Universidade de Lisboa

Faculdade de Farmácia

Development and characterization of mannose-targeted liposomes for antitumoral drugs delivery

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Mestrado Integrado em Ciências Farmacêuticas

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Resumo

Segundo a Organização Mundial de Saúde, em 2018 morreram perto de 9,6 milhões de pessoas em todo o mundo devido à muitas vezes assumida como "a doença do século": o cancro.

Apesar do contínuo esforço da comunidade científica para o conhecimento das causas e mecanismo de desenvolvimento dos tumores malignos, existem ainda muitas perguntas por responder, o que torna difícil encontrar a terapêutica ideal. As quimioterapias padrão pecam pela sua estreita janela terapêutica e por não serem seletivas para as células cancerígenas, fazendo com que estes medicamentos tenham efeitos secundários sistémicos apenas toleráveis pelos doentes quando são administradas em doses baixas, o que diminui a eficácia do tratamento e muitas vezes força a interrupção prematura do mesmo. Por este motivo, a investigação na área do cancro aponta cada vez mais para o desenvolvimento de sistemas de veiculação de fármacos que favoreçam a interação com as células cancerígenas, aumentando a sua captação por parte das mesmas e diminuindo o seu efeito em células saudáveis. Estas terapêuticas avançadas permitirão assim, uma diminuição dos efeitos secundários e da dose que é necessário administrar para que haja o efeito terapêutico desejado, não comprometendo a eficácia do tratamento.

A Università degli Studi di Firenze idealizou um projeto com a pretensão de desenvolver um veículo para um fármaco anti-cancerígeno comum (não seletivo), como a doxorrubicina, e funcionalizado com vista a um efeito terapêutico restrito à célula ou tecido alvo. Para isso, o projeto incluía duas vertentes: i) *design*, síntese e caracterização das nanovesículas- lipossomas e niossomas; ii) *design*, síntese e caracterização de recetores para manosídeos biomiméticos modificados. Estes recetores de manose modificados são utilizados uma vez que as células cancerígenas sobre expressam este açúcar, tornando-se este o fator diferenciador em relação às células saudáveis.

Esta abordagem teórica visa estudar algum do imenso trabalho que tem sido desenvolvido na área dos lipossomas, os seus métodos de produção e caracterização, aplicações e particularmente o seu funcionamento como veículo de fármacos citotóxicos. Por fim, faremos uma projeção do desenvolvimento do projeto da Univesità "*Development and characterization of functionalized liposomes for mannose-targeted antitumoral drugs delivery*".

Palavras-chave: Lipossomas, nanovesículas, veiculação de fármacos, doxorrubicina, cancro

Abstract

According to World Health Organization, nearly 9.6 million people died worldwide in 2018 due to what is often assumed to be “the disease of the century”: cancer.

Despite the continuous effort of the scientific community to understand the causes and mechanisms of development of malignant tumors, there are still many open questions, which makes it difficult to find the ideal therapy. Standard anti-cancer chemotherapies have a very narrow therapeutic window and are not selective for cancer cells. This leads patients to experience intense systemic side effects, such as nausea and hair loss, and therefore they only tolerate low doses of drugs, decreasing the effectiveness of the treatment and many times leading to its interruption. For this reason, cancer research increasingly points to the development of Drug Delivery Systems that offer preferential interaction of the drug with cancer cells, thus increasing their uptake in these cells and decreasing their effect on healthy cells. The consequences are the reduction of the side effects and in the dose that needs to be administered without compromising the treatment efficacy.

Università degli Studi di Firenze thought of a project with the aim of developing a DDS loaded with a common (non-selective) anti-cancer drug like doxorubicin and functionalized to selectively target tumor cells. This project included two major tasks: i) design, synthesis, and characterization of nanovesicles- liposomes and niosomes; ii) design, synthesis, and characterization of modified biomimetic receptors for mannosides. These modified mannose receptors enables the selective targeting of cancer cells overexpressing mannose with this being the distinction in relation to healthy cells.

This thesis covers the recent developments in the liposomes field, namely by addressing the methodologies used for their production and characterization, and major applications, particularly as anti-tumoral vehicles. Lastly, we will provide an overview of the major goals established for the Università’s project “Development and characterization of functionalized liposomes for mannose-targeted antitumoral drugs delivery”.

Key-words: Liposomes, nanovesicles, drug delivery, doxorubicin, cancer

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Às minhas orientadoras, Doutora Marzia Cirri e Doutora Helena Florindo.

Aos meus professores do agora e do antes, da escola e da vida.

Aos meus amigos.

À minha família.

Para a Ana do “Buçado”, para o Avô Alexandre,

e para todos os que travam esta luta contra cancro.

List of Abbreviations

API	Active Pharmaceutical Ingredient
DDS	Drug Delivery System
EPR	Enhanced Permeability and Retention Effect
CHL	Cholesterol
PEG	Polyethylene Glycol
MPS	Mononuclear Phagocytic System
DR	Drug Release
DOPE	1, 2-dioleoyl-glycero-3-phosphoethanolamine
CHEMS	Cholesteryl Hemisuccinate
ISO	International Organization for Standardization
PDI	Polydispersity Index
ULV	Unilamellar Vesicles
SUV	Short Unilamellar Vesicles
LUV	Large Unilamellar Vesicles
MLV	Multi Lamellar Vesicles
OLV	Oligo Lamellar Vesicles
GUV	Giant Unilamellar Vesicles
DD	Drug Delivery
ZP	Zeta Potential
DOTAP	1, 2-dioleoyl-3-trimethylammonium
DSPG	1, 2-distearoyl- sn-glycero-3-[phospho-rac-(1-glycerol)]
TME	Tumor Microenvironment
T_c	Transition temperature
EE	Entrapment Efficiency

HPLC	High Resolution Liquid Chromatography
FFF	Field- Flow Fractionation
CMC	Critical Micelles Concentration
$scCO_2$/W	Super Critical CO_2/Water
PBS	Phosfate Buffered Saline
ICH	International Conference on Harmonization
FDA	U.S. Food and Drug Administration
W/O/W	Water/Oil/Water emulsion
cryo-TEM	Cryo-Transmission Eletronic Microscopy
DLS	Dynamic Light Scattering
ELS	Electrophoretic Light Scattering
AFM	Atomic Force Microscopy
DNA	Desoxyribonucleic Acid
ROS	Reactive Oxygen Species
UniFi	Università degli Studi di Firenze
RNA	Ribonucleic Acid
TLE	Thin Layer Evaporation
SALT	Solvent-assisted Active Loading Technology
CBA	Carbohydrate Binding Agent
MBL	Mannose Binding Lectin
MPEG-DSPE	N-(carbonyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine
PPE	Palmar Plantar Erythrodysthesia
ABC	Accelerated Blood Clearance

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Introduction

1.1 Liposomes and its composition

Liposomes are biodegradable and biocompatible enclosed, colloidal, phospholipidic spherical vesicles formed by one or multiple lipid bilayers, spontaneously produced when these lipids are dispersed in an aqueous medium and a certain energy is provided (1–4). Liposomes were first synthetically made in England in 1961 by Bangham *et al* (1,2). These carriers have structural features that allow the incorporation of drugs into their inner compartment- aqueous core- or dispersed within the lipidic bilayer, strongly increasing the stability of entrapped active pharmaceutical ingredients (API) against enzymatic degradation and chemical or immunological inactivation which constitutes one of the major advantages of this Drug Delivery System (DDS) (1). The core and the bilayer, can respectively transport hydrophilic or hydrophobic drugs and have large surface to volume ratio, which enhances drug loading, thereby increasing their therapeutic index (1–3). Moreover, these vesicles are particularly interesting for hydrophobic drugs that cannot solubilize in blood, which bioavailability is increased while delivered within the liposomal lipid bilayer (2).

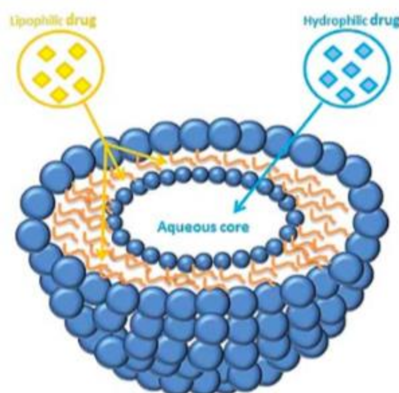

Figure 1. Schematical representation of a liposome. Adapted from (1).

Furthermore, liposome surface modification allow drug controlled release, including in response to biological alterations (1). Liposomes also have the ability to passively accumulate in cancer cells because of the enhanced permeability and retention effect (EPR)- a consequence of the nanometer size of the vesicles and the large vascularization of tumoral and inflamed tissues (5,6).

Liposome composition includes cholesterol (CHL) and lipids that vary from natural to synthetic (1,2). Nevertheless their main component is usually soy or egg extracted phospholipids, solid lipids relatively biocompatible, biodegradable and non-immunogenic, making them a very versatile and safe DDS (1).

Phospholipids, the major component of liposomes, are amphiphilic *triglyceride-like* molecules that have a polar head with a phosphate-containing group, and two fatty acid's hydrophobic tails (4,7). The factor that drives the phospholipid arrangement in aqueous solution is the tails turning to each other through hydrophobic interactions and Van der Walls forces, forming the bilayer in order to minimize unfavorable interactions between tails acyl-chains and the surrounding aqueous medium. Also, the polar heads using hydrogen bridges and polar interactions, turning both to that same aqueous medium, form the hydrophilic core of the particle (1,7). Most of the phospholipids have a cylindrical shape, with the polar head occupying a molecular area similar to the chains, which results in their arrangement in bilayer instead of micelles, usually made of cone shaped lipids or detergents (6). The most used phospholipids are the phosphatidylcholine (or lecithin), phosphatidylethanolamine and phosphatidylserine, being the first two, the main components of biological membranes that explains the low chronic toxicity of this DDS (1).

CHL diminishes permeability of hydrophilic molecules through the liposome but enhances vesicles stability and lowers the leakage of the particles (1,4,8). This happens because CHL impedes crystallization of the lipid's acyl chains and creates a steric hindrance to their movement making the molecule more rigid (4).

Adding non-physiological components to liposomes may boost their efficiency. For example, polyethylene glycol (PEG) can be used as a stabilizer molecule that improves liposomes' pharmacokinetics and allows them to stay in the blood stream for longer periods (6,9). PEG creates an aqueous extra layer around the liposome that shields liposomes surface charge, preventing their interaction and recognition by cells and therefore their opsonization by mononuclear phagocytic system (MPS) cells (6,9,10). Consequently, vesicles will have more time to reach their target and exert their pharmacological effect (9,10). These are called long-circulating liposomes, one of the many existing types of bilayer vesicles (11).

Stimuli-responsive liposomes enables a controlled drug release (DR) that is triggered by changes in pH (*e.g.* tumors acidic environments in relation to normal tissue), redox potential,

hyperthermia, ultrasound etc. (6,8). Oleic acid, 1, 2-dioleoyl-glycero-3-phosphoethanolamine (DOPE), cholesteryl hemi succinate (CHEMS), phosphatidyl ethanolamine and derivatives are the most used compounds to develop pH-sensitive liposomes because they can trigger structural alterations in the liposomes' membrane by being (de)protonated under certain pH levels (6,12). As an example, despite its' conical shape, we can stabilize a DOPE bilayer with CHEMS' negative charge that repulses the DOPE's polar group making them big enough for the complex shape to become cylindrical and create a lamellar (liposome) phase. When in an acidic environment, CHEMS is protonated and loses his anionic character becoming unable to stabilize the bilayer which is disarranged and thereby releases the loaded drug (6).

1.2 Liposomes' properties

1.2.1 Size, polydispersity index and lamellarity

A nanomaterial is defined as a material in which 50% or more of the constituent particles (by number) have one or more external dimensions in the size range 1-100 nm or the volume-specific surface area is larger than 60 m^2 cm^{-3}. However, International Organization for Standardization (ISO) also includes larger materials if they are made of internal or surface structures in the nanoscale spectra (13). Accordingly, as most of the legislative frame concerning medicines, cosmetics and food products does not explicitly cover nanomaterials, most of the time liposomes are considered nanoparticles even knowing that their size ranges from 50 to approximately 400 nm (7,14).

The average size and size distribution- measured by the polydispersity index (PDI)- are crucial parameters, especially for DDS intended to be administered by parenteral route. Accordingly, a PDI lower than 0.3 indicates an homogenous population for colloidal systems; a particle size under 100 nm enables an extensive accumulation at tumor site, while liposomes with an average size close to 250 nm will be more recognized by cells from the MPS (1).

Liposome size usually is proportional to the drug loading and also the amount of CHL present in the formulation (3,12,15).

Regarding liposome classification based on the number of bilayers -lamellarity- there are Unilamellar Vesicles (ULV's) that usually have mean size diameters around 250 nm, one

bilayer and a large core (2). These ULV's can be divided into Small Unilamellar Vesicles (SUV's) and Large Unilamellar Vesicles (LUV's) (2,12). SUV have 20 nm to 100 nm, and therefore are not removed from the blood stream, thus having greater chances of entering tissues and having high therapeutic indexes (1,2). The LUV present mean diameters between 100 nm and 250 nm (1). Multilamellar Vesicles (MLV's) have an *onion arrangement* with two or more bilayers, diameters higher than 500 nm, and are more stable and easier to produce. In this last type of liposomes, although the EE is low, the drug is slower released because it has to cross additional lipid layers, permitting DR modifications (2). Less commonly used are the Oligolamellar vesicles (OLV) (100 to 500 nm) and the Giant Unilamellar Vesicles (GUV) which size is usually higher than 1000 nm (1). Different levels of lamellarity can be achieved by different preparation procedures or lipids used in the formulation. Concluding, the size and number of bilayers influence the drug EE, drug delivery (DD) and circulation half-life of liposomes because it changes the surface-to-volume ratio, stability and release kinetics (3,5).

1.2.2 Zeta Potential and Charge

Particle Zeta Potential (ZP) is the global charge of this carrier in a certain medium (1). It is determined by dispersing the particles in a buffer and measuring their mobility, once in an electric field. The mobility is then converted in ZP using mathematical models (15).

This is a very useful physical property that helps predicting the stability of colloidal particles: values higher than +30 mV or lower than -30 mV indicates that there is stability, because in this conditions particles will tend to repel each other enough not to aggregate, while also avoiding phase separation (1,4).

The phospholipidic fraction of liposomes, particularly the saturated fatty acids chains of phosphatidylcholine, rises the melting point of these vesicles, thus improving their overall stability once those temperatures are more difficult to achieve. Contrarily, phospholipids with unsaturated chains improves vesicles' permeability (2). The natural phospholipids' heads are

neutral and provide ZP of -10mV to +10 mV that can be altered by adding cationic or anionic lipids (16).

Positively-charged liposomes contain in their composition cationic lipids- DOPE, 1, 2-dioleoyl-3-trimethylammonium (DOTAP) or others- or involve the modification of lipids by a cationic functionality (like ammonium or phosphonium ions), which turns the surface of the vesicle positively charged (12,16,17). These positively charged polymers can increase the loading of negatively charged molecules like nucleic acids and endothelial angiogenic tumor cells (12,17).

On the contrary, negatively-charged liposomes are formed following the addition of anionic lipids- 1, 2-distearoyl- sn-glycero-3-[phospho-rac-(1-glycerol)] (DSPG)- to the formulation, or adding an acidic group (carboxylic acid, phosphoric acid) to lipid components (16,17). The anionic liposomes can load and preferably interact with positively charged molecules and so, they are carried through the blood without significant interaction with the negatively-charged blood components (16).

There is also zwitterionic liposomes with both acidic and alkaline functions. These are negatively-charged under physiological pH conditions and positively-charged in an acidic medium. Just like anionic liposomes, zwitterions are easily transported through the blood stream without interacting with the blood components. However, major interaction with the membranes will occur when in the acidic tumor micro environment (TME), favoring DR (16) .

1.2.3 Phase Transition Temperature (T_c)

The temperature at which the liposome formation occurs, is based on the T_c of the constituent lipids, which is the temperature below the melting point, at which the permeability of a phospholipidic layer increases because of a disarrangement in the previous ordered bilayer (4). The experiment has to occur at a temperature superior to the T_c in order to achieve the thermodynamic equilibrium that allows lipids to arrange in spherical bilayers (18). This temperature depletes with the decrease of the acyl chains size, with its unsaturation , branching and bulky polar groups (4). All of these characteristics can be optimized to produce liposomes

with certain technical features: for example, a semi-rigid and less leaky liposome is achieved when adding high-T_c lipids to the formulation (4,12).

1.2.4 Stability

The stability of a product -storage with quality- must encompass the analysis of physical, chemical, and microbial parameters from the manufacture to the storage and delivery in order to avoid unexpected side effects (1,15). Also, as researchers attach different drugs, targeting molecules or modify manufacturing processes and composition of the nanomedicines, their clinical use gets more dependent on an extensive assessment of their properties, making it fundamental to identify and control the critical points during their production in order to ensure security, quality, and smooth the scaling-up process (19).

The average size distribution of liposomes changes upon their storage: these vesicles tend to aggregate into bigger carriers, because it is thermodynamically more favorable and this may lead to drug leakage. Thus, visual appearance and size distribution are important features to assess physical stability (1,12).

Other stability problem comes from the liposomes' phospholipid sources that contain unsaturated fatty acids which undergo oxidative reactions, and which products can cause permeability changes in the vesicle bilayer (12,15). Furthermore, interactions of the drug with the phospholipids also modify liposome chemical stability by altering the bilayer fluidity (1).

Liposome leakage during storage is another matter of concern regarding the stability of these carriers. The drug leakage can have different sources and therefore can be assessed by evaluating distinct liposome features. For example, a fluid membrane vesicle leads to a more extensive DR. Also, the hydrolysis of phosphatidylcholine enhances liposome's permeability and so, drug leakage (12).

Finally, as a parenteral product, we have to be sure that the liposomal suspension is sterilized and endotoxin-free removing the possible microbial contamination (1).

1.2.5 Encapsulation Efficiency, Drug Delivery and Drug Release

Encapsulation Efficiency (EE) is the total amount of drug found in the liposomal suspension versus the total amount used in the formulation process, and it can be determined using the equation (Equation 2) (1):

$$\mathbf{EE\%} = \frac{[\textbf{total drug}] - [\textbf{free drug}]}{[\textbf{total drug}]} \times \mathbf{100} \tag{1}$$

Where total drug is the total amount of drug used in the liposomal formulation ($\boldsymbol{\mu g/mL}$). The free drug is determined following the separation of the liposomes from the medium by dialysis or ultracentrifugation. The amount of drug present in the supernatant can be subsequently quantified by different methodologies, such as spectrophotometry, fluorescence, High Performance Liquid Chromatography (HPLC) or Field Flow Fractionation (FFF) (1,9,15). As an alternative, the total amount of drug encapsulated in the liposomes can be directly determined following the disruption of the lipidic bilayer with ethanol (15).

The release of the drug from the liposomes depends on several factors, including the surface modification of these vesicles with targeting moieties that enforce the delivery of the encapsulated drug to the target site. When arriving into the site of action liposomes can be adsorbed to cell membranes by electrostatic or hydrophobic forces releasing the drug into the extracellular space, which usually occurs for neutral liposomes due to the weak interaction with the cells (2). When loaded with hydrophobic API's- like doxorubicin- liposomes can exchange components with the cells' membrane and fuse, leading to the immediate release of the drug into cell cytoplasm (2,10). By these two mechanisms, the liposomes disruption is performed by lipases, mechanically or following a chemical or physical stimulus, resulting in DR. If the liposomes' hydrodynamic diameter is lower than 150 nm and the drug is acid-resistant, these vesicles can also be endocytosed by MPS, being further degraded in the lysosomes, which results in DR (2).

The lipidic composition itself affects bilayer fluidity and partition coefficient, and thus liposome ability to be permeable to the drug (3). This coefficient is defined as the logarithm of the ratio of the sample concentration in an hydrophobic phase *versus* that same sample's concentration in an hydrophilic phase- Log P. Log P is a measure of the hydrophobicity of a

molecule: higher Log P, indicates that the molecule is more hydrophobic (10). Hamidreza Farzaneh *et al* studied the impact of CHL and phosphatidylcholine in various features of liposomal doxorubicin, and found out that when entrapping API's that are not extremely hydrophilic or hydrophobic, like doxorubicin, it usually benefits from CHL addition once it balances the LogP of the other highly hydrophobic phospholipids- e.g. phosphatidylcholine- and allows doxorubicin to pass through the liposome (10). Also, when using saturated phospholipids, the better option in order to have a high EE, is to use longer hydrophobic chains (10,20). When the chain length is identical, trapping efficiency increases with the incorporation of a double bond into the main chain (20). However, DR diminishes or takes longer to occur when adding CHL because of its resultant higher stabilization, reducing liposome interactions and drug leakage (10).

Besides it's many advantages, some of the liposomes characteristics are major setbacks for their production. Niosomes urge as an equally biodegradable, non-immunogenic and biocompatible alternative, but composed of non-ionic surfactants and CHL. Like liposomes, niosomes encapsulate both hydrophilic and lipophilic drugs, but also overcome some of the liposome limitations (Table 1.) (9,15).

Table 1. Liposomes vs. Niosomes: a comparison. Adapted from (15).

Parameter	**Liposomes**	**Niosomes**
Major components	Phospholipids, CHL	Non-ionic surfactants and CHL
Encapsulated drugs	Hydrophilic and lipophilic	Hydrophilic and lipophilic
Encapsulation Efficiency	Variable	Variable
Stability of the encapsulated drug	Susceptible to hydrolysis, oxidation and leakage	Susceptible to leakage but less susceptible to hydrolysis, and oxidation
Production cost	Higher	Lower
Sterilization	Possible with filtration, γ-irradiation and aseptic processing	Possible with filtration, γ-rays and aseptic processing

1.3 Novel methods for liposomes preparation

In general, the principle behind every liposome method of production relies on hydrophilic/hydrophobic interactions between their components, and on an input of energy that is required to turn the flat and more favorable disposition of lipid bilayers, into a curved particle (18).

Among all possible procedures for nanovesicle manufacture, liposomes area has been constantly offering innovative methods (21). These techniques were not chosen to be used in this project since they all present disadvantages that a University laboratory could not pass through, like the need for specialized equipment, currently not available at the laboratory and high costs of the procedures (15). In other hand, these relatively recent methods are being used to overcome the conventional methods' weaknesses: vesicles with enormous size or broad size distribution, formulation with organic solvents, and the fact that they are not sterile nor automatic methods and so, greater the probability of non-conformities (22). The use of organic solvents is particularly non-desirable once they cause harm on human health and so, it is mandatory to completely remove all the residues, adding another step and costs to the process (1,20). The threats of these solvents include enzyme inhibition, protein denaturation, membrane alterations and extraction of nutrients (18). Besides being directly risky to the human body, organic solvents are as well indirectly harmful once they influence liposomes stability and contribute to environment contamination (20).

1.3.1 Heating method

Besides the overcoming of the organic solvent use, this procedure also goes over a vital issue, especially when a DDS is expected to be administered through intravenous injection: sterility (1). Liposomes can only be sterile if treated by filtration since the classical heating procedure destroys the lipidic packaging and distorts the vesicle structure. However, filtration is often non-sufficient for viruses and similar particles elimination and makes it needed to do an extensive microbiological control, increasing time and costs of the process. The heating method makes it possible to apply a controlled temperature that exempts further sterilization but also does not affect lipids structure (18).

This method consists in using an aqueous medium to hydrate solid lipids and heating them in the presence of glycerol at a temperature above the T_c of the mixture, under agitation. Glycerol is an hydrophilic and bio acceptable molecule that increases the lipid stability by dispersing its components preventing aggregation, without being harmful like organic solvents, and therefore excusing removal (1,4,18).

1.3.2 Microfluidic-based methods

Microfluidic methods are based on the principle of ethanol injection (a conventional method) and it takes advantage of microfluidic channels within an interaction chamber, to project the lipidic and aqueous phase into each other at ultra-high velocity in a laminar trajectory, supplying the energy enough to the mixture to become a liposomal suspension (1,4,9,23).

The advantages of the method are the particles uniformity, smaller size, procedure reproducibility and above all, no use of toxic organic solvents and suitability for scaling-up (9,21). However, the use of extreme pressures can negatively influence the liposomes structure (4).

1.3.3 Detergent dialysis

Operating a detergent dialysis, lipids solubilize in detergent- at their critical micelles concentrations (CMC)- that is subsequently removed by controlled dialysis, in order to progressively form phospholipid richer LUV's (1,2,7). The detergents used, are cationic, anionic or neutral surfactants with high CMC making them easier to be removed (2).

This method has the benefits of producing highly homogenous populations of carriers and being reproductible (2). Yet, the final concentration of liposomes and the EE is very low and detergents remain in the formulation, requiring further treatment-dialysis- to be removed (2,7).

1.3.4 Supercritical fluid - based methods

A supercritical fluid is formed when a gas is submitted to a temperature and pressure beyond its critical values, turning it into a non-condensable fluid (20). Supercritical carbon dioxide ($scCO_2$) was found to be a great substitute for organic solvents in vesicles development, for its similarity regarding solubility but its negligible toxicity and low cost (7,20,22).

One of the methods based on supercritical fluid technology, is the equivalent to reverse phase evaporation technique (a conventional method) but with no consumption of organic solvents. The process involves adding an aqueous solution- may be phosphate buffer saline (PBS)- to the solid components of liposomes and place the mixture into a sealed viewing cell, under stirring. As the CO_2 is introduced in the cell, the temperature and pressure are adjusted to values that allows CO_2 to become a supercritical fluid, forming a $scCO_2$/W emulsion, maintaining the phospholipids in the interface. When lowering the temperature and pressure, CO_2 escapes as a gas, and highly stable ULV's are formed (20).

This method has the advantages of being fast, high EE, produces liposomes with low PDI and high stability (15).

1.3.5 Freeze drying double emulsion method

In this method, cryoprotectants are added to the formulation and the key goal is to form a W/O/W emulsion (22). This purpose is achieved dissolving the lipids in an organic solvent and then dispersing an aqueous phase in the lipidic mixture. Then, agitation provides energy to the mixture creating the primary W/O emulsion. The next step requires emulsification of the previously formed primary emulsion with another aqueous phase (that has the cryoprotectant dissolved in) forming a W/O/W final emulsion that will be set in the rotavapor to eliminate the organic solvent (2).

The final step involves liposomes turning into a lyophilized powder by the process of freeze drying, solving the possible long-term (in)stability and sterility issues (1,7,22). The liposomes prepared by this method have a size around 100-200 nm, are highly effective in encapsulating drugs and very stable (7,22). One major setback is that cryoprotectants are

carbohydrates like sucrose and trehalose, and that makes this kind of liposomes non-suitable to be used by patients with diabetes (15,22).

1.4 Post-production processing for liposomes improvement

According to the aim of the liposomes we construct, after their production we may have to lower their size, uniformize the shape or PDI, or remove organic solvents. In order to do that, some methods were developed and optimized, to be an indispensable part of the production methods (7).

Table 2. Post-production methods for lipossomes improvement.

Method	Method Description	References
Sonication	Probe sonication uses ultrasonic radiation to disrupt MLV's by shearing, preferably with low frequency and so, high amplitude and microjet streaming. The size and position of the probe inside the sample, and the number of cycles also influences the final size of the liposomes. The inconveniency it the possibility of high PDI, phospholipid degradation because of the probe heating, and low EE. Also, the probe tends to release its metal particles into the lipid suspension.	(2,4,5)
High pressure extrusion	Involves vesicles subdivision by passing the suspension through small openings of a polycarbonate membrane, at high pressure. Produces larger vesicles than sonication but is simple, fast and reproductible.	(2,4,7)
Homogenization	A MLV suspension is pumped continuously through an opening and collides with the homogenizer system at high pressure, reducing liposome size.	(7)
Gel filtration, vacuum, dialysis	These generic techniques are used for organic solvent removal.	(18)

1.5 Liposome methods of characterization

According to this analyses, it is possible to conclude that many features have massive impact on the way liposomes act on human body and so, on their quality, safety and efficacy. Furthermore, in this project it is particularly meaningful to characterize the liposomes produced in the laboratory, in order to compare between the different batches generated by the different techniques and so, select the better conditions to fabricate the better carriers. For this reason, it is critical to know which characteristics are imperative to evaluate, and how can we assess them.

1.5.1 Cryo- Transmission Electronic Microscopy (cryo-TEM)

Cryo-TEM allows direct visualization of vitrified particles, obtained by nitrogen freezing (5). This method advantages rely on the fact that it determines the size of the liposomes with high precision and allows individualized visualization of the particles without the need to assume its spherical shape (5,7). Nevertheless, it is costly, time consuming, requires expertise and sample preparation that may generate artefacts and induce shrinkage or shape distortion (1,7). Also, the results of this measurements are statistically weak as they are subjective to the human eye analysis (5).

1.5.2 Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS)

When experiencing Brownian motion, the difference between indexes of refraction of a particle and the vehicle in which it is suspended, deviates a light beam when it is pointed through the suspension and hits the particle (1). DLS, turns time-dependent differences of light scattered from vesicles into size, assuming a spherical shape of the particles and according to Stokes-Einstein equation (Equation 3)(5):

$$D = \frac{k_b T}{6\eta\pi r} \quad (2)$$

Where D is the diffusion coefficient, r the hydrodynamic radius of the particle, k_b the Boltzmann constant, T the absolute temperature and η dynamic viscosity of the suspension (5).

ELS, in other side, is used to measure ZP because the scattered light will have frequency fluctuations proportional to the speed of the particle, when we apply an electric field to the environment (24). These moves are called electrophoretic mobility (μ) and applying a mathematical model (Equation 4), it is possible to calculate the ZP (25).

$$ZP = \frac{\mu\eta}{\varepsilon} \tag{3}$$

Where η is the viscosity of the sample and ε its permittivity (25).

These methods are sensible to a small amount of sample, the device that performs them is easy to find in every laboratory and they require minimum preparation of the sample making them very fast methods (1,7,24). However light scattering methods do not provide shape information, there is a certain bias when there is aggregation or the population is very heterogenous, multiscattering phenomena makes it mandatory to dilute de sample and we have to assume spherical shape of the particles (1,7).

1.5.3 Field-flow Fractionation

FFF uses a semipermeable membrane through which only passes the sample (channel flow) and perpendicularly, an injected liquid (cross flow) that separates liposomes based on their size (1,3). With a centrifuge or electric force, the cross flow stops the laminar flow and projects the larger particles to the channel walls delaying their retention (1,3,7). This procedure is rapid, convenient and non-invasive as it only applies a mild force (1). It works with a wide range of sizes with high resolution, however it is complex and expensive (1,7).

1.5.4 Atomic Force Microscopy (AFM)

AFM is centered on fast scanning by a nanometer sized probe over the fixed sample and makes it possible to visualize small liposomes with high resolution in a three dimensional way offering information on morphology, size, as well as on the possible aggregation processes of liposomes during their storage and so, their stability (1,7).

This process is carried out with no sample manipulation and it is rapid, but has the disadvantage that liposomes in water dispersion maintain their integrity for only few minutes, and so the liposomes images have to be obtained within 10 min after deposition (1).

1.5.5 Dialysis

Dialysis is carried out by placing an aliquot of sample in the dialysis bag (that has no drug adsorption and is freely permeable to the drug), hermetically tied and immersed in a receptor compartment containing a dissolution medium magnetically stirred at a predetermined temperature. Samples of the dialysate are taken at various time intervals and examined for the drug (by HPLC, spectrophotometer or other approved method). The sample volume is always replaced with fresh dissolution medium so that the volume of the receptor compartment remains constant. The experiment stops when concentration drug values maintain constant for successive tries which means that there is no more concentration gradient and the drug concentration can be calculated (26).

1.5.6 Visual monitoring of physical properties

At the laboratory scale, different liposomal suspensions should be kept at 4ºC and at 25ºC for one month and be monitored at certain periods of time (may it be every week, every fifteen days etc.) to assess its physical stability evaluating the visual and size aspects to verify stability and also EE (21).

Table 3. Summary of the methods mostly employed to characterize the physicochemical properties of liposomes.

Characteristic vs. Method	Size and PDI	Lamellarity and Morphology	Zeta Potential	Stability	EE and DD
(cryo-)TEM	X	X			
DLS	X				
ELS			X		
FFF	X				X
AFM	X	X		X	
Dialysis					X
Visual monitoring				X	

1.6 Liposomes applications

Liposomes can be liquid (suspension), solid (dry powder) or semi-solid (gel or cream) pharmaceutical preparations and they can be administered parenterally, orally and topically (27).

When injected intravenously, these vesicles are recognized as foreign and therefore endocytosed by the MPS. Liposomes can serve as an excellent drug-delivery vehicle to the locations in which there is more expression of this phagocytic system cells, like liver and spleen (1,8). However some other drugs that are supposed to exert their effects in other organs, have their distribution reduced by the taken up via MPS (8). There is also some liposomes that extravasate- for their smaller size or characteristics that allows them greater time of circulation

escaping from phagocytic cells and accumulating in sites of trauma, such as tumor, infected and inflamed cells (1).

Liposomes' encapsulation ability, protects drugs against enzymatic degradation then for this reason, they are also candidates for oral delivery of enzyme-degradable peptides and proteins like insulin and vaccines (1).

Lipid-based vesicles can also be used in order to enhance solubilization and drug uptake by the cells because encapsulating the lipophilic drug promotes cellular penetration and makes it to be much more efficiently delivered, significantly reducing toxicity (1).

Cationic liposomes can complex negatively charged desoxyribonucleic acid (DNA) plasmids and transfect cells *in vitro*, causing the expression of the protein encoded in the targeted cells (1,2,4).

Dermatological and cosmetic applications of liposomes are based on the similarity between the vesicles bilayer and natural membranes structure enabling them to modify cell's membrane fluidity and passing through it (1,2). Once in the appropriate location, these carriers apply their moisturizing and restoring properties fruit of a natural ability to humidify the skin and provide lipids that are essential to prevent skin aging (1). This way, liposomes overcome the problematic of most transdermal DD: restriction of macro or hydrophilic molecules to pass through the stratum corneum (2).

In other area of study, the addition of nitrates to cheese milk to suppress the growth of spore-forming bacteria is not recommended once its effect on health is questionable. Avoiding the use of nitrates, liposomes can occupy the cheese water spaces that allow microorganisms to develop preventing that phenomena to occur (1). Other technique used in cheese is adding encapsulated flavor-producer enzymes that usually get lost in the curd, reducing the cost and time of production, and improving cheese elasticity and flavor (1,2,4). Liposomes can also transport enzymes that are missing in some health problems, like lactose intolerance, making some food products suitable for those populations (2). Still in the area of food production, biocides encapsulated into liposomes have shown to be improved due to extended presence of fungicides, herbicides, or pesticides with reduced damage to other life forms, once their surface can be made to be sticky so that they remain on the leaves for longer times and do not wash

into the ground (1). In resume, it is possible to alter flavors, textures and colors by using entrapped antimicrobials, antioxidants and other molecules (2).

The crossing of the possible formulations with the possible routes of administration and the different sites of action depending on liposomes own characteristics, makes them very adaptable for new drug designs and different areas of application (1). However, liposomes can also serve science without being used as DDS. This vesicles can simulate a cell and be used to study many of their behavior, specially the membranes' behavior (4).

Table 4. Examples of commercialized liposomal products.

Product name and manufacturer	Properties	References
Capture® Christian Dior	The first cosmetic using liposome technology launched in 1986, is a line of cosmetics that uses liposomes alone to prevent skin aging	(1)
Regain® Johnson & Johnson	Line of products that uses liposomal minoxidil, a vasodilator claimed to prevent or slow hair loss	(1,28)
ELA-Max® Ferndale Laboratories, Inc.	Liposomal lidocaine topical preparation with enhanced anesthetic effect because of the augmented drug absorption and protection against its rapid metabolism	(1,25)
Doxil® ALZA Corporation	First nano-drug approved by FDA, Doxil® consists in liposomal doxorubicin with 4.5 times less cardiotoxicity due to prolonged half-life in circulation, high stability of the drug, high targeting	(3,11)
AmBisome® Gilead	A liposomal formulation that stabilizes and so diminishes the neuro and nephrotoxicity of amphotericin B	(1,30)
Epaxal® Berna Biotech	Hepatitis A virus vaccine in a liposomal formulation that reduces the side effects because of the non-use of aluminum	(1,31)
Other products	• Atopic dry skin treatments that restore barrier function and deliver drugs at the same time • Corticoids with less side effects • Retinoids like tretinoin with less local skin irritation	(1)

1.6.1 Liposome applications against cancer

Beyond the routes of administration and the advantages of each one, liposomes can be used for drug targeting, increasing efficacy and decreasing toxicity (1). This fact is particularly important in cancer treatment: chemotherapy is only effective with doses that cause severe toxic side effects, and liposomal encapsulation of the anti-tumoral drug can significantly reduce the side effects and increase the efficacy of the treatment (1,2). This drug targeting can be done, apart from the size and characteristics that already described along this review, by targeting specific receptor ligands to antigens or other molecules produced by the tumor cells (1,2).

A tumor is a complex group cells that have uncontrolled proliferation if in the presence of the right microenvironment. The TME components, modulate the growth of the cancerous cells by suppressing or enhancing it (32). TME is, therefore, one of the most important considerations to take in account when designing an anti-tumoral DDS.

TME cells produce cytokines, chemokines, growth factors, inflammatory and matrix remodeling enzymes that build de microenvironment itself, holding cells like fibroblasts, endothelial and immune cells, and causing suppression and/or promotion of oncogenes, hypoxia, acidic pH, nutrient deprivation, neovascularization, high interstitial fluid pressure and inflammation. Without the right TME, no tumor can persist (32).

As the tumor grows in volume, some cells become distant from the blood vessels, and the apport of oxygen progressively decreases. These cells cannot actively proliferate and the hypoxic and less nourished regions of the tumor undergo hypoxia-induced changes in gene expression that aims survival advantage, resulting in the exchange to a fermentative metabolism that does not need oxygen. As we know, this type of mechanism to obtain energy, end up with the synthesis of protons and lactate that will make the microenvironment acidic (32). Another changes in gene expression and damage in DNA happen because of the reactive oxygen species (ROS) formed during this hypoxic status. Also, the dropping in the pH, induces several signal pathways that selectively stimulate or suppress gene expression to promote survival of the cancerous cells, including the apoptosis suppression- making cells grow and multiply indeterminably- allowing metastasis, escaping the immune system and resisting to the anti-cancer therapies (32,33). At last, the cells that are closer to the vessels can grow rapidly with sufficient apport of oxygen and nutrients, and their growth promotes an increasing pressure on the interstitial fluid (32).

Angiogenesis- a phenomenon based on growing of the vascular network- occurs to serve the uncontrolled growing of the cells and lack of nutrients, and leads to leaky vessels with gaps with up to 800 nm. Furthermore, the risen interstitial pressure puts more effort into the lymphatic drainage that becomes compromised, not being able to remove nanoparticles from the TME (6,32). This EPR has been considered the key mechanism for the prolonged half-life of liposomes that show themselves suppressive effects on this angiogenic process. One more time, it all starts with the hypoxic conditions that induce the secretion of cytokines and growth factors that initiate the neovascularization and inflammation of this tissues (32).

TME is, additionally, crowded in immune cells: macrophages, neutrophils, dendritic and natural killer cells. In a situation of acute inflammation, it can be more easy to think that the more immune cells present the better, so that the inflammation can be *fought*. However, in chronic inflammation, the presence of macrophages promotes angiogenesis and metastasis by releasing the cytokines, growth factors and enzymes that we have been shown to be tumor progressing agents (33).

Apart from the TME that can be targeted by numerous means, cancer therapy has other characteristics that can be influenced to be perfected. Chemotherapy generally follows a treatment scheme based on the administration of the maximum tolerated dose followed by a recovery period. In this recovery period, the body's healthy cells are supposed to regrow. However it has been shown that some cancer cells also recuperate. *Metronomic dosing* has been used in order to reduce the recovery time, using a submaximal dose administered by long infusion times. This way, the drug concentration is persistent but lower, and results show effective competence to eliminate tumor endothelial cells, reduce hypoxia and increase apoptosis in those cells. Assuming that a liposome mimics this metronomic dosing- because it slowly releases the drug according to a specific mechanism during a longer half-life period- better results are automatically achieved thanks to the DDS *per si* (32).

Some liposomes were studied to be used in cancer therapy to induce cancer cells differentiation to get to apoptotic events. For this aim, liposomes were loaded with retinoic acid and other vitamin A derivatives in combination with traditional chemotherapeutic drugs (33). Some other vesicles respond to the acidic conditions of the TME, or its' higher temperature, therefore releasing the drug selectively in those sites. Nanoxel® is a paclitaxel micellar formulation composed of a pH sensitive co-polymer (N-isopropyl acrylamide and vinylpyrrolidone) that is degradable under acidic conditions, releasing paclitaxel to the TME.

It is approved for the treatment of the breast cancer, lung carcinoma, and Kaposi's sarcoma. Responding to the higher temperature, we have Thermodox®, a liposomal formulation containing doxorubicin and among other lipids, 1-stearoyl-2-hydroxy-sn-glycero-3-phosphatidylcholine, a lysolipid that lowers the liposome's T_c making it easier to achieve and less probable to cause damage to the healthy cells (34). Thermosensitive liposomes rapidly release the drug because the *trans* conformation of the lipids molecules undergo a transition to *gauche* conformation creating leaky regions. To trigger DR, helping the already warmer conditions of the TME, we can use mild hyperthermia, increasing the desired temperature up to 43ºC that has itself a cytotoxic effect on tumorous cells that, for being debilitated are less able to tolerate extreme conditions (35).

All of these features- stimuli-responsive liposomes sensitive to the acidic environment of tumors, liposomes loaded with TME modulating molecules, metronomic dosing and others, makes liposomes one of the most wanted molecules to perform anti-cancer therapy.

Table 5. Approved liposomal formulations for cancer treatment. Adapted from (32).

Encapsulated Drug	Commercial name	Types of cancer involved	Mechanism of action
Cytarabine+ Daunorubicin	Vyxeos®	Acute myeloid leukaemia in adults	Anti-mitotic and cytotoxic activity (40)
Doxorubicin	Doxil®/Caelyx®	Breast cancer, ovarian carcinoma, AIDS-related Kaposi' s Sarcoma	Antiangiogenic activity and enhanced tumor-associated immune response (intercalation between DNA/RNA base pairs) (38)
	Myocet®	Breast cancer	Cytotoxic activity (39)
Irinotecan	Onivyde®	Adenocarcinoma of the pancreas	Antiangiogenic activity and topoisomerase inhibition (37)
Mifamurtide	Mepact®	Osteocarcinoma	Enhanced tumor-associated immune response
Vincristine	Marqibo®	Philadelphia chromosome-negative acute lymphoblastic leukaemia	Anti-mitotic activity (36)

2 Project main goals

It is widely known that standard anti-cancer chemotherapy have a very narrow therapeutic window and it is not selective for cancer cells, which causes patients to experience side effects, such as nausea, hair loss, cardio and neurotoxicity. Also, patients suffering from these systemic-related side effects will only tolerate low doses of drugs, decreasing the effectiveness of the treatment. Drug targeting to specific sites of action provides many advantages over non-targeted drugs, such as the enhancement of the drug uptake, reducing the required dose to be administered as well as the systemic toxicity that causes the undesirable effects (1,2). Section 1.6.1. discusses the passive delivery that takes advantage of the EPR effect, as well as the active targeting achieved by modifying the carrier surface with ligands to specific receptors/markers on target cells (12).

For the past few years, in order to solve these problems, significant advances have been made in the development not only of new anti-cancer selective drugs, but also of new delivery technologies that allow the administration of non-selective drugs, thus constituting a targeted therapy. These delivery technologies use nanometer-sized vesicles, such as liposomes, functionalized to preferably deliver a target to a specific type of cells- in this case, cancer cells (41). To develop a DDS that hopefully would make cancer treatment simpler, Università degli Studi di Firenze (UniFi) designed a project structured in two lines of research.

The Department of Pharmaceutical Chemistry developed a receptor based on the lectin-carbohydrate interaction: recognition and binding to specific glycoproteins that are frequently over-expressed or that have different structures in malignant cells. They took advantage of the high-mannose expression in these cells, to produce biomimetic synthetic lectin receptors for mannose that use non-covalent interactions to bind to saccharides and discriminate tumor from healthy cells (42,43).

The aim of the project is focused on the design, synthesis and characterization of nanovesicles loaded with a non-selective anti-cancer drug, being functionalized with the biomimetic mannose-receptors already prepared by the Pharmaceutical Chemistry Department. Two types of mannose-targeted vesicles will be tested- liposomes and niosomes- to select the one that will enable higher drug loading (e.g. doxorubicin) and surface-modification with artificial receptors, while presenting physicochemical properties suitable for intravenous

administration. This study also includes the evaluation of the methods of preparation and vesicle composition on the main features of these vesicles.

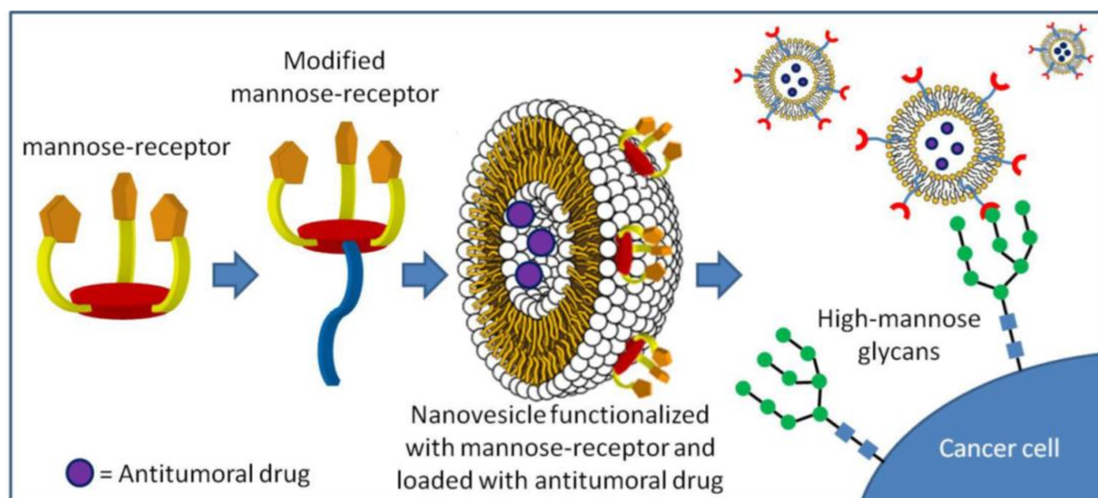

Figure 2. Graphical representation of the main topic of the project.

Provided by Dra. Marzia Cirri

2.1 Laboratorial work design

The first step of this project involves the selection of the lipidic components of the liposomal formulations, including the assessment of the most suitable molar ratios considering the targeted specifications.

Four conventional methods were selected to prepare the liposomes, based on the previous laboratory experience and literature evidence. Also, these methods only require the use of basic laboratorial equipment, which are easily available making them easier and less expensive to do (1). However, these techniques include the use of organic solvents, that has to be removed from formulation to avoid environmental and human health damage (1). Furthermore, these methods are in general very difficult to scale up due to the numerous steps involved in the liposome production, and lead to non-homogenized and/or big particles that have to be subsequently treated to fit the correct range defined for size and PDI (1).

Two different molar ratios (1:1 and 2:1) of CHL and phosphatidylcholine will be tested for the development of highly stable liposomes prepared by each of the four methodologies

(4,12). Phosphatidylcholine was chosen because of its biocompatibility, low toxicity and possibility to produce neutral liposomes, that in this case will only benefit of the targeting ability of the mannose receptors that will be developed (1). As for the CHL, similar to what is fully explained in the section 1.2.5, when entrapping an API like doxorubicin that is not extremely hydrophilic or hydrophobic, CHL addition balances the LogP of phosphatidylcholine and allows doxorubicin to be entrapped (10).

2.1.1 Thin Layer Evaporation-Vortex (TLE-Vortex)

TLE-vortex, thin-film hydration with vortex, or Bangham method is performed by starting to weight every component into a rounded bottom flask. The solid lipids are after dispersed a minimum amount of chloroform (an organic solvent) and kept under stirring using a rotary evaporator at reduced pressure and constant rotation and temperature above the T_c (see section 1.2.3.), to form a thin lipid film on the flask wall and enable the removal of any trace of organic solvent (1,2,26). After evaporation, PBS (aqueous phase) is added to the flask to hydrate the film under agitation (1,2). At this point, a vortex or a paddle may be used to agitate. In this specific case, cycles of warming in the bath/vortex mixing are carried out (44). This hydration allows the lipid layer to separate very slowly and merge again faster, producing heterogenous MLV's (7). The final step aims to decrease the size of the particles, starting with a sample centrifugation, followed by supernatant sonication (1,15).

This is the oldest method, initially performed by Bangham *et al* and most probably the simplest at the laboratory level, but it results in lipidic vesicles with low EE, high PDI and cannot be scaled-up without having a portion of complications to solve (1,2,15,21).

2.1.2 Thin Layer Evaporation -Paddle (TLE-Paddle)

Similarly to the previous method, in the TLE-Paddle each component powder is weighted into a rounded bottom flask and manually dispersed in a minimum quantity of chloroform required for dissolution. The organic solvent is subsequently evaporated in the rotary evaporator, then being hydrated with PBS (1,2). At this point, the agitation is carried out by a

paddle while the flask is soaked in the bath at a temperature above the T_c (44). The final step necessary to decrease particle size is similar to the one used in the TLE-Vortex.

The TLE methods, both vortex and paddle, are difficult to scale up, and require additional procedures to reduce the size of the liposomes (2). However, it is a methodology easily established at the laboratory scale (21).

2.1.3 Reverse Phase Evaporation

After weighting every component into a rounded bottom flask and dissolving them in the minimum volume of chloroform, the PBS is added before evaporating the chloroform (2). Then, the mixture is kept in an ultrasound bath for 3 hours, which leads to the formation of inverted micelles (2,12). Just then, the chloroform is evaporated by keeping the mixture at the rotavapor under a temperature higher than the T_c (2). The rotavapor agitation helps turning the micelles into a liposome emulsion (12). This methodology requires a final step to reduce the particle size similarly to the two previous described methodologies.

This method produces MLV's with high EE due to the large aqueous space-to-lipid ratio (2,15). Moreover, it is relatively easy to scale-up, even if it makes use of organic solvents (2).

2.1.4 Solvent Injection

This method involves the direct hydration of the lipids dispersed into an organic phase, which can be done using a syringe to introduce the lipid solution into the aqueous phase (PBS) previously warmed-up and kept under magnetic stirring (15,21). One of the solvents used in this method is the ethanol. Each component is weighed into a beaker and ethanol is subsequently added. Chloroform is added into another beaker, and kept under agitation using a magnetic agitator (21). The lipid solution is after injected to this second beaker using a syringe (1). After this operation, the chloroform is evaporated in a rotavapor and the size of the particles subsequently reduced by employing the methodologies mentioned in the three methods described above (21). Liposomes are formed because the ethanol mixes in the water and forces the lipids to leave the dispersion, aggrouping as SUV's (7).

This is a method frequently used to scale-up liposome production, leading to liposomes of low mean diameters and PDI (1,21).

2.2 Liposomes characterization

To measure particle size and PDI, ICH (International Conference for Harmonization) and FDA (U.S. Food and Drug Administration) recommends the DLS method (24). Using the same device that processes both electrophoretic and dynamic light scattering, ZP can be assessed by ELS, streamlining the process. Particle size, PDI and ZP are crucial physicochemical properties, considering their impact on liposome biological function. The size of the liposomes has to be controlled in order to be maintained in a range that allows their delivery through the vessels and further reach the mannose residues. Additionally, ZP should ensure carrier stability in blood flow. To process the samples, it is necessary to dilute the previously sonicated mixture, to prevent the multiscattering effect (44). Multiple scattering is the phenomenon wherein there is a re-scattering of the photons already scattered from adjacent particles making them appear to be moving much faster than they truly are. The size measurements will then appear to be smaller than they really are (45).

Stability must be also ensured while addressing liposome manufacture. At the laboratorial level, the measurement of liposome size, PDI and ZP every fifteen days provides an indication of the stability of each formulation. Throughout this period, the samples have to be stored at 4ºC and any variation in mean average diameters and/or ZP values close to zero may indicate vesicle aggregation and therefore, formulations instability. Besides, assessing the presence of organic solvents and characterizing the impact of the loaded drug and surface functionalization on liposomal physicochemical properties, namely by assessing the liposome EE and in vitro DR are also some of the required procedures to perform (21,44,46).

This study aims the selection of the manufacture method and molar ratio (CHL: Other Lipids) that allows the production of liposomes meeting the targeted specifications: size under 200 nm (7); PDI as low as possible (< 0.3); and ZP below -30mV or above +30mV (1).

2.3 Doxorubicin encapsulation

Ultimately the drug-loaded liposomes will be prepared employing the best formulation versus production process. Doxorubicin is a very well-known drug, being considered one of the most powerful and versatile anti-cancer molecule, as it effective against many types of cancer (3,44). However, its application is limited since it has powerful side effects: myelosuppression, nausea, vomiting, stomatitis, alopecia, skin hyperpigmentation and cardiotoxicity (3,11).

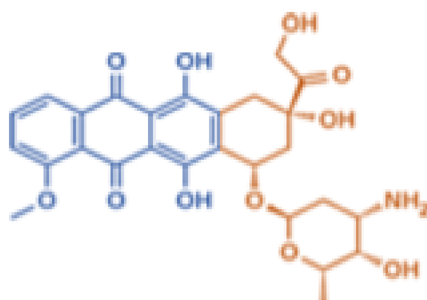

Figure 3. Doxorubicin's chemical structure. Adapted from (17).

The anthracycline is an amphiphilic antibiotic divided in two chromophores that provide its anti-tumoral activity (47). The pharmacophore represented in blue (Figure 3) intercalates between the negative base pairs of DNA through the ester group, thus inhibiting its replication, transcription and overall synthesis (11,47). The orange represented pharmacophore (Figure 3), inhibits the topoisomerase II, an enzyme that promotes relaxing of the DNA structure making the replication process possible (11). Additionally, it forms iron-mediated free radicals: the quinone structure (blue representation in Figure 3) participates in the redox reactions led by several mitochondrial enzymes, as an electron acceptor producing ROS causing oxidative damage (11,47). Mitochondria is the most affected organelle, as it is rich in cardiolipin, which is an anionic molecule to which the cationic form of doxorubicin has high affinity (11). The cardiac muscle has high mitochondrial levels, and therefore this organ is highly affected by the systemic distribution of this drug. The increased permeability of the oxidative damaged membranes allows the release of Ca^{2+}, which leads to mitochondrial disfunction, loss of myocytes and cardiac failure (11,47).

Doxil® is an example of a liposomal doxorubicin formulation with 25 years of success related to its long circulating half-life in the bloodstream that results from its high drug loading (about 15.000 doxorubicin molecules/vesicle) and ability to escape MPS cells, improving the delivery of doxorubicin to tumor cells (37). The passive targeting of doxorubicin to tumor cells results in high accumulation of the drug in the tumor site, which prevents the massive cardiotoxicity resultant from the systemic effect of this cytotoxic drug (11).

Doxorubicin vesicle development started with the preparation of anionic OLV passively loaded with this drug during the hydration process (11). It is the easier, cheaper and faster way of loading, as it just encompasses the dissolution of lipophilic drugs in the organic solvent, hydrophilic drugs in the aqueous phase (48). Unloaded drug can be removed using methods mentioned above, like dialysis, centrifugation, chromatography and filtration. However, it has some disadvantages that the investigation teams rapidly understood: the "burst release" phenomenon (11,48). This occurrence is due to the low solubility of doxorubicin that only allows passive entrapment into the outer lipidic layer of the liposomes making the drug more available to the leakage during the rapid dilution that takes time throughout the administration process (8,11).

Doxil® was then successfully designed by using remote loading, another method that actively places the drug into a preformed liposome (8,11). This was guaranteed by preparing liposomes in an ammonium sulfate buffer- $(NH_4)_2SO_4$, which creates a pH gradient with higher buffer concentration in the liposome core. Triggered by this gradient, a molecule of NH_3 crosses the liposome bilayer leaving a H^+ and SO_4^{2}. As an amphipathic drug, doxorubicin pass through the phospholipidic bilayer in its molecular shape and form aggregates in the core by precipitating with sulfate ions- $(DOX\text{-}NH_3)_2SO_4$ (34). The drug/lipid molar ratio also has a great impact on liposomal drug loading: values lower than 0.95 will lead to high EE and so, it is preferable to use less concentrated solutions for longer incubation periods (11,17).

There are several techniques that can be used to achieve remote loading taking advantage of pH gradients. Generically, we can form liposomes in an acidic pH solution, and further incubate these vesicles into an alkaline medium containing the amphipathic drug that will then cross the lipidic bilayer and ionize when in the acidic core of the vesicle. Accordingly, the protonated molecule no longer can cross the lipid bilayer and will retain the drug entrapped until liposome disruption. This is the technique used in Myocet®, a non-pegylated liposome

which composition is based on CHL and egg phosphatidylcholine and that shows better tumor accumulation and lower toxicity than free doxorubicin (37).

Solvent-assisted active loading technology (SALT) is a novel approach that allows insoluble drugs to be trapped into the aqueous core of a liposome. A trapping agent is added to the liposomal formulation, and a water miscible solvent is after added to the drug to rise its solubility and allow its penetration into the vesicle's core, where it will interact with the trapping agent and form a precipitate. The solvent can then be removed by dialysis or gel filtration, as it is done for the organic solvents (48).

For all off these reasons, and giving the great experience of Doxil®, and many others, the doxorubicin is the selected anti-cancer drug to be actively loaded, may it be via ammonium, other pH gradient, or using other technologies.

2.4 Functionalization

Cell surface has many glycans- *chain-like* structures constituted by monosaccharides- associated with lipids or proteins, which forms the glycocalyx or extracellular matrix essential for signaling, adhesion and recognition molecules involved in embryonic development and differentiation, cell-cell recognition, host-pathogen interaction and among others, metastasis (42,48,49). Glycocalyx is built through the process of glycosylation, a post-translational modification that concerns enzymatic addition of glycans to proteins (42). Its alterations are often due to misregulation of glycosyltransferases and glycosidases- the enzymes responsible for acting on glycans- in the TME (43,50). High-mannose glycans present in glycocalyx are extremely important to protect proteins from degradation during their intracellular transport, during which these glycans are cleaved and replaced by more complex structures. Most of the matured glycoproteins exiting in the Golgi have complex glycans: high levels of high-mannose glycans suggest a premature termination of the glycosylation pathway, which occurs in cells that are under uncontrolled and rapid growth- like tumor cells (42,50).

The Chemistry Department of UniFi produced biomimetic synthetic lectin receptors for mannose that use non-covalent interactions to bind saccharides and discriminate tumor from healthy cells. Lectins are carbohydrate binding agents (CBA), or in other words, proteins that

have the ability to recognize glycans with high specificity, based on hydrogen bonding and hydrophobic interactions, and potentially alter the biological processes in which they are involved. Mannose binding lectins (MBL) belong to the c-type lectin family and some of them, like Concavalin A, *Sophora avescens* and *Polygonatum cyrtonema* lectins show themselves cytotoxic activity (49,51). However, these are expensive, hard to produce, poorly stable, have low bioavailability and can be immunogenic. Thus, biomimetic molecules are preferable to perform this reverse targeting, where an exogenous lectin mimetic is recognized by the endogenous glycans (41,49,51).

The most important aspects that should be considered while designing a biomimetic CBA are the structure and the nature of the binding interaction with the carbohydrate. Regarding the structure, the design of a CBA can follow two approaches: 1) preorganization, in which a rigid conformation maximizes the affinity and selectivity, lowering the energy cost required to the event of binding. However, it reduces the flexibility of the molecule, which can be a limitation when the exact architecture of the complex is not known; or 2) adaptivity, in which flexible structures that can achieve the correct binding geometry upon recognition of its receptor are designed. Otherwise, mimicking a lectin receptor implies that the binding will occur via hydrogen bonds and CH- π and so, the MBL must be designed in order to be able to donate and accept H^+ from the -OH groups of the mannose residues (51).

Francesconi O. team have a strong expertise in CBA, and recently have made some advancements in this area. While examining macrocyclic, acyclic, oligomeric and charged structures, the group found that benzene substituted with hydrogen-bonding fractions is the most promising scaffold for a biomimetic MBL. Additionally, they found that amino-pyrrolic tripodal substitution is the most effective and added *trans*-1,2-diaminocyclohexane units that provided selectivity for mannosides and a recognition of the *trans*-1,2-diol group of sugars, thus enhancing the binding ability. The receptors (2) and (3) were then designed and showed the best affinity to mannose ever reported in a biomimetic CBA, as well as the best cytotoxic activity via caspase-cascade activation of the apoptosis (Figure 4). The study of these receptors was carried out considering chirality as a major feature: as carbohydrates are chiral molecules, their best match is probably a chiral receptor, indicating that the enantiomers (*R*)-(2) and (*S*)-(3) are the most selective towards α and β mannosides respectively, a change provided by the bridging of two arms of the tripodal structure (51–53).

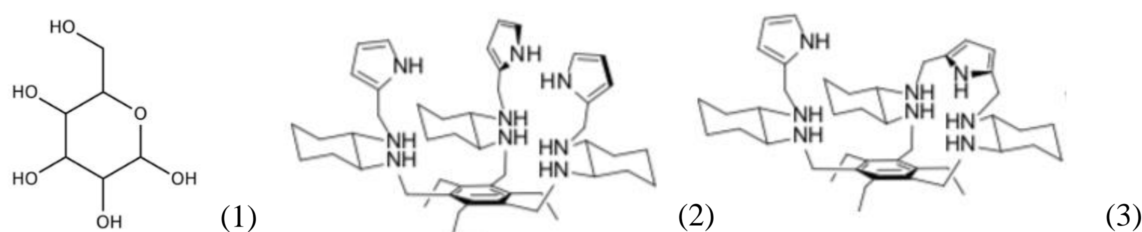

Figure 4. Chemical structures of mannose (1) and two tested CBA's (2 and 3).

Adapted from (51).

The modification of the surface of nanoparticles by receptors specifically aimed at certain ligands at cell surface, will ensure the active targeting of these cells. However, these carriers still have to overcome physiological barriers and reach the target tissue, and the particles have to be able to release the encapsulated drug (49).

Doxil® is a good learning model for the development of liposomal formulations. The functionalization of this liposome with N-(carbonyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine sodium salt (MPEG-DSPE) is based on the use of PEG, a molecule extensively used for the delivery of proteins. PEG is covalently bound to liposome formulation components, being exposed at the surface of these carriers, which improves the stability of these formulations by counteracting their recognition by cells from the MPS (11).

Despite the undoubtly therapeutic effect of Doxil®, the goal of the project in discussion is to improve the limitations of this formulations, such as its accumulation in skin causing PPE (Palmar Plantar Erythrodysthesia), the infusion-related reaction (flushing and shortness of breath) and improve doxorubicin accumulation, which is currently limited by the large size of PEG that hampers the drug entry in the TME (11,34). These side effects are attributed to PEG molecule, since Myocet® that is also a doxorubicin-loaded liposome but without PEG on its composition, do not have the above mentioned limitations (34). The receptors (2) and (3) (Figure 4) will then be added in the attempt to improve the therapeutic efficacy of the liposomal doxorubicin.

3 Conclusion

Liposomes have several advantages that makes them one of the most applied- or studied to apply- DDS nowadays. However, the successful pathway of a liposome research and design hangs not only on its superiority in technological and clinical terms, but also on its economic viability, which is underlying the non-exponential success of this type of DDS. At one hand, the stepping up from laboratory to industrial scale is very expensive and sometimes not possible at all due to its lipidic composition that is sensible to sterilization by heating and hard to stabilize. In the other hand, the mechanisms by which liposomes are considered a superior DDS, are themselves controversial: the EPR effect that promotes tumors' invasion by nanoparticles, has been considered heterogenous and irregular; the presence of the nanovesicles in the tumor, does not mean that the drug is there or if so, that it is available to the tissue; the type of cancer, immune system condition, physiological state, and other variabilities affects the overall effect; improved liposome formulations have not led to fully positive outcomes as for example, the repeated treatment with pegylated liposomes has increased liposome clearance from the circulation by the ABC (accelerated blood clearance) phenomenon as a result of the anti-PEG IgM response produced following the previous exposure to PEG.

Despite the fact that we neglect that liposomes have disadvantages that are hard and expensive to evade, the truth is that scientific community cannot look away, and other types of DDS have been developed, like niosomes that overcome some of the liposome limitations, such as physical and chemical instability, complexity, high costs of production, and versatility.

As the clinical success for a DDS requires extensive monitorization, it has been made unmistakable the lack of specific regulatory protocols for characterization of these nanomedicines, which might be responsible for some failures in the marketing authorization process. Legislative frame concerning medicines, cosmetics and food products mostly covers nanomaterials *in principle,* which means that if it does not excludes those materials, in practice it includes them, revealing a huge lack of guidance for manufacturers and for evaluation agencies as well. The demand starts with the definition of nanomaterial, a material in which 50% or more of the constituent particles (by number) have one or more external dimensions in the size range 1-100 nm or the volume-specific surface area is larger than 60 m^2 cm^{-3}. Nevertheless, ISO also includes materials larger than 100 nm, when made of internal or surface

structures in the nanoscale spectra, making the inclusion on nanoparticles legislation or regular medicines legislation a challenging task itself.

The multiplicity of nanomedicine products studied in the last decades is bringing together all kinds of sciences to provide better health care, and UniFi took advantage of its' interdisciplinary departments to project the *Development and characterization of functionalized liposomes for mannose-targeted antitumoral drugs delivery*. In 2020, this project started by the production of niosomes. All the TLE-paddle and vortex, reverse phase evaporation and ethanol injection methods were tested for these particles but unfortunately, the project had to be interrupted due to the COVID-19 outbreak, and liposomes were not tested. Therefore, the steps of loading and functionalization of the carriers were not even fully projected. However the expectations are high, the prospects point to a successful pathway and there is still the willing to battle a disease that, dozens of years of investigation later, continues to escape all the progresses made.

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