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Profiling pharmacokinetic parameters of liposomal formulations optimized for nanotherapy purposes.

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SUMMARY

Making a property profile for the drug in its formulation is a very important step in the current drug designing process. It consists of for example the partition coefficient, biophysical influences of the drug and its formulation on the membrane and binding to plasma proteins. With these parameters, the drugs and its formulation can be altered. The overall quality of the drug in its formulation can be improved by for example lowering toxicity and improving the absorption.

Cancer is a worldwide endemic and a broad variety of medicines are available. They are however very toxic and show a lot of adverse effects. They might also not have ideal pharmacokinetic properties in terms of absorption. This is why in this work the drug property profile of 2 drugs, Paclitaxel and Resveratrol, are partially determined. Since both compounds are hydrophobic, they are formulated in cationic liposomal formulations, DODAC:MO (1:2) and DODAB:MO (1:2) respectively. The partition coefficient was determined at different pH and different temperatures for both compounds in their formulation. This was done by derivative spectroscopy. Fluorescence quenching was used to determine the binding of PTX to the human serum albumin protein. Furthermore the influence of PTX on the microviscosity and order of the liposomes is predicted. For RSV and PTX, we found that the transition from the aqueous phase to the DODAB:MO and DODAC:MO liposomal phase was spontaneous. The temperature range studied (30°C-60°C) contains the phase transition temperature of the DODAB:MO liposomes and as a consequence, the results for RSV were different depending on the rigidity of the state. The more rigid the liposomes, the more energy was needed to separate the bilayers and hereby the less spontaneous the reaction. The DODAC:MO liposomes have lower T_m so results were the same at all temperatures. The drugs were found in both the aqueous as the membrane phase. However concentrations in the membrane phase were higher. Because of the high Kp, we can conclude that DODAB:MO (1:2) and DODAC:MO (1:2) liposomal formulation are adequate nanocarriers for RSV and PTX. In PTX we didn't find a great change in the T_m of DMPC liposomes but a big change in the cooperativity of the phase transition when PTX was added. This proves that PTX is located deeply in the bilayer, as expected. There was only 1 binding site for PTX on HSA and the binding was rather weak. But addition of PTX to HSA stimulated separation of the HSA dimers into monomers.
SAMENVATTING (Summary in Dutch)

Het maken van een geneesmiddel eigenschapsprofiel in zijn formulering is in de huidige geneesmiddelontwikkeling van groot belang. Het bestaat uit bv de partitie coëfficiënt, biofysische invloeden van het geneesmiddel and zijn formulering op de membraan en de binding aan plasma proteïnen. Met deze parameters kunnen het geneesmiddel en zijn formulering worden aangepast. De gehele kwaliteit van het geneesmiddel en zijn formulering kunnen worden verbeterd door bv het verlagen van de toxiciteit en verhogen van de absorptie.

Kanker is wereldwijd een endemie en een grote variëteit aan geneesmiddelen zijn hiervoor beschikbaar. Ze zijn echter zeer toxisch en vertonen vele nevenwerkingen. Verder kunnen de farmacokinetische eigenschappen ook te wensen overlaten op het vlak van absorptie.

Daarom is hier getracht om het geneesmiddelen eigenschapsprofiel van 2 geneesmiddelen, PTX en RSV, gedeeltelijk op te stellen. Aangezien beide componenten hydrofoob zijn, werden ze geformuleerd in kationische liposomen, respectievelijk DODAC:MO (1:2) en DODAB:MO (1:2). De partitie coëfficiënt werd bepaald bij verschillende pH waarden en bij verschillende temperaturen voor beide componenten en in het formulering. Dit was door afgeleide spectroscopie. Fluorescente quenching werd gebruikt voor de bepaling van de binding van PTX aan het humaan serum albumine. Verder werd de invloed van PTX op de microviscositeit en de ordening van de liposomen voorspeld. Voor RSV en PTX vonden we dat de transitie van waterige fase naar de liposomale fase spontaan verliep. Het bestudeerde temperatuurgebied (30°C-60°C) bevat de fase transitie temperatuur van de DODAB:MO liposomen en hierdoor waren de resultaten voor RSV afhankelijk van de rigiditeit van de liposomen. Hoe rigider, hoe meer energie nodig was om de bilayer te scheiden en hierdoor ook hoe minder spontaan de reactie. De DODAC:MO liposomen hebben lagere Tm dus de resultaten waren gelijk bij alle temperaturen. De geneesmiddelen werden beiden in zowel de waterige fase als de membranaire fase teruggevonden. Maar de concentratie in de membranaire fase waren hoger. Door de hoge Kp, kunnen we besluiten dat DODAB:MO (1:2) en DODAC:MO (1:2) liposomen adequate nanocarriers vormen voor RSV en PTX. In PTX werd geen verandering van Tm gevonden wanneer toegevoegd bij DPMC liposomen. Maar er werd wel een significant verschil gevonden in de coöperativiteit. Dit bewijst dat PTX zich diep in de bilayer bevindt, zoals verwacht. Er was slechts 1 bindingsplaats aan HSA voor PTX.
en de binding zelf was eerder zwak. Maar toevoeging van PTX aan HSA stimuleerde de scheiding van de HSA dimeren in monomeren.
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LIST OF ABBREVIATIONS

ACR: average count rate

DLS: Dynamic Light Scattering

DMPC: Dimyristoylphosphatidylcholine

DODAB: Dioctadecyldimethylammoniumbromide

DODAC: Dioctadecyldimethylammoniumchloride

DOPE: 1,2-dioleyl-3-phosphatidylethanolamine

DPH: Diphenylhexatriene

ΔG: Gibbs free energy

ΔH: variation in enthalpy

HSA: Human serum albumin

Kp: partition coefficient

Lα: liquid-crystal state

Lβ: gel state

λmax: wavelength corresponding to a maximum in absorption

λmin: wavelength corresponding to a minimum in absorption

LUVs: large unilamellar vesicles

MLVs: multilamellar vesicles

MO: monoolein

NSLC: non-small cell lung carcinoma

PCS: Photon Correlation Spectroscopy

PTX: Paclitaxel
RSV: Resveratrol

ΔS: variation in entropy

\( T_{m} \): gel to liquid-crystalline phase transition temperature

UV-VIS: Ultraviolet- visible
1 INTRODUCTION

Worldwide, cancer is one of the most deadly diseases. In 2012 there were 8,2 million deaths that can be related to cancer according to the World Health Organization [1]. This illness is caused by multiple factors such as the alteration of the cell division. One cell starts multiplying rapidly and uncontrollably and hereby invades surrounding tissue. Current treatment mainly consists of chemo- and/or radiation therapy in hopes of shrinking the tumor. If the complexity of the tumor allows it, doctors will attempt to remove the cancerous tissue using surgery. All of these techniques are invasive, show a lot of adverse effects and develop resistance during time of use. Since we are aware of these flaws, innovative treatments are highly demanded [2].

1.1 PACLITAXEL

For the past 20 years new therapies have been trending. Whereas previous treatments focused on killing rapidly multiplying cells, some of the more recently developed drugs such as Paclitaxel (PTX) (Figure 1.1) also interfere with the angiogenesis. This is the process where new blood vessels are being formed from the existing vasculature in the human body. This mechanism does not only occur in pathogenic tissue, it can also be found in healthy cells. Because tumor cells are dividing more frequently and rapidly than normal endothelial cells, they need more nutrition and oxygen to be able to do so. The oxygen and other nutritional elements are supplied by the red blood cells. When interrupting the angiogenesis, the cancer cells are likely to die due to lack of nutrition and a build-up of metabolites [3] [4].

![Figure 1.1: The molecular structure of Paclitaxel obtained with Marvin sketch calculator by Chemaxon™](image-url)
In the battle against ovarian cancer, PTX is often used since its approval by the Food and Drug Administration in 1992. It is also administered for breast, prostate and pancreas cancers, non-small cell lung carcinoma (NSCLC) and AIDS related Kaposi’s sarcoma. The drug is especially recommended for previously treated patients who did not respond to chemotherapy [5]. Both the concentration and the duration of exposure influence the biological and pharmaceutical effects of the drug as seen in preclinical and clinical trials [6].

PTX reversibly binds to β tubulin, one of the dimers of cylindrical tubes called the microtubule. They are vital for cell mitosis because amongst other things, they are responsible for capturing and aligning the chromosomes [7]. The dynamic instability, or the constantly growing and shortening of the microtubule are crucial for the proper functioning of the microtubule [8]. This happens by adding dimers to the plus end of the microtubule. By binding to the tubulin, PTX will disrupt this equilibrium process and form unnaturally stable and dysfunctional microtubule. Therefore PTX was the first molecule to be categorized as a microtubule-stabilizing cytotoxic drug. In normal conditions, the microtubule need GTP to attach new dimers and polymerize the tubulin [9]. When PTX is present, the cell doesn't need this GTP. Paclitaxel is proven to lower the velocity of expanding as well as shrinking the ends of the tubule [10]. PTX will reduce the number of protofilaments from a normal 13 to 12 per cylinder. Results show that PTX can also disrupt the microtubule functions in other ways that could cause their anticancer activity. For example, by inactivating the proteins that prevent cell apoptosis in the cancer cells, by phosphorylating them [2].

Paclitaxel is hydrophobic, which makes it difficult to transport in the human body that consists up to 65% of water. Therefore an environment needs to be created in such a manner that the drug can be dissolved in aqueous solutions. This is why after discovering this compound in the bark of the Western Yew tree, Taxus brevifolia, it took about 10 years before the drug was made soluble for human use [11]. When the drug is administered intravenously, multiple side effects are experienced [6]. Most commonly used at the moment is 50 % Cremophor EL, a polyethoxylated castor oil carrier as a non-ionic surfactant and 50 % dehydrated ethanol. It is prepared in a ratio of 30 mg PTX to 5 mL of this Cremophor EL solution. This is an unavoidably high amount of vehicle in comparison with other drug formulations using this compound [12]. And when administering this formulation to humans, it caused severe to life-threatening side effects such as hypersensitivity,
neurotoxicity and neutropenia. The first signs of these complications are visible in the bone marrow. Most of the clinical side effects occur because of the toxic surfactant needed to solubilize the drug. This is believed because other drugs formulated with polyoxyethylated caster oils such as vitamin K and cyclosporine, revealed similar complications. They induced histamine release and hereby caused severe hypersensitivity in treated patients. Even with prophylactic high dose of corticosteroids, H₁ and H₂ receptor antagonists, rashes and flushing appear frequently. The neurotoxicity is also most likely due to the vehicle. This conclusion comes from studies where rats treated with PTX formulations without this surfactant do not experience the neurotoxic side effect [11].

Renal clearance of PTX is rather low, the drug is mostly eliminated from the body by hepatic metabolism, excretion via bile ducts and elimination in feces [5]. Paclitaxel's bioavailability is low and often variable. Part of this changing availability is due to P-glycoprotein, which actively pumps the drug out of the cells. This is why research is conducted using new vehicles that are stable enough to be used in clinical context. Formulations considered are liposomes, co-solvents, emulsions, cyclodextrins, nanosuspensions and micelles in hopes of increasing the drug concentration obtained in the target cells and reducing the toxicity without losing antitumor effect [13]. When using nanocarriers, such as liposomes, the PTX encapsulated is expected to be located in this carrier according to the idea of 'like dissolves like'. In correlation with its lipophilic nature, with a the K_{ow} of 3.60 as estimated by Pubmed, PTX will occupy the inner hydrophobic parts of liposomal bilayers.

Studies also revealed resistance for the drug in cancer cells. There have been many different ways discovered how the cancer cells develop this resistance and some tumors are never sensitive for the drug to begin with. To obtain resistance, there are usually multiple factors in play, some more important than others. For PTX we see for example an over expression of P-glycoprotein, changes in the genes and proteins that regulate cell apoptosis or alterations in the binding of the drug to its target (the microtubule in the case of PTX). The latter is a mechanism often seen in drug resistance in general [14].
1.2 RESVERATROL

Cancer is a disease that acquires a certain time to manifest. The transformation from healthy cells to tumorous ones does not occur overnight. This is why it is important and cost-effective to invest in cancer prevention. Especially since some of the measures are fairly simple and at no or very low cost. For instance, avoiding presumably cancer triggers, such as: smoking, unprotected sunbathing and exposure to certain chemicals or radiation [15].

One naturally appearing substance that can be used for these purposes is 3,5,4’-trihydroxy-trans-stilbene or Resveratrol (RSV) as seen in Figure 1.2. It is a polyphenolic phytoalexin found in grape skin, peanuts, cocoa beans and mulberries where it is adaptively produced as a natural defense against injury and pathogens. Traces of this substance can also be found in nutritional products such as chocolate and (mostly red) wine. For centuries this product has been used for its anti-inflammatory purposes in Asia and China as a medicine called Ko-jo-kon. The health benefits were discovered in the Western civilization thanks to the French paradox. This is the idea that the French population has bad dietary habits but do not display a higher risk for cardiovascular complications. So assays were conducted searching for the incentive of this advantage in their lifestyle or diet [16].

Besides being an anti-oxidant, RSV is neuroprotective, can increase metabolic health and acts as an anti-tumoral cyclooxygenase inhibitor. As an inhibitor of cyclooxygenase-1 and cyclooxygenase-2, RSV prevents the conversion of arachidonic acid in prostaglandins which can stimulate cancer cells and prevent effective immune response. 

For a few years now RSV is used as a chemoprotective agent against the growth of cancers in the breast, prostate, lung and skin in particular. More recently, RSV is also used as a drug once the cancer has already established in the human body. For the anticancer effect RSV can be administered alone or in combination therapy with, for example, gemcitabine resulting in a synergistic antitumor effect and possible lower resistance and toxicity [17] [18].
However, this does not apply to all anticancer drugs. Multiple researchers have obtained contradictory data that are cell- and drug dependent. For example, if PTX and RSV are administered together in breast cancer treatments, the anticancer effect of PTX will be reduced [19].

RSV is easily absorbed after oral administration compared to other polyphenols. Also RSV is usually well endured by patients without registered toxicity and therefore not much adverse effects even at high doses. This is not unusual since it is a natural compound. Despite its advantages, RSV has however a strong affinity towards protein binding; isomerizes into its less active cis form in contact with light; has poor water solubility and low bioavailability. RSV is rapidly metabolized, it has a plasma half-life of 8-14 minutes, to sulfate or glucuronide compounds in our body with half-lives of about 9,2 hours. It is not only absorbed in the gastrointestinal duct and converted in stable components by intestinal flora, but hepatic enzymes such as cytochrome P450 subtype 1A2 also modify the molecule. On the other hand, RSV itself inhibits multiple subtypes of the cytochrome which may lead to higher concentrations of drug who are metabolized by these hepatic enzymes [20]. Multiple ways to prevent the metabolism and to solve the problem of poor water solubility are examined. One of them is to encapsulate RSV in nanocarriers such as liposomes.

When encapsulated in liposomal formulations, RSV will be located in the inner hydrophobic parts of the membranes. This is as expected from a compound with a lipophilic nature (K_{ow} is 3,08 as estimated by Pubmed) since substances are dissolved according to the theory 'like dissolves like'. RSV has also been described as presenting fluidizing effects on the lipid membranes, being located across the lipid bilayers [21].

1.3 LIPOSOMES
Liposomes, or synthetic lipid vesicles are made up out of what is called amphiphile molecules. They consist of a polar head group and hydrophobic tails. When dispersed in aqueous solutions, they form a population of vesicles, which may vary in size from nanometers to microns in diameter. Due to their biphasic structure, they are able to accommodate different kinds of components in different parts of the bilayer (Figure 1.3).
Additionally they are biodegradable, biocompatible and affordable which makes them promising for further studies on their use as catalysts or biological membrane mimicking systems to conduct *in vitro* research. They are also suited as nanocarriers of various compounds in our body. [22].

To improve the poor solubility in water, Paclitaxel is currently formulated with Cremophor EL as vehicle. But Cremophor EL has shown high toxicity when administered both in animals as in humans. If liposomes are used to transport the drug, there is no need for Cremophor EL. In animal models, lower toxic side effects without loss of anticancer activity can be seen in the absence of Cremophor EL [23].

Depending on the pH and composition of the head group, the surface charge of liposomes will differ from a negative to a neutral or a positive charge. Studies indicate that cationic liposomes have a higher affinity for the negatively charged cells found in cancerous tissue as opposed to the healthy cells of the patients. The effect is also prolonged. These results are not found in anionic or neutral liposomes [24]. Encapsulation of Paclitaxel in cationic liposomes makes the anti tumor activity greater [25].

When making liposomes focus needs to be on the size, stability, structure and ability to encapsulate materials rather than the construction of the membranes. These will form spontaneously because of the unfavorable environment created combining hydrophobic (here the hydrocarbon tails) and hydrophilic (the solvent: water) components. Various methods to manufacture liposomes have been designed. A commonly used way is Bangham's method. In this mechanical dispersion method, the lipids are dissolved in an
organic solvent before being dried down onto a solid support. Later they are rehydrated above the transition temperature of the lipids. Only a small amount of the volume used for hydration, about 5% - 10%, is enclosed within the lipid membrane. To ensure the desired size, the liposomes are passed through a membrane filter of defined pore diameter multiple times [26].

1.3.1 Dioctadecyldimethylammonium bromide and chloride

The synthetic lipids Dioctadecyldimethylammoniumchloride (DODAC) and Dioctadecyldimethylammoniumbromide (DODAB) form positively charged double layer organized in large unilamellar vesicles (LUVs) when dispersed in water above the gel to liquid-crystalline state transition temperature (T_m). Even though the only difference between these lipids is their counter ion, Cl⁻ for DODAC and Br⁻ for DODAB, the characteristics of the formed cationic unilamellar vesicles are greatly different [27]. Studies concerning the diversity between these lipids speculate about the possible reasons for the difference in for example size, phase transition temperature (T_m) and curvature defining the vesicle structure. Research indicates that DODAB has a lower T_m than DODAC. A decrease in phase transition temperature equals a decrease in size or an increase in curvature [28]. Perhaps it is due to the binding specificity and affinity for the vesicle interface being stronger for Br⁻ as opposed to Cl⁻. Thus Br⁻ is more tightly bound to the interface of the DODAB vesicles than the Cl⁻ counter ions are to the DODAC interface. Furthermore, the Cl⁻ and Br⁻ ions do not have the same size, which may also affect the properties of the vesicles [29]. The bromide ion is smaller and is less hydrated so the H₂O mantel surrounding the ion is smaller. This might make it easier for the vesicles to bind more closely [27]. The vesicles that are formed when these lipids are dissolved in water can be used as transporters of drugs, enzymes or vaccines because they are able to contain small molecules [30].

1.3.2 Monoolein

Monoolein (MO), or 1-monooleoyl-rac-glycerol, is a neutral unsaturated fatty acid with one tail and of natural origin. When low concentrations are dispersed in an excess of water at room temperature, the lipids will form double inverted cubic or hexagonal shape. The reason for this can be explained by examining the structure of monoolein (Figure 1.4.).
At C9 monoolein has a *cis* double bound, which requires a certain steric formation in the tails of the molecule. These vesicles have a high hydrophobic character [31].

MO is mixed with DODAB and DODAC as a helper lipid. It is less toxic than other helper lipids such as for example 1,2-dioleyl-3-phosphatidylethanolamine (DOPE) [27]. It will organize itself between the other lipids and hereby influence the overall structure of the vesicles. Previous research has shown that this mixture of lipids, when MO is in excess, will produce lamellar bilayers of DODAB/DODAC enclosing inverted nonlamellar structures. As a drug delivery system, this is a very interesting structure because it will be able to accommodate higher concentrations of drugs within the internal lipidic content [32]. Another benefit is the stability. The liposomal formulation will be less tempted to release the drugs instantly [33]. Besides, MO is responsible for the lower rigidity of the bilayer, which will boost its mobility [27] [34]. Even though the DODAC:MO liposomes show more homogeneous formulations and are prone to release its contents more easily in the cell cytoplasm as opposed to the DODAB:MO. The latter has a higher ability to encapsulate compounds [27].

1.4 RATIONALIZATION OF DRUG DEVELOPMENT PROCESS

To have a better understanding of why and how a specific drug works; a drug property profile is made by studying the physical, chemical and biological properties of the molecule. With the knowledge of these properties, the drug or the formulation can be altered and hereby improved. Not only will it empower scientists to optimize the structure of the product, it also enables them to develop the best matching carriers and discover the optimal route of administration [35].

The development of nanocarriers such as liposomes is a very complex and demanding enterprise. But the advantages concerning not only the pharmacokinetics and pharmacodynamics, but also toxicity of the formulations outweigh this. Although the drugs encapsulated might have been already studied, the encapsulation in new formulations will change the features completely. The drug property profile will not only reveal the drug like
nature of the compound but can also help predicting possible difficulties in formulation and safety. In this manner, the development time and cost can be reduced and a delay of clinical introduction can be prevented.

To achieve a drug property profile important parameters should be determined like the partition coefficient of the drug, the biophysical effects of drugs in membranes and the binding of drugs to plasma proteins. All these parameters will influence drug absorption, distribution, accumulation and ultimately effect and toxicity.

The partition coefficient ($K_p$) is the ratio of equilibrium concentrations of a compound between two immiscible phases, here the aqueous phase and the membrane or liposomal phase. It demonstrates if the compound will prefer to be located in aqueous or in lipid phases. With this information, the lipophilicity of the drug will be established and hereby also the interaction of the drug with the lipids in the formulation. For drug development, this is very important since we want molecules who are enough solubilized to be transported in the body. On the other hand, the drug still needs to be released at the site of the target and be able to pass through the local membrane barriers. In 1997 Christopher A. Lipinski designed the rule of 5. If a drug does not apply the guidelines given by Lipinski, the probability of problems with oral administration is higher. However, compliance with the rules does not warrant successful oral administration.

Human serum albumin or HSA is one of the most found proteins in our bloodstream. This protein, synthesized in the liver, counts 585 amino acids. It binds and transports a significant amount of molecules throughout our body. The molecules that HSA can bind are very diverse. Going from endogenic ligands such as fatty acids until exogenic drugs like ibuprofen and warfarin. It has been proven many times that the pharmacokinetics of drugs can vary significantly due to their binding to this protein. HSA protein binding can affect the distribution, free concentration and metabolism of the compound [36]. A high affinity binding site on HSA for PTX has been described in literature [37].

Finally, the evaluation of drug effects on membrane biophysics can be studied by means of effects of drugs in the microviscosity of the lipid bilayers.
1.5 TECHNIQUES

1.5.1 Spectrophotometry for Kp determination

In this assay UV-VIS spectrophotometry is used to determine the partition coefficient of the drugs Paclitaxel and Resveratrol.

Spectroscopy is a widely used method for quantitative and qualitative measurements. Absorbance is the phenomenon where the energy of a photon is transmitted to another atom or molecule that is able to absorb it. The atom or molecule will hereby get into an excited state. The energy transfer is quantitative, because all the energy from the photon is carried to the receiving atom or molecule. The reciprocal action between energy and matter can be registered in a spectrum where the wavelength is plotted against the absorption.

By monitoring the change in absorbance at a fixed concentration of drugs, PTX or RSV, with increasing amounts of liposomes, the Kp can be determined using the following equation 1:

\[ D_T = D_W + \frac{(D_m - D_w)K_p[L]V_\rho}{1 + K_p[L]V_\rho} \]  

(equation 1)

Where \( D_T \), \( D_w \) and \( D_m \) represents the second or third derivative of the absorbance of respectively the compound: total, in the aqueous phase and in lipid phase. \([L]\) is the concentration of lipids in M and \( V_\rho \) is the lipid molar volume of the liposomes in L.mol\(^{-1}\). The derivative spectra were used to eliminate the light scattered by the liposomes that would make difficult and inaccurate quantification.

Once the \( Kp \) of the compounds are known, thermodynamic parameters of the system can be calculated. Such as the enthalpy change (\( \Delta H \)) between the membrane and the water phase, which is the energy released from the system when it remains under constant pressure. Another important parameter is the entropy change (\( \Delta S \)) of the liposome/water system, which is a measure for the disorder of the system. Using the Van’t Hoff equation (equation 2) these parameters can be obtained with information that is available:

\[ \ln(Kp) = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \]  

(equation 2)

Where, \( R \) is the gas constant (8,314 Jmol\(^{-1}\)K\(^{-1}\)) and \( T \) the temperature in Kelvin. The enthalpy is in kJ.mol\(^{-1}\) and the entropy in kJ.mol\(^{-1}\).K\(^{-1}\). Ultimately with the enthalpy in kJ.mol\(^{-1}\) and
entropy in kJ.mol\(^{-1}\).K\(^{-1}\), the Gibbs free energy (\(\Delta G\)) in kJ.mol\(^{-1}\) can be calculated using equation 3.

\[
\Delta G = \Delta H - T\Delta S
\]

(equation 3)

The Gibbs free energy provides information on whether or not the changes occur spontaneously.

1.5.2 Fluorescence quenching

To determine the binding of PTX to Human Serum Albumin (HSA) fluorescence quenching is used. Emission is the exact opposite of absorption. Energy from an excited atom or molecule is released in the shape of a photon or radiation. Depending on the nature of the excited state (singlet or triplet), there are 2 categories; respectively fluorescence and phosphorescence. The driving force of these mechanisms is the desire of particles to exist in their form with the lowest energy possible. The relaxation from excited state back to the ground state can occur in many different ways but mostly happens by the graduate loss of energy because of collisions with others. Most of the time the energy is lost by heat but sometimes will produce a photon.

Fluorescence quenching occurs when a compound, other than the fluorescent molecule studied, interferes with the measurements and weakens the observed fluorescence. Fluorescence quenching can be used in different manners to determine different variables. In steady state fluorescence quenching for example, a membrane bound fluorescent probe (called fluorophore) is used to determine the location of a compound such as a drug in the membrane. The position of the fluorophore is well known and it emits constant fluorescence. When however there is interference with their environment, the emitted fluorescence will be reduced. In steady state fluorescence quenching, this interference is caused by the addition of a drug. When this compound is partitioned into the membrane and its location is close to the one of the fluorophore. The magnitude of the decrease is an indication of its accessibility to the drug [38].

In this project, the fluorescence quenching method was used to calculate the binding of HSA to the drugs, PTX or RSV. Of the 585 the amino acids in HSA, there is only one tryptophan at position 214 [38]. This is in the hydrophobic cavity and is the reason for its fluorescence. HSA
also has other fluorescent amino acids, phenylalanine and tyrosine. But the quantum yield of phenylalanine in the protein is very low and insufficient. The amino groups close to the tyrosine will cause its fluorescence to almost completely be quenched by them. Therefore it is acceptable to assume that the fluorescence of HSA is only linked to that of the tryptophan [39].

Usually, fluorescence quenching is categorized as either static quenching or dynamic also called collisional quenching. The former is when the fluorophore and the quencher form non fluorescent complexes. When light is absorbed, the complex will return to its ground state without the emission of a photon. In the latter, as says the name, the relaxation to the ground state is caused by a collision of the fluorophore with the quencher. Hereby a photon will be emitted.

Fluorescence quenching is characterized by the Stern-Volmer equation (equation 4).

\[
\frac{F_0}{F} = 1 + K_{SV} [Q] 
\]  
(equation 4)

In the particular case of HSA binding assay, \( F_0 \) and \( F \) represent the fluorescent intensity originated from HSA without and with the drug respectively. \( K_{SV} \) is the Stern-Volmer quenching constant in L.mol\(^{-1}\) and \([Q]\) is the concentration of the quencher, in this case the drug (PTX), in mol.L\(^{-1}\).

1.5.3 Dynamic Light Scattering

With Dynamic Light Scattering (DLS) or Photon Correlation Spectroscopy (PCS), the size of the liposomes is evaluated. This analysis is based in the random movement of particles in a fluid due to collisions between the molecules surrounding them. This is called the Brownian motion. The Stokes-Einstein equation (equation 5) defines the association between particle size and its diffusion coefficient \(D\) because of Brownian motion. Namely the larger the particle, the slower it will move.

\[
d(H) = \frac{kT}{3\pi\eta D} 
\]  
(equation 5)

With \(d(H)\) the hydrodynamic radius of the particles, \(D\) called the translational diffusion coefficient, \(k\) is Boltzmann’s constant, \(T\) is the absolute temperature and \(\eta\) is the viscosity. As seen in the equation, the temperature will influence the measurement. Not only will it affect
the speed of the particles movement and the viscosity of the solvent but an unstable
temperature can induce non-random movements as a result of convention currents [40].

With DLS the average count rate, this is the average number of photons detected per second
or ACR, can be measured as a function of temperature variation. This value is representative
of an emerging macroscopic phenomenon, but not directly size dependent. It may be
adapted to detect changes in the measured scattering intensity due to phase transitions
occurring in the liposomal systems. Thus, discontinuity in the ACR, as the temperature is
altered, corresponds to a change in optical properties of the material studied (i.e. transition
from initial state to another one). Thus, by plotting ACR versus the temperature (T), the
phase transition (Tm) of the liposomal systems produced by a variation of the temperature
can be easily characterized with this technique (Figure 1.5.).

![Figure 1.5: Plotting of the normalized average count rate in function of the temperature in degrees celcius](image)

By fitting experimental data with a modified Boltzman equation 6:

\[
ACR = r_{s1} + p_1T + \frac{r_{s2} - r_{s1} + p_2T - p_1T}{1+10^{(T-Tm)/r_s}}
\]

(equation 6)

Where \( r_{s1} \) and \( p_1 \) are respectively the slope and ordinate at origin of the linear trend
observed before \( Tm \) and \( r_{s2} \) and \( p_2 \) are respectively the slope and ordinate at origin of the
linear trend observed after \( Tm \), it is possible to calculate the main phase transition
temperature \( Tm \) and the cooperativity of the transition (B).
1.5.4 Laser Doppler electrophoresis

This technique is used to determine the surface charge of the liposomes. A charged particle has several ions on its surface which will attract counter ions, ions of the opposite charge. Surrounding the particle there is an electrical double layer. The inner layer, or Stern Layer, consists of very tightly bound ions. The outer layer, the Diffuse layer, contains counter ions less firmly restrained to the particle. Within this diffuse layer not all the ions will show the same behavior. Some, who are closer to the core of the particle, will move with this particle, and others will not. The potential measured at this layer, known as the slipping plane, is the Zeta-potential (Figure 1.6). Besides giving information regarding the surface charge of the particles the zeta-potential gives an idea about the particles stability. The higher the Zeta-potential, the higher the particles’ charge and the least chance that the particles will aggregate [40].

![Figure 1.6 The Zetapotential of an anionic particle [40]](image)

The Zeta-potential is measured by applying an electric field with electrodes at opposite side of the cell where the sample containing the particles is. This will create a potential and cause for the particles to migrate in the direction of the charge opposed to their surface charge. Using Laser Doppler electrophoresis, the speed of this movement is measured. A laser will illuminate the particles and the dispersion of the light will be measured at an angle of 17°. The fluctuations in light scattering are proportional to the velocity and ultimately correlated with the charge of the particles [40].
1.5.5 Steady-state anisotropy measurements as a function of temperature

The steady-state fluorescence anisotropy \((r)\) is based on determining the degree and extent of rotational diffusion of the fluorophore (probe) during the lifetime of the excited state. Small changes in the stiffness of the matrix surrounding the probe produce changes in the rotational movement of the probe and, as such, cause changes in the anisotropy [41].

To determine the steady-state anisotropy, the sample is excited with vertically polarized light and fluorescence intensities are measured with the emission polarizer oriented parallel \((I_\parallel)\) and perpendicular \((I_\perp)\) to the excitation polarizer.

The steady-state anisotropy \((r)\) is then defined by the following relationship (equation 7) between the relative intensities of fluorescence:

\[
r = \frac{I_\parallel - GI_\perp}{I_\parallel + 2GI_\perp}
\]  
(equation 7)

where \(G\) is an instrumental correction factor, given by the ratio of the sensitivities of the detection system for vertically and horizontally polarized light (equation 8) [41]:

\[
G = \frac{I_\perp}{I_\parallel}
\]  
(equation 8)

The determination of steady-state anisotropy involves the use of probes (extrinsic fluorophore) inserted into the membrane, whose photoselective excitation is performed by polarizers. If the molecules of fluorophore are present in a highly ordered membrane, as seen in the gel or solid-crystalline state or in a viscous solvent, their movement is highly restricted. A parallel orientation of the fluorophore molecules is hereby induced to the vertical excitation polarizer. As a result, the molecules of fluorophore emit polarized light because they remain immobile during the lifetime of the excited state. However, if the environment surrounding the fluorophore is the fluid state, the notorious free rotation of the fluorophore molecules pushes a random fluorophore orientation, resulting in a decrease in the emission of polarized light. The explanation for this decrease is based on the lack of alignment with the vertical excitation polarizer (Figure 1.7) [41].
The application of studies of steady-state anisotropy to membrane models allows the determination of the main phase transition temperature of the lipid. The essence of this technique consists on monitoring the anisotropy in a range of temperatures, of a labelled suspension of liposomes. In this work, large unilamellar vesicles (LUVs) of Dimyristoylphosphatidylcholine (DMPC) labelled with Diphenylhexatriene (DPH) were used. Additionally, the liposome suspension was incubated with the drug PTX in order to study their effect.

Typically, sigmoid curves are obtained which show the variation of the anisotropy of fluorophores in liposomes with the temperature, allowing analysing the influence of the drug on the phase transition temperature \( T_m \) and influence on the anisotropy before and after the transition and the transition profile. The parameters of cooperativity \( B \) and \( T_m \), are calculated from the slope and the inflection point of the data fitted to sigmoid curves, respectively, using equation 6 where ACR has been replaced by anisotropy values \( r \).

Due to strong packing, the lipid molecules are unable to disorder gradually, and thus, when the phase transition occurs, there is a sudden increase in the movements of phospholipids. Therefore, the phase transition is a cooperative process where all the lipid molecules are involved. The presence of a foreign molecule in the acyl chain region of the membrane decreases cooperativity, turning the lipid melting into a more gradual, smooth and therefore less cooperative process. Therefore, it is more frequent that the drugs decrease the cooperativity of a process. However, some drugs also increase the cooperativity of the transition, from which can be inferred that the drugs are located within the lipid bilayer but

---

**Figure 1.7** Effects of polarized excitation and rotational diffusion on the anisotropy of the emission. Adapted from [41].
closer to the polar zone, so its presence does not cause any delay on the lipid melting process [42].
2 OBJECTIVES

The drafting of a drug property profile is an important aspect in the drug development process. Such a profile consists of chemical, physical and biophysical properties of the drug in its formulation. With the knowledge retrieved from these analyses, the drug or the formulation containing the drug can be altered to improve its pharmacokinetic properties and hereby predict possible complications in the further development.

DODAB and DODAC are cationic synthetic lipids that only differ in their counter ion being respectively bromide and chloride. Formulated with monoolein (MO) in liposomal formulations, they are used to help dissolve and transport miscellaneous compounds entrapped in the liposome. Paclitaxel and Resveratrol are respectively an anticancer drug and a bioactive compound used in clinical practice against various different cancers. However, both are too lipophilic to ensure optimal absorption and distribution to the cancerous tissues without adjuvant carriers. The purpose of this project is to investigate the influence of the liposomal formulation on the drug property profiles of the encapsulated compounds. Therefore, the partition coefficient of the drug in its nanocarrier was determined at different temperatures and at different pH values. The data was treated using the spreadsheet Kp Calculator, developed by the research team. The results retrieved from the temperature assays were used to calculate the change in Gibbs free energy ($\Delta G$), the change in enthalpy ($\Delta H$) and the change in entropy ($\Delta S$) when drug is transferred from an aqueous phase to a lipid phase. The different pH analyses were used to get insights on the behavior of the formulation at different relevant physiological pH (namely blood pH 7.4 and tumor acidic pH 5).

To evaluate the influence of the liposomes on the binding of the drug to endogenic proteins such as human serum albumin (HSA), a fluorescent quenching assay is conducted in which the fluorescence is evaluated for HSA in the absence and presence of increasing drug concentrations. Also the UV-VIS spectrum is taken to investigate the structural changes PTX induces in HSA. Furthermore the binding to HSA plasma protein was also achieved by Fluorescence quenching assays.
Finally the influence of the drug in the biophysical stability of the formulations was also studied for Paclitaxel by fluorescence anisotropy studies and dynamic light scattering technique.

To sum up the specific objectives of this project were:

1) Determination of partition constants of RSV and PTX in liposome formulations/water by derivative spectroscopy at different interesting physiological pH values (pH 5 and pH 7.4) and at different temperatures;
2) Prediction of plasmatic proteins binding (human serum albumin) to PTX (alone or included in liposome formulation) by fluorescence quenching assays and/or by DLS;
3) Prediction of PTX effect on microviscosity and order of the liposomal system by DLS and/or anisotropy studies.
3 MATERIALS AND METHODS.

3.1 MATERIALS
For the exact weighing of solid specimen the analytical Denver Instrument balance from New York, USA was used. During all the assays 1500 µL eppendorfs were used and the VWR Ultra high performance micropipettes from VWR Prolabo in Pennsylvania, USA with the pipet tips from the same company. For the extrusions of the liposomes, the LIPEX 10 mL Thermobarrel extrudor from Northern Lipids Inc (Burnaby, Canada) were used with the Nucleopore Track-Etch Membrane filters from Whatman (Kent, United Kingdom). For the incubation the bath was brought to the correct temperature using the water bath, VWB series from VWR Prolab (Pennsylvania, USA). The spectrophotometer was from Shimadzu, namely the UV-2101*3101 PC spectrophotometer system from Kyoto, Japan with the UVProbe software. In the temperature controlled assays the TCC-Controller 260 of Shimadzu (Kyoto, Japan) was used to ensure the correct temperature at all time. The Fluorolog-3 spectrofluorometer system by the Horiba Scientific (Kyoto, Japan) was used for all the fluorescence measurements. Because of the low volume of the samples the High Precision cell made of quarts suprasil from Hallma Analytics (Müllheim, Germany) were used to measure in the spectrophotometer and also in the Fluorolog-3. To adjust the pH of the Trizma and acetate buffer the Metrohm pHmeter was used. To perform the Laser Doppler Velocimetry, a Folded capillary cell by Malvern was used in the Zetasizer Nano ZS also by Malvern (Worcestershire, UK). The same machine was used to conduct the DLS experiments with the exposable polystyrol/polystyrene cuvettes of the German company Sarstedt (Nümbrecht, Germany).

For stock solutions, Ethanol pro analysis was used purchased from Merck KGaA (Darmstadt, Germany) and is minimum 99,9% pure. Also the components for the acetate buffer, Na-acetate (>99,5%) and Acetoacetate (>99,8%) are acquired from Merck KGaA. Trizma base (>99,9%) and the human serum albumin (97-99%) are from Sigma-Aldrich (St-Louis, Missouri, USA). When not used this product was stored at a temperature of 4°C.
3.2 METHODS

3.2.1 Preparation of liposomes
Liposomes were prepared using Bangham's method. In both cases (DODAB and DODAC) a ratio of cationic lipid: MO of 1:2 was used. After precise weighting, previously calculated amounts of DODAB, DODAC and MO were dissolved in ethanol to make 20 mM stock solutions. The ethanol was completely evaporated using N₂ to form lipid films on the inside walls of the glass container. The lipid films were stored in the glass container protected from light with aluminum foil in the freezer at -20°C.

On the day the liposomes were used, the films were hydrated with ultra pure water at 60°C, which is above the transition temperature (≈38°C) of the lipids [43]. After vortexing and quick heating to make sure that all the film was removed from the walls, the multilamellar vesicles (MLV) were extruded 5 times through a filter of 200 nm pores diameter and 10 times with a 100 nm pores diameter, all at 60°C. Currently extrusion is one of the best methods to ensure a group of vesicles with a certain homogeneous size and geometry if compared to other ways such as sonication and ethanolic injections [44].

3.2.2 Kp determination

3.2.2.1 Kp determination at different temperatures.
[DODAC:MO](1:2) liposomes (5000 µM) prepared as explained above, were cooled down. The eppendorfs were filled with 128 µL of PTX₄E₉₀ (234,22 µM) and the ethanol was evaporated. The final concentration of PTX in the eppendorfs of 1500 µL was 20 µM. After this, they were filled with increasing concentration of liposomes. There were 3 samples who did not contain any liposomes, to be able to measure the absorbance of the PTX in water (D₆₀). Then the volume of the eppendorfs was completed up to 1500 µL with ultra pure H₂O. The exact same procedure was followed to make the references but without the PTX. The eppendorfs were incubated at 60°C for 30 minutes to let the drug and the liposomal formulations interact. The absorbance of the samples and references were measured in the spectrophotometer using the Uvpc program and the TCC Controller 260 at 30°C, 37°C, 50°C, 55°C and 60°C. For PTX the wavelengths between 200 and 300 nm were measured at medium scan speed with a slit width of 2. For RSV (100 µM), which is dissolved in ultra pure water, no evaporation was needed. The eppendorfs were filled with 600 µL RSV (100 µM),
making a final concentration of 40 μM RSV in each eppendorf. In analogy of the PTX, samples with [DODAB:MO](1:2) liposomes (5000 μM), without liposomes but with RSV to measure D_w and another series of eppendorfs without RSV with liposomes were made as references. Before measuring, all the eppendorfs were placed in a bath of 60°C for 30 minutes. In the UV-spectrophotometer wavelengths between 200 and 400 nm were measured at slow scan speed with a slit width of 1 in the same temperature conditions as used for PTX. Before each measurement, we waited about 5 minutes for the temperature to stabilize inside the cell. The data was treated with Excel 2007, OriginPro 9.0 and the spreadsheet Kp Calculator, a tool developed by elements of the research group that represents a step forward for fast determination of this parameter, as data analysis is also a time-consuming step.

3.2.2.2 Kp determination at different pH values
The eppendorfs were prepared the same way as for the measurements at different temperatures but with the use of different buffered solutions instead of water. 1 Liter of the Trizma buffer (0,1 M), with a pH of 7,4 was made by weighing 24,2284 g of Trizma base and adding 1L of H_2O ultrapure. Then the pH was checked and adjusted using HCl (6 M). The buffer to create a pH of 5, acetate buffer (0,2 M), was made by combining 2 compounds. Exactly 2,353 mL acetic acid (0,2 M) in 197,647 mL and the exact measurement of 8,203 g sodium acetate (0,2 M) in 500 mL H_2O ultrapure. These were combined on the day of use by adding respectively 14,8 mL and 35,2 mL in a 100 mL volumetric flask and completing this with ultrapure H_2O.

UV measurements and data treatment were performed in the same way as described in 3.2.2.1. but at room temperature.

3.2.3 HSA binding
Preparation of stock solution of HSA was made by weighing exactly 11,97 mg of the HSA stored at 4°C and adding up to 10 mL H_2O ultrapure in a volumetric flask at the day of use. This results in a solution of HSA with a concentration of 18 μM. After the preparation of the liposomes as described before, 8,520 mL of the [DODAC:MO](1:2) liposomes (6000 μM) were transferred to a falcon that contained 4355 μL of EtOH evaporated PTX (234,22 μM). In the obtained 2mol % PTX suspension of liposomes and PTX;, the concentration of PTX was 120 μM. Different sets of eppendorfs were prepared, some with the liposomes with PTX
(120 µM) and some with the residual liposomes. This resulted in eppendorfs with HSA (18 µM) and liposomes with PTX; liposomes with PTX (120 µM) without HAS; HSA (18 µM) and liposomes without PTX; and liposomes without PTX or HSA. The difference within 1 series of eppendorfs was the increase of the amount of liposomes, and hereby also in PTX if present. There was a last series of eppendorfs prepared without liposomes, but with HSA (18 µM) and different concentrations of PTX. These samples concerning PTX were all measured in the spectrophotometer from 200 to 300 nm at medium scan speed and slit width of 2. In the fluorimeter the wavelength of excitation was 260 nm and the range of 290 until 400 nm was measured. The size of the particles was measured with the Zetasizer Nano ZS at 37°C, performing 5 measurements per sample and an equilibration time of 120 seconds per sample before the first measurement was made. To know the surface charge, the same machine was used but performed only 1 measurement per sample also at 37°C and with 60 seconds of equilibration time. Later the data was treated using the Zetasizer software, Excel 2007 and OriginPro 9.0.

3.2.4 Effect of PTX in membrane microviscosity

3.2.4.1. Effect of PTX in membrane microviscosity evaluated by DLS

Preparation of DMPC liposomes in water were made by the film hydration method followed by extrusion to obtain LUVs of 100 nm as previously described. After the preparation of the liposomes as described before, 5 mL of the DMPC liposomes (1000 µM) were transferred to a falcon that contained 107 µL of EtOH evaporated PTX (5 µM). This originated liposomes of DMPC containing 0,5mol% of PTX, added by incubation.

For both liposomes of DMPC without or with PTX (0,5 mol%) DLS measurements were performed at different temperatures (15-40°C). A first optimization stage was performed, where the cell positions, compensation and attenuator settings for the cell, sample and measurement type were determined. By default the software adjusts these values by itself—based on sample optical properties such as turbidity, etc. This stage was repeated three times so as to achieve accurate reproducibility in the intensity of scattered light. Parameters were then introduced and locked manually (overcoming the default software settings) for the second stage of experimentation. In this step, software was used in trend mode that allows multiple measurements to be made over a range of temperatures.
Parameters were selected with the first and final temperatures of the trend (15-40°C), with temperature intervals of 1°C and stabilization times of 5 min before each measurement.

Data were collected as ‘average count rate versus temperature’ or ‘normalized average count rate versus temperature’ for comparison purposes and treated with a modified Boltzmann regression equation (equation 6) to determine the transition temperature $T_m$ and cooperativity ($B$).

3.2.4.2. Effect of PTX in membrane microviscosity evaluated by steady-state anisotropy

For DPH labeled liposomes, DPH probe was co-dried with the lipid to give a lipid/probe molar ratio of 300:1. Typically a stock ethanolic solution 33,90 mg of DMPC in 15 mL ethanol was prepared, and 2,32 mg of the probe was prepared in 2 mL ethanol. Then 1000 µL of ethanolic lipid solution was co-dried with 10 µL of probe stock solution.

After labeling the lipid with the probe and hydration of the thin lipid film with 10 mL of ultrapure water, the preparation of labeled MLVs follows the procedure previously described. Large unilamellar liposomes (LUVs) were then prepared by extrusion of the MLVs suspension at 37 °C (temperature above the phase transition temperature of the lipid). After the preparation of the liposomes as described before, 5 mL of the DMPC liposomes (1000 µM) were transferred to a falcon that contained 107 µL of EtOH evaporated PTX (5 µM). This originated liposomes of DMPC containing 0,5mol% of PTX, added by incubation. At the end we have 5 mL of LUVs of DMPC labeled with DPH probe not containing PTX and 5 mL of LUVs of DMPC labeled with DPH probe containing 0,5 mol% PTX. Both lipid samples were analyzed by the measurement of the steady-state fluorescence anisotropy at several temperatures: 15-40°C, with temperature intervals of 1°C and stabilization times of 5 min before each measurement. Steady-state anisotropy values were calculated after insertion of horizontal and vertical polarizers and measuring fluorescence emission of the probe at $\lambda=444$ nm using an excitation of $\lambda=380$ nm. Steady-state anisotropy was calculated for each temperature according to equations 7 and 8.
4 RESULTS

4.1 KP DETERMINATION

4.1.1 Kp determination of RSV

4.1.1.1 Kp determination at different pH values

Two different pH conditions were studied, namely pH 7,4 and pH 5. The collected data was used to calculate Kp with the spreadsheet Kp calculator. When measuring the UV-VIS absorption of the PTX in DODAC:MO liposomal formulation, background signals are visible. These are due to the scattering of the DODAC:MO liposomes in the solution. To reduce the effect caused by the scattering of liposomes, the 1st, 2nd and 3rd derivative of the spectra are taken. The quantification of Kp is made in the maximum or minimum of the derivative spectra, in a wavelength where the scattering is eliminated. This will result in higher reproducibility and ensure the desired signal to background noise is as high as possible. In Figure 4.1 and Figure 4.2., respectively the 2nd and 3rd derivative spectra of the DODAB:MO (1:2) liposomes with RSV in the acetate buffer pH 5 are illustrated with different liposomal concentrations. With increasing lipid concentrations a shift in the spectra maximum is visible. This is a shift towards a higher wavelength, namely from 343 nm when RSV is in water in the absence of liposomes to 352 nm when RSV is in the presence of the highest liposome concentration at [DODAB:MO] = 3 x 10^{-3} M.
Figure 4.1 Second derivative of resveratrol spectra at pH 5 and at different liposome concentrations (M): (A) 0, (B) 5x10^{-5}, (C) 1x10^{-4}, (D) 2x10^{-4}, (E) 4x10^{-4}, (F) 5x10^{-4}, (G) 6x10^{-4}, (H) 8x10^{-4}, (I) 1x10^{-3}, (J) 2x10^{-3}, (K) 3x10^{-3}. The derivative spectra of the liposomes without resveratrol are represented in black.

Figure 4.2 Third derivative of resveratrol spectra at pH 5 and at different liposome concentrations (M): (A) 0, (B) 5x10^{-5}, (C) 1x10^{-4}, (D) 2x10^{-4}, (E) 4x10^{-4}, (F) 5x10^{-4}, (G) 6x10^{-4}, (H) 8x10^{-4}, (I) 1x10^{-3}, (J) 2x10^{-3}, (K) 3x10^{-3}. The derivative spectra of the liposomes without resveratrol are represented in black.
Using OriginPro 9.0 Figure 4.3. was constructed for the data collected with the resveratrol DODAB:MO (1:2) liposomes in acetate buffer (pH 5) at λ=311 nm. This was done for all data at pH 5 and 7,4 at all λ<sub>max</sub> where the scattering was eliminated. The λ<sub>max</sub> chosen at both pH values were for the second derivative 352 nm and for the third derivative 311, 324, 343, 352, 359 and 367 nm. These plots for pH 5 and 7,4 can be found in appendix I and II.

Results are summarized in Table 4.1. for pH 5 and in Table 4.2. for pH 7,4. The average of these values was taken and used to calculate firstly the average K<sub>p</sub> in mol<sup>-1</sup>L, and then the adimensional value by multiplying the previous by the lipid molar volume (in Lmol<sup>-1</sup>). For the liposomal formulation at pH 5 the final adimensional value of logK<sub>p</sub> is 3,34 ± 0,071. For the formulation at pH 7,4 the final adimensional value of logK<sub>p</sub> is 3,31 ± 0,031.

**Table 4.1** K<sub>p</sub> values for Resveratrol DODAB:MO (1:2) at pH 5 at different wavelengths ± SD.

<table>
<thead>
<tr>
<th>2&lt;sup&gt;nd&lt;/sup&gt; Derivative</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt; Derivative</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ=352 nm</td>
<td>λ=311 nm</td>
</tr>
<tr>
<td>1004 ± 285</td>
<td>1288 ± 127</td>
</tr>
<tr>
<td>λ=324 nm</td>
<td>λ=343 nm</td>
</tr>
<tr>
<td>1338 ± 138</td>
<td>1487 ± 271</td>
</tr>
<tr>
<td>λ=352 nm</td>
<td>λ=359 nm</td>
</tr>
<tr>
<td>1334 ± 150</td>
<td>1427 ± 197</td>
</tr>
<tr>
<td>λ=367 nm</td>
<td></td>
</tr>
<tr>
<td>1594 ± 375</td>
<td></td>
</tr>
<tr>
<td>2nd Derivative</td>
<td>3rd Derivative</td>
</tr>
<tr>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>λ=352 nm</td>
<td>λ=311 nm</td>
</tr>
<tr>
<td>905 ± 325</td>
<td>1189 ± 107</td>
</tr>
</tbody>
</table>

**4.1.1.2 Kp determination at different temperatures**

The determination of the Kp of RSV in [DODAB:MO 1:2] liposomal formulations gives the opportunity to calculate thermodynamic parameters of the system. Following Figure 4.4. illustrates the 3rd derivative of the UV-spectrum obtained at a wavelength of 311 nm as function of increasing concentration of the liposomes at 30°C, 37°C, 50°C, 55°C and 60°C.

![Graph](image_url)

*Figure 4.4 Third derivative spectrophotometric data collected at λ = 259 nm for resveratrol and DODAB:MO (1:2) liposomes (M) at different temperatures with black: 30°C, red 37°C, green 50°C, dark blue 55°C and light blue 60°C.*

The phase transition temperature of the liposomal formulation will vary because of the presence of the drug. The transition temperature in absence of the RSV is 38°C. The temperatures measured are above and below this value. As a consequence, the liposomes will be in different states according to the temperature. If it is below the Tm, the liposomes will be in a more rigid, gel state (Lβ). Above the Tm they will be in a liquid crystalline or fluid
state (Lα). Therefore the Van’t Hoff equation (equation 2) will present a biphasic appearance with two linear plots corresponding to each phase. This can be seen in Figure 4.5. Kp was calculated for each temperature using the same methods explained in 4.1.1.1.

![Figure 4.5: Van’t Hoff Plots of resveratrol in LUVs of DODAB:MO (1:2). The pink square represents the Lα phase and the blue square represents the Lβ phase](image)

Using this graph, it is possible to calculate the phase transition temperature of the DODAB:MO (1:2) liposomes with encapsulated resveratrol by determining the intersection of the two linear plots. Relying on the collected data, we can conclude that RSV increases the disorder of the lipid system which is related with an increase of the entropy of the system which is a favorable situation. Because of this, the liposomal formulation will have a smaller tendency to transition into a state with more energy and higher entropy.

The thermodynamic parameters obtained for resveratrol partition between aqueous phase and the DODAB:MO (1:2) were calculated based on the temperature dependence of Kp value using the Van’t Hoff equation (equation 2). The gathered results are shown in Table 4.3.
As can be seen in Table 4.3., the change in Gibbs free energy ($\Delta G$) is negative for each temperature. The change in enthalpy ($\Delta H$) when transitioning from the aqueous to the DODAB:MO liposomal phase, is greater than zero as is the entropy ($\Delta S$) of the partitioning for each of the temperatures studied. The data collected below transition temperature have a very much lower change in enthalpy change ($6,00 \pm 0,68$ KJ.mol$^{-1}$) when compared to the data collected at higher temperatures ($43,8 \pm 5,05$ KJ.mol$^{-1}$). The calculated entropy change is greater at lower temperatures ($0,2 \pm 0,005$ KJ.mol$^{-1}$.K$^{-1}$) than at temperatures above $T_m$ ($0,083 \pm 0,0022$ KJ.mol$^{-1}$.K$^{-1}$).

### 4.1.2 Kp determination of PTX

#### 4.1.2.1 Kp determination at different pH values

The results of the Kp calculations of PTX in DODAC:MO (1:2) liposomes at different pH values were treated by firstly choosing the wavelength ($\lambda$) with a minimum or maximum where the background signal is sufficiently reduced. The background signal is produced by the lipid scattering of the DODAC:MO (1:2) liposomes. To diminish this scattering effect the 1st, 2nd and 3rd derivative of the UV-spectra were taken. For both pH 5 and pH 7,4 the $\lambda_{\text{max}}$ at 256 nm of the 2nd derivative and the $\lambda_{\text{min}}$ at 265 nm of the 3rd derivative were used (Figure 4.6).
Figure 4.6: Third derivative of paclitaxel spectra at pH 5 at different liposome concentrations (M): (A) 0, (B) 5x10^-5, (C) 1x10^-4, (D) 2x10^-4, (E) 4x10^-4, (F) 5x10^-4, (G) 6x10^-4, (H) 8x10^-4, (I) 1x10^-3, (J) 2x10^-3, (K) 3x10^-3. The derivative spectra of the liposomes without paclitaxel are represented in black.

With increasing concentration of DODAC:MO (1:2), the $\lambda_{\text{max}}$ shifts from 265 nm when PTX is in water in the absence of liposomes to 262 nm when PTX is in the presence of the highest liposome concentration at [DODAB:MO] = 3x10^-3 M.

The $K_p$ was calculated with the same techniques as described for RSV. Also using OriginPro 9.0, UV-spectrophotometric data was plotted against different liposomal concentrations as in Figure 4.7 and Figure 4.8. As an example the 2$^{rd}$ and 3$^{rd}$ derivative spectra of PTX at respectively $\lambda = 256$ nm and $\lambda = 265$ nm at different concentrations of DODAC:MO (1:2) in H$_2$O ultrapure are shown. For the plots made with the data collected at pH 5 and pH 7.4 see appendix III and appendix IV respectively.
This analysis was repeated in acetate buffer creating a pH of 5 and in Trizma base, resulting in a pH of 7.4. Measurements were taken at the same wavelengths as were done for pH 5. The final adimensional values for logKp in acetate buffer, in Trizma base and in H2O ultrapure are summarized in Table 4.4.
Table 4.4. The final Kp and logKp values for PTX in DODAC:MO (1:2) formulations at pH 5; pH 7.4 and in ultrapure water

<table>
<thead>
<tr>
<th></th>
<th>Acetate buffer (pH 5)</th>
<th>Trizma base (pH 7.4)</th>
<th>H2O ultrapure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kp PTX (M⁻¹)</td>
<td>542 ± 18</td>
<td>603 ± 339</td>
<td>1807 ± 485</td>
</tr>
<tr>
<td>logKp PTX</td>
<td>2.39 ± 0.014</td>
<td>2.43 ± 0.276</td>
<td>3.26 ± 0.119</td>
</tr>
</tbody>
</table>

4.1.2.2 Kp determination at different temperatures

The Kp of PTX in [DODAC:MO 1:2] formulation was determined to be able to calculate the thermodynamic parameters associated with the drugs partition in the biphasic system. Firstly, with the knowledge of the Kp in dependence of different temperatures, the membrane to water variation in the enthalpy (ΔH) and in the entropy (ΔS) can be learned using the well known Van’t Hoff equation (2). In Figure 4.9, the Van’t Hoff plot of the PTX-DODAC:MO liposomal formulation is shown. The Kp was calculated as explained above at 30, 37, 50, 55 and 60°C.

![Van't Hoff plot of paclitaxel in LUVs of DODAC:MO (1:2).](image)

In this temperature range, the liposomes are in liquid-crystalline or fluid phase (Lα). This is because the phase transition temperature of the DODAC:MO (1:2) formulation without the drug is below the lowest temperature measured, being 303 K. This is why the Van't Hoff plot (figure 4.9.) has a monophasic appearance with one linear plot. Using the slope and
interception of this plot, the aqueous to DODAC:MO variation of the enthalpy and of the entropy respectively can be calculated. The thermodynamic parameters in Table 4.5. were however acquired using equation 2 and 3.

Table 4.5. The variation of the enthalpy (ΔH) ±SD, entropy (ΔS) ±SD and Gibbs free energy (ΔG) obtained for the PTX partition between the aqueous phase and DODAC:MO (1:2) liposomes at different temperatures

<table>
<thead>
<tr>
<th>Temperature (K)</th>
<th>ΔH w→m (KJ.mol⁻¹)</th>
<th>ΔS w→m (KJ.mol⁻¹.K⁻¹)</th>
<th>ΔG w→m (KJ.mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>303</td>
<td>-4,23 ± 2,05</td>
<td>0,049 ± 0,0064</td>
<td>-19,13</td>
</tr>
<tr>
<td>310</td>
<td></td>
<td></td>
<td>-19,47</td>
</tr>
<tr>
<td>323</td>
<td></td>
<td></td>
<td>-20,11</td>
</tr>
<tr>
<td>328</td>
<td></td>
<td></td>
<td>-20,35</td>
</tr>
<tr>
<td>333</td>
<td></td>
<td></td>
<td>-20,60</td>
</tr>
</tbody>
</table>

As shown in Table 4.5. the change in enthalpy for each of the temperatures is negative. The change in entropy is greater than zero. The resulting change in Gibbs free energy is negative. At higher temperatures, the ΔG becomes more and more negative.

4.2 HSA BINDING

4.2.1 HSA binding of PTX in water

Whether the quenching mechanism of a certain fluorophore with a certain quencher is static or dynamic can be distinguished by their dependence on temperature. When dealing with static fluorescence quenching, an increase in temperature will cause dissociation of the weakly bound complexes formed. This will result in lower quenching quantity and a lower $K_{sv}$ value. When the mechanism is dynamic or collisional, an increase in the diffusion rate of the quencher to the fluorophore occurs. This will ensure higher rate of collisions and hereby increase the quenching. The $K_{sv}$ will also increase with increasing temperature.
To determine the fluorescence quenching, firstly the fluorescence spectra of the human serum albumin-paclitaxel system were taken with increasing concentrations of PTX (Figure 4.10 A). The relative fluorescence intensity is $F_0/F$ with $F_0$ being the fluorescence intensity of HSA without PTX and $F$ the fluorescence intensity of HSA with PTX.

Using the Stern-Volmer equation (equation 4), the Stern Volmer constant ($K_{sv}$) can be calculated when $F_0/F$ is plotted in function of the concentration of $Q$, in this case PTX. This is called the Stern-Volmer plot and is depicted in Figure 4.10 B. The value found for $K_{sv}$, $327 \pm 71 \text{ L.mol}^{-1}$, is the slope of the graph with intercept at $F_0/F = 1$. This is very similar to results found in other literature [45]. In those data, the $K_{sv}$ showed to be inversely proportional to the temperature. This is proof that the mechanism behind the binding of HSA to PTX is static, meaning that the quenching is caused because of the formation of HSA-PTX complexes rather than the collision between the molecules. For this mechanism the binding constant ($K_b$ in $\text{M}^{-1}$) and number of binding sites of PTX on HSA ($n$) can be calculated using equation 9.

$$\log \frac{F_0-F}{F} = \log K_b + n \log [PTX]$$

(equation 9)
Where \( F_0 \) and \( F \) still represent the steady-state fluorescence intensity of HSA and HSA with PTX respectively and \([\text{PTX}]\) is the concentration of the drug in M. When plotting \( \log \left( \frac{F_0 - F}{F} \right) \) against \( \log [\text{PTX}] \), \( K_b \) and \( n \) can be calculated (Figure 4.11 and Table 4.6). The intercept of the plot is \( \log K_b \) and the slope is the number of binding sites or \( n \).

\[
\log ((F_0 - F)/F) = \log K_b + \log [\text{PTX}] (M)
\]

**Figure 4.11.** The Hill plot of the HSA–paclitaxel system at room temperature with \([\text{HSA}]=9 \mu M\), \([\text{paclitaxel}]= 0, 10, 24, 72, 96, 120, 144, 168, 192, 216 \mu M\)

**Table 4.6:** The Stern-Volmer constant and binding parameters for the binding of HSA to PTX. With R the linear correlation coefficient.

<table>
<thead>
<tr>
<th>Stern-Volmer constant</th>
<th>Binding parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_{SV} ) (M(^{-1}))</td>
<td>( R )</td>
</tr>
<tr>
<td>327 ± 71</td>
<td>0,85</td>
</tr>
</tbody>
</table>
Uv-Vis absorption spectra were also acquired because they are an easy way the investigate the structural changes of HSA caused by the addition of PTX. In static quenching, there is a complex being formed between the fluorophore (HSA) when in its ground state and the quencher (PTX). This complexation will influence the UV-VIS absorption spectra. In Figure 4.12 this spectrum is illustrated.

When the concentration of PTX in the HSA-PTX system increases, the intensity at $\lambda_{\text{max}}$ seen at about 230 nm also increases. Also a red shift, towards a higher wavelength is observed.

The binding of PTX to HSA was also evaluated analyzing the same samples used for fluorescence quenching studies by DLS. The goal was to evaluate if the binding from PTX and the protein HSA resulted in some changes in size of HSA protein and surface charge. Figure 4.13. presents the changes in size (decrease of sizes upon PTX binding) and zeta-potential values (zeta-potential values become more negative upon PTX binding) upon PTX binding.
4.2.2 HSA binding of liposomes containing PTX

The determination of HSA binding to the liposomes containing PTX will be achieved by evaluating the fluorescence obtained in increasing concentrations of LUVs of DODAC:MO (1:2). HSA was added to liposomes containing a fixed concentration enzyme and increasing concentrations of liposomes (in the range of 10–1500 µM). The correspondent reference solutions were identically prepared in the absence of HSA. The absorption spectra of samples and reference solutions were recorded using quartz cells with 1 cm path length, in the 200–400 nm range. After measurements the spectrum for the reference was subtracted from that of the sample to obtain corrected absorption spectra. Derivative spectra were calculated using the Savitzky–Golay method in which a second-order polynomial convolution of 13 points was employed. Binding constants of HSA were calculated by the non linear fit of the values of derivative spectra at λ where the liposome scattering was eliminated according to equation 1. This process is illustrated in Figure 4.14
The binding of HSA to empty liposomes of DODAC:MO (1:2) (without PTX) will be achieved by the same method previously described at 4.2.2. HSA was added to liposomes containing a fixed concentration enzyme and increasing concentrations of liposomes (in the range of 10–1500 µM). The correspondent reference solutions were identically prepared in the absence of HSA. The absorption spectra of samples and reference solutions were recorded using quartz cells with 1 cm path length, in the 200–400 nm range. After measurements the spectrum for the reference was subtracted from that of the sample to obtain corrected absorption spectra. Derivative spectra were calculated using the Savitzky–Golay method in which a second-order polynomial convolution of 13 points was employed. Binding constants of HSA were calculated by the non linear fit of the values of derivative spectra at λ where the liposome scattering was eliminated according to equation 1.

**4.2.3 HSA binding of Liposomes without PTX**

The binding of HSA to empty liposomes of DODAC:MO (1:2) (without PTX) will be achieved by the same method previously described at 4.2.2. HSA was added to liposomes containing a fixed concentration enzyme and increasing concentrations of liposomes (in the range of 10–1500 µM). The correspondent reference solutions were identically prepared in the absence of HSA. The absorption spectra of samples and reference solutions were recorded using quartz cells with 1 cm path length, in the 200–400 nm range. After measurements the spectrum for the reference was subtracted from that of the sample to obtain corrected absorption spectra. Derivative spectra were calculated using the Savitzky–Golay method in which a second-order polynomial convolution of 13 points was employed. Binding constants of HSA were calculated by the non linear fit of the values of derivative spectra at λ where the liposome scattering was eliminated according to equation 1.
After fitting the experimental values with equation 1 it is possible to calculate $K_b$ of HSA to liposomes of DODAC:MO (1:2) with or without 0.5 mol% of PTX (Table 4.7.)

<table>
<thead>
<tr>
<th>Liposomes of DOBAC:MO (1:2)</th>
<th>Liposomes of DODAC:MO (1:2)+0.5 mol% of PTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_b \text{ (M}^{-1}\text{)}$</td>
<td>$R^2$</td>
</tr>
<tr>
<td>719 ± 281</td>
<td>0.986</td>
</tr>
</tbody>
</table>

4.3 EFFECT OF DRUGS IN THE MICROVIS COSITY OF MEMBRANES

4.3.1 Effect of PTX in membrane microviscosity determined by dynamic light scattering

The transition temperature of DMPC liposomes in the absence and presence of 0.5 mol% of PTX were analyzed by plotting the ACR as a function of temperature (Figure 4.15).

![Figure 4.15](image-url)
After fitting the experimental values with equation 6 it is possible to calculate $T_m$ and $B$ for liposomes of DMPC with or without 0.5 mol% of PTX (Table 4.8.)

Table 4.8: The main phase transition temperature ($T_m$) and the cooperativity of the phase transition ($B$) of liposomes of DMPC without or with 0.5 mol% of Paclitaxel (PTX).

<table>
<thead>
<tr>
<th>Liposomes of DMPC</th>
<th>Liposomes of DMPC+0.5% mol% of PTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_m$ (°C)</td>
<td>$T_m$ (°C)</td>
</tr>
<tr>
<td>24.01 ± 0.029</td>
<td>23.58 ± 0.646</td>
</tr>
<tr>
<td>$B$</td>
<td>$B$</td>
</tr>
<tr>
<td>580 ± 37</td>
<td>358 ± 254</td>
</tr>
<tr>
<td>$R^2$</td>
<td>$R^2$</td>
</tr>
<tr>
<td>0.999</td>
<td>0.972</td>
</tr>
</tbody>
</table>

4.3.2 Effect of PTX in membrane microviscosity determined by fluorescence anisotropy

The transition temperature of DMPC liposomes in the absence and presence of 0.5 mol% of PTX were analyzed by plotting the steady-state anisotropy values as a function of temperature (Figure 4.15).

![Steady-state anisotropy values (r) as a function of temperature of liposomes of DMPC in the absence (black dots) and in the presence of 0.5 mol% of paclitaxel (red dots). Fitting lines were obtained by fitting experimental data with equation 6 where ACR was substituted by r values. Each value is expressed as the mean ± S.D. (n = 2).](image)

After fitting the experimental values with equation 6 after substituting ACR by $r$ values it is possible to calculate $T_m$ and $B$ for liposomes of DMPC with or without 0.5 mol% of PTX (Table 4.9.)
Table 4.9: The main phase transition temperature ($T_m$) and the cooperativity of the phase transition ($\theta$) of liposomes of DMPC without or with 0.5 mol% of Paclitaxel (PTX) determined by steady-state anisotropy measurements.

<table>
<thead>
<tr>
<th>Liposomes of DMPC</th>
<th>Liposomes of DMPC+0.5 Mol% of PTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_m$ (°C)</td>
<td>B</td>
</tr>
<tr>
<td>26.46 ± 0.271</td>
<td>500± 37</td>
</tr>
</tbody>
</table>
5 DISCUSSION

In the medicinal chemistry, the thermodynamics of the transfer of drug components from one medium into another are useful parameters. They can be calculated when the rational partition coefficient (Kp) is measured as a function of different temperatures. The retrieved information can be used to predict the absorption, membrane permeability and the in vivo distribution of the drug. The Van’t Hoff analysis of the Kp in dependence of the temperature allows the calculation of the membrane-water variation of enthalpy (ΔH), entropy (ΔS) and hereby also the variation in Gibbs free energy (ΔG).

When studying RSV in the DODAB:MO (1:2) liposomes at different temperatures, the Kp was calculated as a way to know the thermodynamics of the system. As depicted in Table 4.3, the variation in Gibbs free energy (ΔG), when transitioning from one phase to the other, is negative for all temperatures measured. This means that the partitioning of the RSV from the aqueous phase to the DODAB:MO liposomal phase occurs spontaneously. This is as expected since the drug is hydrophobic and will preferably be located in the more lipophilic areas of the liposomes. The change in enthalpy (ΔH) when the drug is transferred from the aqueous phase to the liposomal, is positive. Enthalpy depicts the energetic requirement of the system and a positive ΔH means it is an endothermic reaction. This means that the energy of the system after the transition will be greater than before. This is not a favorable situation. Also the entropy of the system for partitioning, which is a measure for the disorder, is greater than zero. It can be explained as followed. In the beginning, the drug is present in only the aqueous phase. In order for the transition into the liposomal phase, which consists of lipids organized in a specific manner, there must be a rearrangement of the molecules. The bilayers must be separated to create space to accommodate the drug. Once the RSV is contained in the liposomal phase, it will interact with the lipid molecules and will release energy. Due to the disorder created in an otherwise very organized bilayer system, there will be an increase in entropy. Now that the drug is (partially) disappeared from the aqueous phase, the space that it was occupying, is now again filled with water molecules. This will create new water-water interactions and hereby release energy. It is however possible that the drug initially, when in the aqueous phase, also interacted with the water,
specially because RSV has hydrophilic hydroxyl groups that can establish hydrogen bounds with water.

Within the temperature range, the values differ greatly depending whether the liposomes are below (303K and 310K) or above (323K, 328K and 333K) the phase transition temperature. At the lower temperatures the liposomes are in a more rigid state as opposed to the temperatures above phase transition temperature. Here the liposomes are in a more fluid state. The more rigid the liposomes are, the more energy will be required to separate the bilayers and accommodate the RSV inside of the liposomes.

The chosen pH values for this assay represent different physiological fluids and organs. A pH of 7.4 is not only encountered in the bloodstream but also in the cerebrospinal fluid. In the small intestine and cancer tissue the pH is 5. The $K_p$ determination using UV-VIS spectrophotometry is based on the fact that the spectral characteristics of the liposomal formulation will change when the drug, RSV, will transfer from the aqueous phase to the liposomal phase. The shift of $\lambda_{\text{max}}$ seen when increasing the concentration of liposomes indicates a decrease in the polarity of the molecule. This indicates the transition of the drug from one phase to the other. It's indicative for the incorporation of the drug in the hydrophobic parts of the liposomes. The isobestic points demonstrate that the drug is present in both phases. When looking at Figure 4.1. and Figure 4.2. the background signal, produced by the scattering of the lipid itself is significantly reduced in the $2^{\text{nd}}$ derivative and practically eliminated in the $3^{\text{rd}}$ derivative.

The values for the log $K_p$ for Resveratrol DODAB:MO (1:2) liposomal formulation at pH 5 and pH 7.4, respectively 3.34 ± 0.071 and 3.31 ± 0.031 are very similar. This is not surprising looking at Figure 1.2. and the acid dissociation constants (pKa) of the drug. Them being 8.73; 9.56 and 10.88 for C10, C3 and C13 respectively calculated with Marvin sketch calculator by Chemaxon™. From this and the Henderson-Hasselbalch equation, we can conclude that the drug will be 99.98% non-ionized at pH 5 and 95.5% at pH 7.4. Since being in the same neutral state in both conditions there will be mainly hydrophobic interactions with the lipids of the membrane. Furthermore we can conclude that Resveratrol has a high partition coefficient in the liposomal formulation. This corroborates the adequacy of the nanocarrier systems to encapsulate the drug. Because this means that the concentration of the drug in the
liposomal phase is greater than the concentration in the aqueous phase, meaning it will be entrapped and transported within the liposomes.

For the PTX in DODAC:MO (1:2) formulation studied at different temperatures, it is interesting to compare the retrieved results with other hydrophobic compounds. In this project the membrane to water variation in enthalpy found for Paclitaxel was $-4.23 \pm 2.05$ KJ.mol$^{-1}$ and a Kp value in water at 37°C of 1807M$^{-1}$. A first example is the Ca$^{2+}$ channel antagonist with a surface charge of +1, Amlodipine. Kp value obtained for this drug are 15500 M$^{-1}$ and a $\Delta H$ of -9,2 kcal.mol$^{-1}$. Another is Flunarizine, a class IV Ca antagonist with a charge of +1, Kp of 28700 M$^{-1}$ and $\Delta H$ of -5,3 kcal.mol$^{-1}$. The electrostatic effects of both examples are corrected for in the calculation of the Kp, which makes it possible to compare the values with the neutral Paclitaxel [46]. All of the $\Delta H$ are greatly negative. This means that for every mentioned drug, heat is released when the molecule migrates from the aqueous medium to the lipid surroundings. Similar results are found for a few other cationic peptides. However, Paclitaxel is the first neutral hydrophobic compound with negative enthalpies. This could be explained by the partitioning into the lipophilic parts of the liposomes. This results in strong Van der Waals interactions being the reason for the negative enthalpy.

With a change in Gibbs free energy of -19,13 KJ.mol$^{-1}$ at 303K up to -20,60 KJ.mol$^{-1}$ at 333K and an enthalpy of only $-4.23 \pm 2.05$ KJ.mol$^{-1}$ it is apparent that the enthalpy will most likely not be the driving force of the reaction. This means that the change in entropy must be the impellent force. When the temperature is raised, the Gibbs free energy becomes more negative, which is a more favorable situation. These findings are contradictory to other research done on the thermodynamics of Paclitaxel, however the membrane system used in the mentioned study was of a different composition [46]. Regarding the negative value of the binding enthalpies obtained in this study, indicating that the binding reaction is accompanied by a release of heat we have to say that this is not common for neutral compounds as PTX, but a molecular interpretation of this finding is suggested by strong van der Waals established between PTX and the lipids in comparison with inclusion compounds formed between cyclodextrins and hydrophobic organic molecules. These complexes are characterized by tight van der Waals contacts between host and guest molecules and, in the majority of cases, by distinct negative binding enthalpies [46].
The shift seen when the concentration of DODAC:MO increases (Figure 4.6.) indicates that the drug knows a change in polarity in its surroundings. This, together with the isobestic point signifies that PTX will reposition from the aqueous phase to the liposomal phase. Isobestic points demonstrate that a compound is in equilibrium in the two phases.

In literature, different Kp values for PTX have been reported, in different liposomal formulations as some examples are summarized in Table 5.1.

<table>
<thead>
<tr>
<th>Binary system</th>
<th>Kp Paclitaxel (M⁻¹)</th>
<th>LogKp Paclitaxel dimensionless</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposomes DOTAP/H₂O</td>
<td>272000</td>
<td>4,73</td>
</tr>
<tr>
<td>Liposomes POPC/H₂O</td>
<td>9500</td>
<td>3,98</td>
</tr>
<tr>
<td>n-octanol/H₂O</td>
<td></td>
<td>3,50</td>
</tr>
</tbody>
</table>

The log Kp values found in this project for the liposome/water system (3,26 ± 0,119) are similar to the ones found in Table 5.1. This could be explained because its pKa of 10,36 according to the Marvin sketch calculator by Chemaxon™. This means that in all conditions studied in this project, the drug was neutral. In addition it is a highly lipophilic compound. Thereby, it can be concluded that there will mostly be hydrophobic interactions with the membrane. Paclitaxel being neutral both at pH 5 and pH 7,4 explains why the log Kp values are so similar (respectively 2,39 ± 0,014 and 2,43 ± 0,276). Furthermore can be noticed that the log Kp of the buffered liposomal formulation is lower (2,39 ± 0,014 and 2,43 ± 0,276) than the log Kp of the PTX in liposomes in ultrapure water (3,26 ± 0,119). This is because the hydration of the former lipid films was with a buffered solution. This results in less stable liposomes as a consequence of a change in ionic strength of the environment. Finally, in analogy with RSV in the DODAB:MO liposomes, the high partition coefficient of PTX shows that the DODAC:MO (1:2) formulation is an adequate nanocarrier for the encapsulation of this anticancer drug.

The Stern-Volmer plot collected from the HSA binding of PTX is a linear plot (Figure 4.10B). This means that there is only one quenching mechanism present. As previously explained this is the static quenching mechanism. The number of binding sites of PTX to HSA in Table 4.6 is 0,95. This means that on the HSA protein there is only one site were PTX will be able to
bind. The $K_b$ value of $327 \pm 3.89 \text{ M}^{-1}$ at room temperature is low. Meaning that the binding is rather weak when compared to other protein-ligand bounds [48] [45]. The peak around 230 nm in Figure 4.12 caused by the addition of PTX in increasing concentrations proves the presence of a HSA-PTX complex. In the same graph, there is a red shift seen when the concentration of PTX is increasing from 0 µM to 216 µM. This indicates that the surroundings of the tryptophan residue of HSA at position 214 are becoming less hydrophobic. Knowing this and that PTX is a highly hydrophobic molecule, its presence near the tryptophan is not likely. The explanation for the change in polarity of the environment of position 214 of HSA is rather because its C=O backbone structure has become loose. At 37 ºC, the z-averaged hydrodynamic diameter of HSA in the absence of PTX was 6 nm, which is consistent with values for HSA dimers described in the literature [49]. Binding of PTX to HSA has been described to occur at the domain IIA binding pocket with conformational changes of loss of helical stability of protein and local perturbation in the domain IIA binding pocket [50]. In Figure 4.13 it is possible to observe that upon addition of increasing concentrations of PTX, HSA size is reduced from 6 nm to 4.17 nm indicating that initially there is 100% of protein in the dimeric form (6 nm = 100% x 6 nm) and upon binding great part of these dimmers are separated in the monomeric forms (4.17 nm = 39% of dimmers 6 nm + 61% of monomers of 3 nm). Probably PTX upon binding to the protein promotes the separation of the dimmers. Furthermore the changes in conformations upon binding of PTX are also confirmed by the surface of the protein which becomes more negatively charged, probably by greater exposition of the negative domain I [51].

When analyzing the binding of HSA to empty liposomes of DODAC:MO (1:2) or the HSA binding to liposomes containing PTX, we conclude that the binding is stronger when PTX is encapsulated in the liposomes. This is probably due to the effects of PTX in the bilayer microviscosity that facilitates membrane interaction with serum albumin. This fact gives us an indication that formulations need to be afterwards pegylated to avoid plasma protein interaction.

PTX effect on the microviscosity of the membrane systems was evaluated by two different techniques that pointed to the same conclusion. PTX did not significantly alter the phase transition temperature of liposomes of DMPC used as membrane model systems, however the cooperativity of the transition was significantly reduced by the presence of PTX. The
absence of changes in Tm means that either a drug does not permeate in the membrane and therefore causes no visible changes in the phase transition, or on the other hand the drug penetrates deeply in the membrane bilayer. In this later case the phase transition changes are not very evident because, at deeper locations, the membrane is very fluid and can accommodate drugs without evident perturbation of microviscosity. However in this latter case the presence of drug is evidenced by changes in the cooperativity of the phase transition. PTX is thus probably deeply located at the membrane bilayer hydrophobic core. The great decrease in the cooperativity of the phase transition induced by PTX mean that the drug is not evenly distributed at the membrane phase. And thus some lipid molecules not influenced by the drug will suffer normal immediate transition, while other lipid molecules that are close to the drug will suffer slower phase transition.
6 CONCLUSION

The $K_p$ was determined at different pH values and different temperatures. With the results of the temperature dependent assays, the change of Gibbs free energy when transferring from the aqueous phase to the liposomal phase, was calculated. For both medicinal compounds, PTX and RSV, it was negative. This means that both reactions will occur spontaneously. The Tm of the formulation used to encapsulate RSV (DODAB:MO 1:2) was within the range studied (30°C-60°C). This was visible in the results because when below Tm the liposomes are more rigid and more energy is needed to separate the bilayers to accommodate the drug. Both drugs appeared in both phases and both compounds were in a higher concentration in the membrane phase as opposite to the aqueous phase. Hereby we can conclude that the nanocarriers chosen, DODAB:MO for RSV and DODAC:MO for PTX, are very capable.

The Tm of DMPC liposomes was studied and the influence PTX has on it. We found that there was no significant change in Tm but there was a change in cooperativity. This indicates that PTX is located deeply in the bilayers of the DMPC liposomes.

Furthermore we found that there was only 1 binding site on HSA for PTX. The binding was also rather weak compared to other molecules. But we found proof that they do form complexes. Also when PTX is added, the size of the HSA was significantly reduced. HSA is a dimer so this can be explained by the separation of the dimers into monomers. Since the size was not completely divided by 2, we can conclude that some of the dimers still remain intact. However it is clear that PTX most likely will stimulate HSA to separate its dimers. Finally, the decrease of the negative surface charge to an even more negative surface charge proofs that the conformation of HSA is changed. The reason for this might be because the negative domain I might be more exposed.

Results for both drugs in these formulations are very promising and considering the need for more, less toxic anti cancer drugs, it could be interesting to investigate both compounds even more profoundly. For example an in dept research in vivo to validate the in vitro results.
REFERENCES


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APPENDICES

Appendix I  Derivative spectroscopic data of RSV at pH 5
Appendix II  Derivative spectroscopic data of RSV at pH 7,4
Appendix III Derivative spectroscopic data of PTX at pH 5
Appendix IV  Derivative spectroscopic data of PTX at pH 7,4
Appendix I:

Derivative spectroscopic data of RSV pH 5

![Graph of derivative at 352 nm]

\[ \text{Chi}^2 = 4.3366 \times 10^{-9} \]
\[ R^2 = 0.94214 \]
\[ K_p = 1004.38626 \pm 285.20147 \]
\[ b = -0.00102 \pm 0.00013 \]
\[ a = 0.0012 \]

![Graph of derivative at 324 nm]

\[ \text{Chi}^2 = 7.9466 \times 10^{-12} \]
\[ R^2 = 0.99115 \]
\[ K_p = 1338.27929 \pm 137.97154 \]
\[ b = -0.00011 \pm 4.5704 \times 10^{-6} \]
\[ a = -4.7475 \times 10^{-6} \]
RD DERIVATIVE AT 343 nm

\[ \text{DODAB:MO (1:2)} \] (M)

Chi² = 5.6918 \times 10^{-11}

R² = 0.9709

Kₚ = 1487.06185 \pm 270.83765

b = -0.00016 \pm 0.00001

a = 0.00018

RD DERIVATIVE AT 352 nm

\[ \text{DODAB:MO (1:2)} \] (M)

Chi² = 2.3359 \times 10^{-11}

R² = 0.98949

Kₚ = 1333.64819 \pm 150.03538

b = 0.00017 \pm 7.8543 \times 10^{-6}

a = -3.8066 \times 10^{-6}
\[ \text{Chi}^2 = 3.1194 \times 10^{-11} \]
\[ R^2 = 0.9836 \]

\[ K_p = 1427.5514 \pm 196.80148 \]
\[ b = 0.00016 \pm 8.6743 \times 10^{-6} \]
\[ a = -0.0001 \]

\[ \text{Chi}^2 = 9.3021 \times 10^{-12} \]
\[ R^2 = 0.95232 \]

\[ K_p = 1593.67933 \pm 375.25527 \]
\[ b = -0.00005 \pm 4.415 \times 10^{-6} \]
\[ a = -0.00004 \]
Appendix II:

Derivative spectroscopic data of RSV pH 7.4

![Graph showing derivative spectroscopic data with fitted lines and parameters](image)

Chi² = 6.0021x10⁻⁹
R² = 0.89031
Kₚ = 904.98276 ± 324.69646
b = -0.001 ± 0.00017
a = 0.0012

![Graph showing derivative spectroscopic data with fitted lines and parameters](image)

Chi² = 3.1812x10⁻¹²
R² = 0.9936
Kₚ = 1189.20316 ± 107.35488
b = 0.00008 ± 3.1395E-6
a = -1.8533x10⁻⁶
**3rd DERIVATIVE AT 324 nm**

- $\chi^2 = 6.6272 \times 10^{-12}$
- $R^2 = 0.99444$
- $K_p = 1337.97077 \pm 109.11793$
- $b = -0.00012 \pm 4.1745 \times 10^{-6}$
- $a = -6.2536 \times 10^{-6}$

**3rd DERIVATIVE AT 343 nm**

- $\chi^2 = 7.0449 \times 10^{-11}$
- $R^2 = 0.95377$
- $K_p = 799.67833 \pm 203.80622$
- $b = -0.00016 \pm 0.00002$
- $a = 0.00016$

**3rd DERIVATIVE AT 352 nm**

- $\chi^2 = 2.1009 \times 10^{-11}$
- $R^2 = 0.99357$
- $K_p = 1579.82197 \pm 136.3548$
- $b = 0.0002 \pm 6.6713 \times 10^{-6}$
- $a = -2.1824 \times 10^{-6}$
\[ \chi^2 = 3.715 \times 10^{-11} \]
\[ R^2 = 0.9774 \]
\[ K_p = 1103.93544 \pm 182.77658 \]
\[ b = 0.00015 \pm 0.00001 \]
\[ a = -0.00009 \]

\[ \chi^2 = 8.601 \times 10^{-12} \]
\[ R^2 = 0.98125 \]
\[ K_p = 1998.37798 \pm 310.09225 \]
\[ b = -0.00007 \pm 3.7102 \times 10^{-6} \]
\[ a = -0.00004 \]
Appendix III:

Derivative spectroscopic data of PTX pH 5

![Graph 1: Derivative at 256 nm](image1)

Chi^2 = 4.3445E-9  
R^2 = 0.85409

K_p = 8378.37171 ± 2360.53237
b = 0.00046 ± 0
a = 0.00067 ± 0

![Graph 2: Derivative at 264 nm](image2)

Chi^2 = 4.9961E-11  
R^2 = 0.71974

K_p = 4998.22561 ± 2275.7117
b = -0.00003 ± 0
a = -0.00006 ± 0
Appendix IV:

Derivative spectroscopic data of PTX pH 7.4

**2nd Derivative at 256 nm**
- Data: der2a_C
- Model: Kp
- Chi^2 = 0.07214
- R^2 = 0.9515
- Kp = 1053.82891 ± 371.79987
- b = -0.00124 ± 0
- a = 0.0012 ± 0

**3rd Derivative at 264 nm**
- Data: DER3A_C
- Model: Kp
- Chi^2 = 0.0002
- R^2 = 0.98595
- Kp = 651.56303 ± 1350.09679
- b = 0.00013 ± 0
- a = -0.00011 ± 0