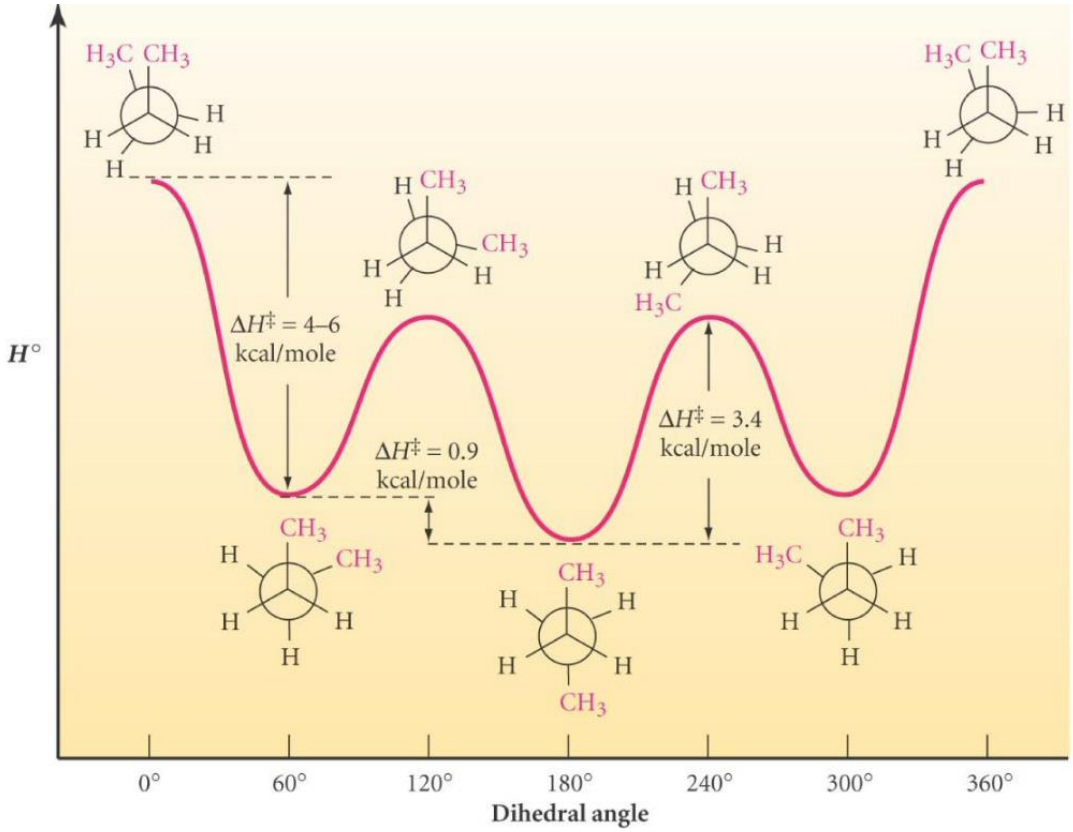

Figure 17: Newman projection of butane
Conformation from left to right: eclipsed, gauche, eclipsed, anti, eclipsed, gauche, eclipsed

The most stable isomer is the anti-conformation (dihedral angel of 180°). As the methyl groups are on both sides of the C2-C3 bond the steric repulsion are the smallest. Hence, the anti-conformation is referred as the zero energy all conformers. Eclipsed isomers (with the highest energy), have greater steric repulsion than gauche isomer therefore the eclipsed isomers are higher in energy then the gauche conformations.