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Dissertation

Development of liposomal formulations for photodynamic therapy of cancer

Manuela Jorge Estevinho Rodrigues

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GENERAL INDEX

INDEX OF FIGURES

INDEX OF TABLES

ABBREVIATIONS

Abs: absorbance
AK: actinic keratosis
AMD: age-related macular degeneration
ALA: 5-aminolevulinic acid
BCC: basal cell carcinoma
BPD: benzoporphyrin derivatives
BPD-MA: benzoporphyrin derivative monoacid ring A
CNV: choroidal neovascular degeneration
DLS: dynamic light scattering
DNA: deoxyribonucleic acid
DPPC: dipalmitoylphosphatidylcholine
DPPG: dipalmitoylphosphatidylglycerol
DSPC: distearoylphosphatidylcholine
DSPG: distearoylphosphatidylglycerol
EPR: enhanced permeability and retention effect
FDA: Food and Drug Administration
HAL: hexaminolevulinate
HBS: HEPES-buffered saline
HDL: high density lipoproteins
HPD: hematoporphyrin derivative
HPPH: 2-(1-hexyloxyethyl)-2-devinyl pheophorbide-alpha
Inc.: incorporated
LDL: low density lipoproteins
logP_{ow}: logarithm of the octanol/water partition coefficient
LT: total lipid
Ltd.: limited
LUV: large unilamellar vesicle
MACE: chlorin e6
M-ALA: methyl aminolevulinate
MLV: large multilamellar vesicle
MPS: monuclear phagocytic system
m-THPC: *meso*-tetra-hydroxylphenyl-chlorin
MVV: multivesicular vesicle
PBS: phosphate-buffered saline

PDD: photodynamic diagnosis

PDT: photodynamic therapy

PEG: polyethylene glycol

PI: polydispersity index

PL: phospholipid

PS: photosensitizer

RNA: ribonucleic acid

ROS: reactive oxygen species

SD: standard deviation

SUV: small unilamellar vesicle

TEM: transmission electron microscopy

T_m: transition temperature

U.S.: United States

USA: United States of America

UV/Vis: ultraviolet-visible

ABSTRACT

This work aimed the development of liposomal formulations with an encapsulated photosensitizer drug. Different passive encapsulation methods were tested, namely, the lipid film hydration method, the reverse-phase evaporation method, the freeze-thaw method and the film loading method. Additionally, different liposome membrane lipid compositions were used. Afterwards, the formulations encapsulating a hydrophobic photosensitizer (PS) were characterized in terms of average size, polydispersity index, loading capacity and encapsulation efficiency. Finally, the validation of the free drug separation method by size exclusion chromatography was made for the most promising liposomal formulation in order to confirm previous results.

Both the average size and size distribution of the vesicles encapsulating a PS were measured by dynamic light scattering (DLS).

For the determination of the encapsulation parameters (i.e. loading capacity and encapsulation efficiency), the PS quantification was made by UV/Vis spectroscopy, using an adequate calibration curve, and the total lipid quantification was performed by the colorimetric method of Bartlett for quantification of inorganic phosphate, being afterwards the concentration of lipid extrapolated from the obtained experimental phospholipid concentration.

Among the different preparation methods, the lipid film hydration method resulted in the best encapsulation parameters (encapsulation efficiency and loading capacity).

The presence of cholesterol seems to have a negative impact on the obtained encapsulation parameters and the presence of DSPG may have a favourable contribution. For that reason, the formulations DSPC : DSPG (9:1) and DSPC : DSPG (7:3) yielded the highest encapsulation efficiency and loading capacity values. Additionally, these formulations also exhibited appropriate average liposomal size for potential intravenous administration.

It is expected that the results obtained in the present work will prove useful in developing new and efficient methodologies for the preparation of liposomal formulations incorporating photosensitizing molecules for photodynamic therapy of cancer.

CHAPTER I – STATE OF THE ART

1- PHOTODYNAMIC THERAPY

1.1- General considerations

Photodynamic therapy (PDT) is a clinical technique that employs a light-sensitive drug (a photosensitizer, PS), in combination with light of a visible wavelength, to destroy target cells, especially cancerous or pre-cancerous cells [1]. An adequate concentration of molecular oxygen is also needed for anomalous tissue damage. If any one of these three components is missing, there will be no effect [2].

PDT requires single administration of a PS followed after a certain time interval by single irradiation with light of specific wavelength corresponding to an absorbance band of the sensitizer. This treatment, very frequently, does not require hospital admission. In comparison, characteristic curative radiotherapy regimes include daily irradiation for a total of 6-7 weeks (once more not requiring hospitalization). Chemotherapy schedules vary, but typically last for several months. Surgery, although a single procedure, requires general anaesthesia and hospitalization for one to several weeks. Cost-effectiveness comparisons have been made for palliative treatment of head and neck cancer with PDT versus extensive surgery or chemotherapy, and for PDT versus esophagectomy or endoscopic surveillance for patients with Barrett's esophagus and high-grade dysplasia. PDT proved to be cost-effective and provided increased life expectancy, compared with other treatment options for these circumstances [3].

While PDT is a completely well-accepted treatment in clinical practice for some types of skin lesion (cancerous or not), it has yet to be explored for other forms of cancer. PDT is normally used either as a primary treatment (usually in skin conditions) or as an adjunctive treatment together with surgery, radiotherapy or chemotherapy [1].

PDT is a local, rather than systemic, treatment; it is appropriate only for localized disease [2]. Light of wavelengths used to excite current PSs can provoke photochemically induced tissue necrosis up to a maximum depth of 10 mm. This signifies that, for superficial illumination, the use of PDT as a primary treatment should be limited to small, accessible tumours. PDT can also be made in combination with surgery for palliative treatment of larger tumours [3].

The technique has the advantage of limited side effects, because phototoxicity is limited to sensitized cells in the area illuminated and the PS tends to accumulate in tumour cells [4]. In fact, some photosensitising drugs can reach higher concentrations in tumour tissue than in surrounding healthy tissue. The accurate mechanisms that drive this process are not totally understood, but the abnormal physiology of tumours, including poor lymphatic drainage, leaky vasculature, decreased pH, increased numbers of receptors for low-density lipoprotein and the abnormal stromal composition, might contribute to the selectivity of PSs [2]. Furthermore, the activation by light at a wavelength matching one of PSs absorbing wavelengths leads to the formation of reactive oxygen species (ROS), mainly the extremely reactive singlet oxygen ($^1O_2$), which travels very short distances and so photodamage mediated by PDT is mainly limited to the site of singlet oxygen generation [4].

Modern fiber-optic technology facilitates delivery of light, of the desired wavelength and fluence rate (light application rate), to tumours located almost anywhere in the body. Localized illumination enables specific tumour treatment without the destruction of critical normal tissues outside the treated area. By contrast, surgery and radiotherapy of tumours located close to critical structures can be very mutilating and lead to loss of function. PDT has the advantage that, although there is sometimes a significant ulceration of the illuminated area immediately after treatment, there is minimal long-term fibrosis, resulting in functional recovery without scarring [2]. PDT spares tissue architecture, providing a matrix for regeneration of normal tissue, since it does not damage subepithelial collagen and elastin and there is preservation of noncellular supporting elements [3].

Another advantage of PDT is that the treatment can be repeated in case of recurrence or appearance of a new primary tumour in the previously treated area, which is difficult with surgery or radiotherapy, without the risk of normal tissue damage [3]. PDT also offers the ability to treat large areas of diseased tissue, areas which are not accessible by surgery and preserves connective tissue within the treated area [1].

A limitation of PDT is that it cannot cure advanced disseminated cancer disease, because irradiation of the whole body with suitable doses is not possible (at least with current technologies). However, for advanced disease, PDT can improve quality of life and extend survival, because it is minimally invasive and it does not restrict the use of other subsequent treatments [2].

Currently, photodynamic diagnosis (PDD), which involves fluorescence to localize abnormal tissue, has been subjected to several clinical trials. In the fluorescence process, an outer electron excited by a photon of appropriate wavelength returns to its ground state emitting a lower energy photon. PDD reveals neoplastic

lesions that cannot be seen by means of conventional methods, representing an additional optical recognition criterion. The advantages features of fluorescence detection are the lack of background signal and specific targeting of a fluorochrome (molecule that makes use of the relaxation path mentioned above). Thus, PDD and PDT allow simultaneous diagnoses and therapy, improving cancer treatment efficiency [5], [6].

1.2- Brief history of photodynamic therapy

The first clinical application of PDT dates 1903, where von Tappeiner and Jesionek attempted unsuccessfully to treat basal cell carcinomas (BCCs) with topical eosin dye followed by exposure of the lesions to sunlight [3], [7]. Von Tappeiner and Jodlbauer later defined PDT as the dynamic interaction among light, a photosensitizing agent and oxygen resulting in tissue destruction [3].

After a hiatus of more than fifty years, in 1960 Lipson synthesised the first PDT drug which he named hematoporphyrin derivative (HPD) [3]. In 1975 Dougherty *et al*. reported that HPD in combination with red light could completely eradicate mouse mammary tumour growth [3]. Clinical trials were afterwards initiated with HPD to treat patients with bladder cancer and skin tumours. After these successful studies, several trials were initiated for a variety of cancers and PSs. This led to the approval of PDT using porfirmer sodium (Photofrin®; Axcan Pharma Inc., Mont-Saint-Hilaire, Canada) for the treatment of bladder cancer in Canada in 1993 [7]. This achievement was followed by approvals for PDT of tumours of lung and esophagus in the U.S. and other countries [7]. However, nearly all patients receiving Photofrin® acquire photosensitivity of their skin to direct sunlight, which may persist for one to three months and so this adverse reaction probably contributed to the low acceptance of PDT by the medical community, although severe reactions in patients have been rare [7].

Nowadays, others sensitizers are approved for clinical use, for example, 5-aminolevulinic acid (ALA, Levulan®; DUSA Pharmaceuticals Inc., Wilmington) is approved for actinic keratosis (AK), the methyl ester of ALA (M-ALA, Metvix®; Photocure ASA, Oslo, Norway) is approved for AK, Bowen's disease, Basal cell carcinoma, and *meso*-tetra-hydroxyphenyl-chlorin (mTHPC, temoporfin, Foscan®; Biolitec Pharma Ltd., Dublin, Ireland) is approved for head and neck cancers. Thus, PDT is becoming an established treatment modality for localized cancers [3]. The term

"PDT" is being used also to describe non-cancer disorders photosensitized by a photosensitizing drug, including AK, psoriasis, acne, and age-related macular degeneration (AMD) [7].

Besides, PSs like ALA and porphyrin hexaminolevulinate (HAL) have obtained approval for the detection of malignant glioma and superficial bladder cancer, respectively, by fluorescence diagnosis in many European countries. Therefore, it is now possible to simultaneously diagnose a suspected cancerous lesion not visible with white light cystoscopy and conduct "curative" therapy, improving survival and quality of life [5], [6], [8].

1.3- Principle of photodynamic therapy

PDT is a developing modality for the treatment of superficial tumours, because the light used with a wavelength of 600-800 nm is not able to penetrate into the tissue more than 1 cm [9], [10].

The principle of PDT is based on the administration of a PS followed by local illumination of the tumour area at an adequate wavelength to activate the specific drug. Activation of the PS upon absorption of the light transforms the drug from its ground state (^{1}PS) into an excited singlet state (^{1}PS*) – Figure 1. From this state the drug may decay directly back to the ground state by emitting fluorescence, which can be used clinically for photodetection, or by internal conversion into heat. However, to obtain a therapeutic photodynamic effect, the excited singlet state must undergo electron spin conversion to its triplet state (^{3}PS*) by a process called intersystem crossing (whereby the spin of the excited electron in ^{1}PS* inverts to form an excited triplet state that has electrons with spin in a parallel conformation). In the presence of oxygen, the excited molecule can react directly with a substrate, such as the cell membrane or other cellular structures, by proton or electron transfer, to produce radicals and radical ions, which can further interact with oxygen to form oxygenated products (type I reaction). Alternatively, the energy of the excited PS can be directly transferred to oxygen to form singlet oxygen (type II reaction), which is the most damaging and cytotoxic agent produced during PDT, since it interacts efficiently with various biomolecules [3], [11], [12].

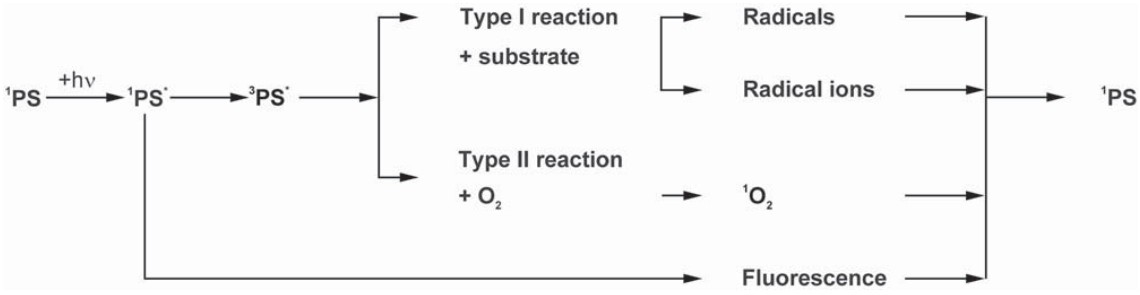

Figure 1: The principle of photodynamic therapy [9].

Both the type I and type II reactions occur simultaneously and the ratio between these processes depend on the type of PS used and also on the concentrations of substrate and oxygen. Due to the high reactivity and short-life of singlet oxygen and hydroxyl radicals, only molecules and structures that are close to the area of its production (i.e. areas of PS localization) are directly affected by PDT and destroyed [11].

1.4- Mechanisms of tumour destruction by photodynamic therapy *in vivo*

The effectiveness of PDT in the treatment of cancer (e.g. tumour cell destruction) depends on the nature of PS, drug concentration, drug intracellular localization, total light dose (fluence), light application rate (fluence rate) and oxygen availability [10]. In general, the rates of singlet oxygen generation and therefore tissue oxygen consumption and depletion within the tumour are significant when tissue PS levels and fluence rate of light are high. An important parameter influencing the rate of tissue oxygen consumption is photobleaching of the PS (PS destruction / alteration by exposure to light or loss of the PS's property of optical absorbance) because the reduction of PS levels also reduces the rate of photochemical oxygen consumption [13].

Three different mechanisms shown in Figure 2 have been assumed to reduce or frequently eliminate tumours when using PDT [11].

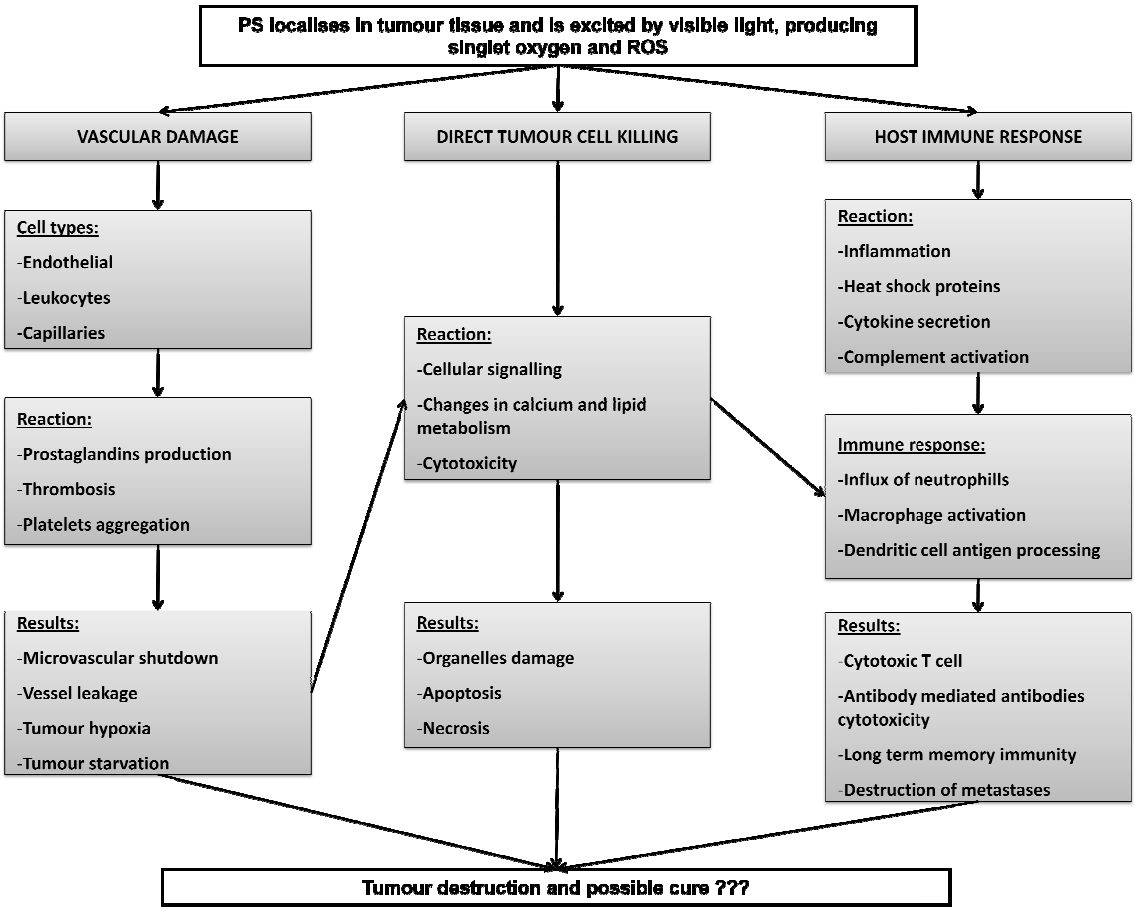

Figure 2: Pathways for PDT-mediated tumour destruction presenting vascular damage, direct tumour cell killing and host immune response as possible contributions [11].

Singlet oxygen produced by the photochemical reaction can directly kill tumour cells by the induction of apoptosis and/or necrosis (Table 1). Singlet oxygen is extremely reactive and can diffuse only 0.01-0.02 µm during its short lifetime [3], [7], [11]. Consequently, damage mediated by PDT is mainly limited to the site of singlet oxygen generation [3], [7], [11]. Alternatively, singlet oxygen damages vascular cells associated to the tumour, which can lead to thrombosis and haemorrhage in tumour blood vessels, resulting in indirect death via the induction of hypoxia and starvation of the tumour. It has been shown that both PS accumulation and tumour cell kill decrease with the distance of tumour cells from the vascular supply [13]. Lastly, the host immune response may occur. After the acute inflammation and subsequent release of cytokines and stress response proteins induced in the tumour by PDT, they are able to initiate an immune response by the attack of cytotoxic T cells against the tumour and tumour cells at isolated locations. The inflammatory signalling after PDT initiates an immense regulated invasion of neutrophils, mast cells, and monocytes / macrophages that may outnumber resident cancer cells too [13]. Therefore, the monocytes can produce

antibodies that mediate cytotoxicity against tumour cells and then the organism acquires some long term memory immunity.

Table 1: Distinction between necrosis and apoptosis – principal mechanisms of cell death in PDT (data was compiled from [7], [11], [14]).

Necrosis or passive cell death	Apoptosis or active cell death
Violent and quick process characterized by gross damage, spillage of intracellular contents and presence of *in vivo* inflammation. It is caused by physical or chemical damage and is considered to be an unprogrammed process. The intracellular targets are the plasma membrane and lysosomes.	Energy-requiring process highly regulated and controlled, characterized by nuclear condensation, cell shrinkage, bleb formation, and absence of inflammatory responses of the affected tissue. It is an indispensible process during normal development, tissue homeostasis, regulation of the immune system... Mitochondria and DNA are the likely targets for the apoptotic response.
Both implicated in the immunological responses to PDT	

The three mechanisms can influence each other and the outcome of the PDT is dependent on all these mechanisms, being the relative contribution of each dependent on the treatment regimen given [3], [7], [11], [12], [15]. The combination of all these mechanisms in PDT is required for optimum long-term tumour regression, especially of tumours that may have metastasized [11].

1.5- Photodynamic therapy photosensitizers

Many PSs, for example, 21-thiaporphyrin and 21,23-dithiaporphyrin have been tested *in vivo* and *in vitro* in PDT experiences and despite several clinically approved PSs, none have shown ideal, safe and selective properties and, for this reason, recent studies have focused on the development and efficacy of new PSs like hexaminolevulinate derivative [11], [16], [12], [17]. The prerequisites for an ideal sensitizer include: i) simple, efficient and economical synthesis; ii) chemical and physical stability, chemical purity and long shelf-life; iii) solubility in biocompatible solvents or vehicles; iv) high absorption coefficient in the “phototherapeutic window”

(600-800 nm); v) short interval required between administration of the sensitizer and its maximal accumulation in tumour tissues; vi) singlet molecular oxygen sensitization (type II process) and/or superoxide generation (type I process) with a high quantum yield; vii) little or no dark toxicity; viii) low skin photosensitization; ix) controlled photobleaching; x) selective accumulation and prolonged retention in tumour tissues and xi) simplistic metabolism or rapid excretion after treatment [11], [12], [15], [18].

PSs can be classified in various ways, all with limitations. Three of those classifications are given below:

- *Chemical structure*

  This is a widely accepted mode to characterize PSs for chemists but has limited utility in the clinical, because alterations in structures by the addition, subtraction or substitution of primary or side chains may sometimes enhance PS activity and may also create toxic substances, being useless for clinic. Fundamentally, most PSs are cyclic tetrapyrroles and are derivates of porphyrins, chlorins and bacteriochorins. Dyes, mainly those used in ink, are also a rich ground to develop PS [15], [18]. Figure 3 presents the chemical structures of these first families and an example of a dye (which corresponds to another type of PS family).

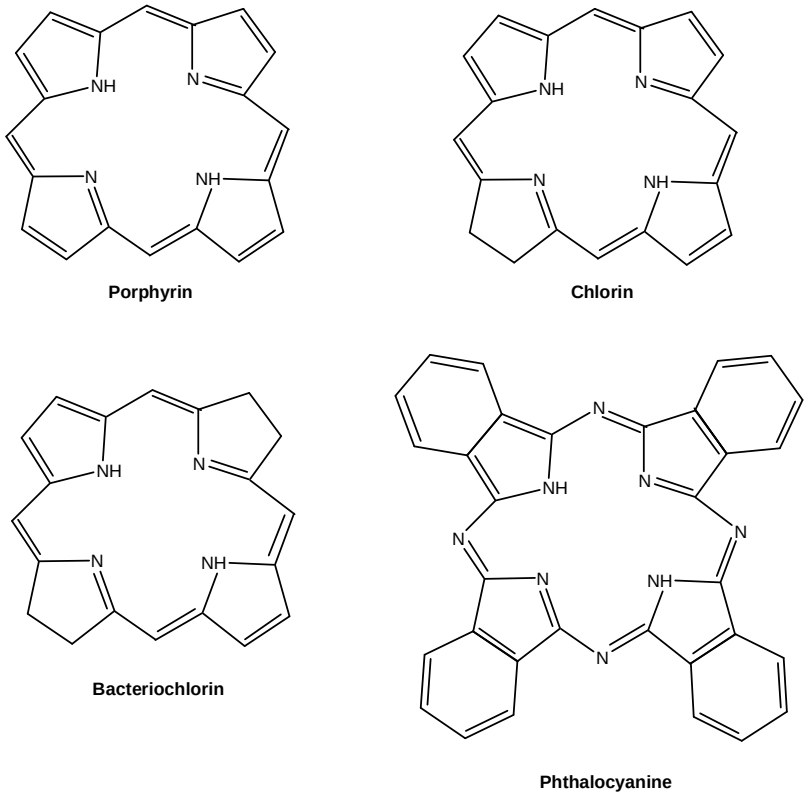

Figure 3: General porphyrin, chlorin, bacteriochlorin and phthalocyanine structures [7], [10].

- *Generation*

  Some attempts to classify PSs are based on when they were generated. First generation PSs are porphyrin based and include hematoporphyrin and its derivatives named hematoporphyrin derivatives. Second generation of PSs were developed based on the supposed deficiencies of the first generation drugs. Thus, second generation PSs demonstrate higher absorption in the 650-800 nm range where tissue penetration is optimal, have higher extinction coefficients of absorption in the red than first generation compounds and their tissue accumulation is much lower and therefore, the treatment can be carried out on the same day as the administration of the drug. Moreover, second generation PSs show lower toxicity [16]. These second generation of PSs have several structures including porphyrins, expanded porphyrins, chlorophyll derivatives and dyes, but most of the compounds are still very hydrophobic and show poor tumour selectivity. Third generation of PS contains fundamentally first and second generation PS conjugated to carrier molecules, which may specifically target the PS to the target cells, resulting in minimized accumulation in healthy tissues [16]. Many drugs of second and third generations are not commercially available [3], [7], [10], [12], [15].

- *Targeting*

  Some PSs preferentially accumulate in tissue while others stay in the vascular supply. Some, such as Photofrin®, may initially circulate extensively than compartmentalize. Clinically, this indicates using vascular PS agents when targeting neovasculature. Though, all invasive tumours have neovasculature and using one PS for this indication may not be critical. An attempt may be made to classify PSs by what they specifically target. Thus, the hematoporphyrin derivatives are composed of monomers, dimmers and oligomers. The two smaller components are brought to mitochondria while the larger components are actively phagocytised by the cell membrane. Chlorin e6 (MACE) is brought to lysosomes by endocytosis. Phatalocyanines concentrate in mitochondria. Benzoporphyrin derivatives (BPD) accumulate in the Golgi apparatus. ALA goes into the cell membranes, lysosomes and mitochondria. By linking these PS to carriers, such as nanoparticles, the accumulation region can be altered significantly [15].

  Note that, the important structural features for different intracellular localizations are the net ionic charge (which can range from -4 to +4), the degree of hydrophobicity expressed as the logarithm of the octanol / water partition

coefficient and the degree of asymmetry present in the PS molecule. Hence, hydrophobic PSs with two or less negative charges can diffuse across the plasma membrane and then relocate to other intracellular membranes. Less hydrophobic PSs with less than two negative charges tend to be more polar to diffuse across the plasma membrane and may be found preferably in lysosomes and in cytoplasm (in general, hydrophilic PSs are taken by endocytosis) [11], [14].

The clinically available PSs (approved and in trials) until the moment are given in Table 2.

Table 2: Clinically available photosensitizers (adapted from [19]).

Chemical family	Product name	Therapeutic substance	Administration route	Manufacturer	Disorder
Porphyrin	Photofrin®	Haematoporphyrin derivative (HPD)	Intravenous	Axcan Pharma Inc.	Lung and esophageal cancers
	Photogem®	Haematoporphyrin derivative (HPD)	Intravenous	Moscow Research Oncological Institute	Bronchus, esophageal and colon cancers
	Levulan®	5-Aminolevulinic acid (ALA)	Topical	DUSA	Skin cancer, AK
	Metvix®	Methyl aminolevulinate (M-ALA)	Topical	PhotoCure ASA	AK, Bowen's disease, Basal cell carcinoma
	Hexvix®	Hexaminolevulinate (H-ALA)	Intravenous	PhotoCure ASA	Bladder cancer
	Visudyne®	Verteporfin (BPD-MA)	Intravenous	Novartis Pharmaceuticals	Macular degeneration, Pathologic myopia, Ocular histoplasmosis
Texaphyrin	Antrin®, Lu-Tex	Lutexaphyrin	Intravenous	Pharmacyclis	Breast cancer
Chlorin	Foscan®	Temoporfin (mTHPC)	Intravenous	Biolitec Pharma Ltd	Head and neck cancers
	LS11, Photolon®	Talaporfin	Intravenous	Light Sciences	Skin cancer, breast cancer, Uterus and rectum cancers
	Litx™, Apoptosin™, Laserphyrin		Intravenous		
	Photochlor	2-(1-Hexyloxyethyl) -2-devinyl pyropheophorbide-a (HPPH)	Intravenous	RPCI	Lung and esophageal cancers
Phthalocianines	Photosens®	Phthalocyanine	Intravenous	General Physics Institute	Macular degeneration
	Pc4	Phthalocyanine	Intravenous or intratumoral	CWRU	Mycosis Fungoides, Sezary syndrome
Bacteriochlorin	Tookad	Palladium-Bacteriopheophorbide	Intravenous	The Weisman Institute of Science	Prostate adenocarcinoma

1.5.1- Topical and systemic photosensitizers for photodymanic therapy

Systemic PSs (administrated in a versatile way that enables binding between PS and serum proteins for effective PDT [3]) have the advantage to be accumulated in multiple lesions by a single administration whereas the topical PSs require their direct application in each lesion. However, topical PSs do not present the disadvantage of photosensitivity (toxic effect) generated by systemic PSs. The photosensitivity is generally a consequence of a slow rate clearance from the skin, persisting during various weeks after treatment. This fact was observed in clinical trials for hematoporphyrin and its more purified form Photofrin®-II [12].

In the clinic, when a topical PS like M-ALA is employed for cutaneous lesions, a series of intensely illuminated PDT sessions may be done, resulting in additionally cell death and increasing PDT efficacy, whereas for systemic PSs only a single powerfully illuminated session can be done [12], [15].

Many primary cutaneous lesions are likely to be treated with topical PSs because of easier application (by a cream / solution) and illumination (higher accessibility of skin to light exposure) and this is the treatment of choice for most patients [20]. In dermatological oncology, PDT is already a routine treatment, and its use will continue to increase. Excellent cosmetic outcomes make PDT suitable for patients with skin cancers. Skin pain during irradiation can be attenuated with local anaesthesia [2].

1.5.2- Photosensitizers conjugates for photodynamic therapy

The extended delocalised aromatic π electron system characteristic of PSs generally makes them extremely hydrophobic and consequently poorly water soluble and prone to aggregation in aqueous solution, which decreases their ability to generate singlet oxygen efficiently. In addition, currently clinically approved PSs have frequently poor bioavailability and unfavourable biodistribution, resulting in lower tumour specificity than the ideal and, thus, in undesirable side effects like prolonged skin photosensitivity and damage to surrounding healthy tissues. These drawbacks have lead to the development of conjugates, and supramolecular carriers like nanoparticles for the systemic delivery of PS (*Vide* 2.2.) [4], [21].

In fact, PSs often possess functional groups to which conjugation is possible by esterification or substitution. Many PSs conjugates have been designed to increase their bioavailability, solubility and target specificity. Distinct sets of proteins, such as receptors and transporters, are often overexpressed at the surface of cancer cells' membrane, and conjugation of PS with solubilising and/or targeting moieties, including sugars, peptides, proteins and antibodies, is the underlying principle to increase target specificity of PDT by receptor-mediated endocytosis (for the cellular uptake of the conjugated PSs) [4].

1.6- Clinical trials for cancer treatment by photodynamic therapy

In the future, PDT treatment regimens still have to be optimized and standardized for better therapeutic effectiveness and for increased safety [3]. Thus, various clinical trials are in development with the aim of determining the best treatment protocol in PDT for various cancer diseases (Table 3) [17].

Table 3: Examples of ongoing clinical trials for cancer treatment by PDT (data was compiled from [17]).

Product name	Therapeutic substance	Therapeutic Indication	Sponsor	Status
Metvix®	Methyl-5-aminolevulinate hydrochloride	Basal cell carcinoma	Roswell Park Cancer Institute	Phase I
-	2-(1-Hexyloxyethyl)-2-devinyl pyropheophorbide-a	Head and neck cancers	Roswell Park Cancer Institute	Phase I
-	Hexaminolevulinate	Cervical intraepithelial neoplasia	PhotoCure	Phase II
Pc4	Silicon phthalocyanine 4	Lymphoma; non-melanomatus skin cancer	Case Comprehensive Cancer Center	Phase I
-	5-Aminolevulinic acid	Non-melanomatus skin cancer	Roswell Park Cancer Institute	Phase II
Hexvix®	Hexaminolevulinate	Bladder cancer	PhotoCure	Phase I
-	2-(1-Hexyloxyethyl)-2-devinyl pyropheophorbide-a	Carcinoma of the oral cavity; oropharyngeal cancer	Roswell Park Cancer Institute	Phase I

2- LIPOSOMES AS NANOSCALE DRUG DELIVERY SYSTEMS

2.1- General considerations

One subject being investigated over this decade is the utilization of nanosystems in different scientific areas. These nanosystems include nanoparticles, which are particles synthesised in the nanoscale with promising applications in various fields of biomedicine, for example, in vectorization of anticancer drugs. They are biodegradable and biocompatible, enhance the drug's biodisponibility and efficiency, reduce the drug's toxicity and its side effects, allow a controlled drug delivery to the target site and enable an increase of the effective concentration of the drug in the target site [22]. Examples of these nanoparticles are quantum dots, liposomes and lipid nanoparticles, polymeric nanoparticles and dendrimers [22].

Only a limited number of the drug-loaded nanoparticles are successful for their clinical applications. An essential parameter of the delivery vehicle pertains to low or no toxicity of the carrier itself *in vivo* or in the environment as a by-product. Therefore, nanoparticles made-up using an assembly of natural biomolecules such as lipids, proteins, and carbohydrates are expected to be a suitable choice for clinical applications [23].

Among various lipid-based formulations, a classical example is liposomes. Liposomes are spherical self-closed structures, composed of phospholipid bilayers, which enclose part of the surrounding solvent into their interior (Figure 4) [23], [24]. The liposome bilayer can be composed of synthetic or natural phospholipids. Examples of phospholipids are: the charge-neutral phosphatidylcholine (neutral liposome), the negatively charged phosphatidic acid (anionic liposome) and the positively charged stearylamine (cationic liposome) [25]. The phospholipids are the major components of biological membranes, having a hydrophilic head and a hydrophobic tail. Thus, the lipid bilayer closes in on itself due to interactions between water molecules and the hydrophobic tails of the phospholipids. This process of liposome formation is spontaneous because the amphipathic phospholipids self-associate into bilayers [23], [26].

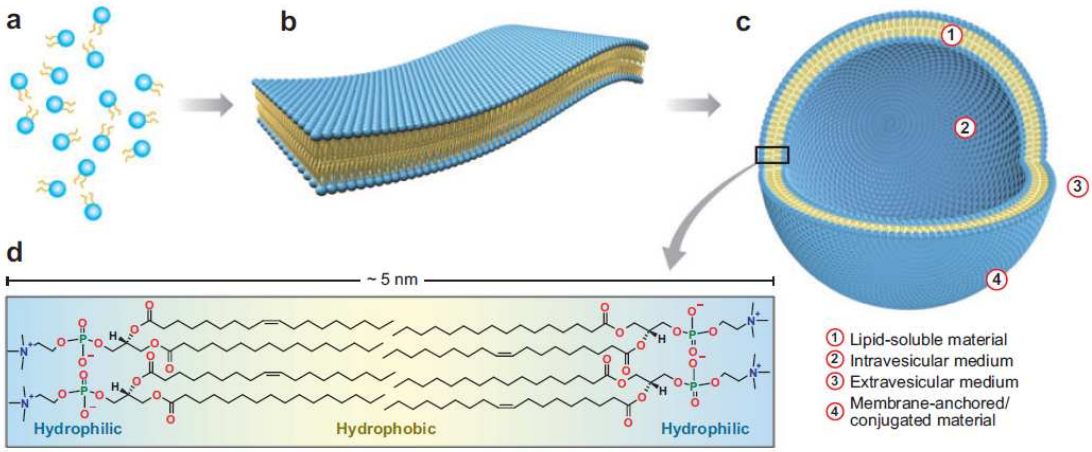

Figure 4: Schematic illustration of the self-assembly process from individual phospholipids molecules (a) to bilayer membrane leaflets (b), followed by transformation into liposomes (c). A single bilayer is typically ~5 nm thick and consists of neatly arranged individual lipid molecules with their hydrophobic tails facing each other and their hydrophilic headgroups facing toward internal and external aqueous medium (d) [25].

Liposomes are easily prepared by various methods (e.g. mechanical methods, methods based on replacement of organic solvents and methods based on size transformation or fusion of preformed vesicle) and can be mechanically stabilized in bloodstream by the inclusion of cholesterol, which also minimizes adsorption of plasmatic proteins by liposomes and controls liposome's membrane fluidity [27].

Drug loading into liposomes can be achieved fundamentally through liposome formation in an aqueous solution saturated with soluble drug, the use of organic solvents and solvent exchange mechanisms and pH gradient methods, depending on the chemical nature of drug [26]. Hence, the encapsulated drug is inaccessible to metabolizing enzymes [27].

The mechanisms to load drugs into liposomes are the passive encapsulation and the active encapsulation. The passive encapsulation is applicable to both hydrophilic and hydrophobic drugs. For hydrophilic drugs, the encapsulation results from the hydration of the dry lipid film with an aqueous solution of drug. The spontaneous formation of liposomes passively captures the dissolved drug. For hydrophobic drugs, the compound is dissolved with the lipid constituents in a suitable organic solvent. Afterwards, the solvent is removed and the film hydrated with an aqueous solution, which result in the entrapment of the drugs within the lipid bilayer [28], [29]. These are the two most common passive encapsulation methods.

Active encapsulation takes advantage of the fact that certain weakly-basic drugs can exist either as neutral or as charged, dependent on the pH of their environment. The molecules are added to preformed liposomes, and in a neutral form, permeate the bilayer lipid through an increasing pH gradient (from outside to inside the liposome) or an ion capable of generating a pH gradient, as with ammonium sulfate or magnesium sulphate. The method of active encapsulation allows higher encapsulation efficiency than the passive encapsulation method [30], [31], [32].

Depending on the processing conditions and the chemical composition, liposomes are formed with one or several concentric bilayers. Liposomes are often distinguished according to their number of lamellae and size. They can be basically small unilamellar vesicles (SUVs, 20-100 nm), large unilamellar vesicles (LUVs, >100 nm), large multilamellar vesicles (MLVs, >0.5 µm), or multivesicular vesicles (MVVs, >1 µm) [27]. The preparation of SUVs starts usually with MLVs, which then are transformed into small vesicles using an appropriate manufacturing technique, e.g. extrusion methods or sonication [22].

Liposomes containing a drug (hydrophilic / hydrophobic) can be administrated by oral or intravenous route in cancer treatment. They generally reach the target site through bloodstream by passive or active (without or with the presence of a ligand on the surface of the lipid bilayer, respectively) targeting strategies. In the passive targeting, the liposome can accumulate in the tumour interstitium owing to a leaky microvasculature and an impaired lymphatic system supporting the tumour area. This effect is often called enhanced permeability and retention effect (EPR) [23], [26], [33]. However, due to the small size of liposomes, they can easily be eliminated by macrophages of the mononuclear phagocytic system (MPS), when liposomes are linked to serum opsonins (which facilitate phagocytosis). This opsonisation process can be reduced by polyethylene glycol (PEG) coating - PEGylation or PEG-coated, reducing clearance by MPS and increasing the circulation half-life [23], [26], [33].

The most widely used polymeric steric stabilizer is PEG, a water-soluble polymer that exhibits protein resistance, low toxicity, non-immunogenicity and antigenicity and can be prepared synthetically with high purity and in large quantities [16].

Given the advantages of liposomes, the major problems associated with them are their stability, poor batch-to-batch reproducibility, difficulty in sterilization and low drug loading capacity [22].

In conclusion, liposomes as drug carriers may avoid the side effects of the conventional cancer treatments and may be a non-invasive, more effective and safe way to treat the disease, leading to better quality of life of patients.

It should be noted that there are various medical applications of liposomes in different areas: drug delivery systems in the treatment of cancer, bacterial infections or ophthalmic disorders. Current clinical applications of gene delivery include liposomes. Also, other applications of liposomes include diagnostic imaging, vaccine adjuvant, photodynamic therapy, dermatology, hemoglobin or chelating agent transporter and enzyme replacement therapy [34].

2.2- Liposomes in photodynamic therapy

Having the concepts above in mind and considering that PDT aims to destroy tumour tissue without affecting healthy tissue (minimizing the risk of unwanted side-effects caused by damage to normal cells), it is still necessary to improve the efficacy and safety of PDT, because during clinical PDT, it is frequent practice to irradiate larger fields that correspond to healthy tissue with microscopic malignant foci and most PSs not seem to exhibit a high selective affinity for tumour tissue [9].

There are various ways to improve the effect of PDT and its safety. Hence, PSs can be loaded in liposomes, which are able to encapsulate hydrophobic as well as hydrophilic drugs without the loss or alteration of their therapeutic activity [16], [21]. Most PSs are usually hydrophobic because of the presence of aromatic rings, presenting low solubility in water. Therefore, liposomal formulations show the ability to decrease the tendency of PS to aggregate in aqueous media, prolonging the circulation of the drug in bloodstream and protecting it [35]. Besides, liposomes improve tumour-selective accumulation as a consequence of the above-mentioned EPR effect [16]. Conventional liposomes exhibit a plasma half-life which is too short for an efficient tumour uptake to occur, but liposomes PEGylated, i.e. long-circulating, and especially actively targeting liposomes, are a better choice in becoming truly tumoritropic carriers of PSs. They increase the tumour accumulation of PS, enhance the controlled release of PS and enhance the efficacy of PDT [9], [36].

Historically, one of the first nanoformulations of PSs was PS loaded into unilamellar liposomes. In the context of non-cancer PDT, Visudyne® (Verteporfin) was the first liposomal drug approved by the Food and Drug Administration (FDA) in 2000 for the treatment of age-related macular degeneration. Another application of this non-PEGylated formulation is in the subfoveal choroidal neovascular degeneration (CNV), which has shown promising results on Indian patients. The therapy was effective and

can cause stabilization or even improved vision [16]. A current Visudine® liposomal formulation in phase I clinical study is described in Table 4 [17].

Table 4: Example of a Visudine® liposomal formulation in PDT undergoing clinical evaluation (data was compiled from [17]).

Formulation name	Therapeutic Substance	Condition	Intervention	Sponsor	Status
Visudyne®	Liposomal benzoporphyrin derivative monoacid termed verteporfin	Port Wine Stains	Combine PDT and pulsed dye laser treatment	University of California, Irvine	Phase I

Foslip is a more recently developed third generation PS based on a dipalmitoylphosphatidylcholine (DPPC) / dipalmitoylphosphatidylglycerol (DPPG) liposomal formulation of 5,10,15,20-tetrakis(*m*-hydroxyphenyl)chlorin (mTHPC). Many studies have been published about Foslip, which present supposed absence of side effects, high efficacy and reduced damage of healthy tissue compared to the non-liposomal Foscan®. Newer studies are related to the potential use of an intratumoral injection of a liposomal formulation of Foslip in a mouse model of local recurrence of breast cancer and its photothrombic activity [16].

For ALA loaded in liposomes composed of phosphatidyl ethanoamine / cholesterol / sodium stearate at the molar ratio 2:1:2.5, improved skin penetration was reported. Likewise, inclusion of ALA esters, especially, of ALA hexyl esters, seemed to result in higher stability upon dilution with cell culture medium [16].

Another approach to tumour specific drug delivery is the use of folate modified liposomes (actively targeted liposomes). Folate receptors are often over-expressed on tumour cells. A comparison of the selectivity of free PS and mTHPC conjugated to the folate receptor for KB tumours with over-expression of folic acid receptors and HT-29 lacking folate receptors was performed. After intravenous injection, the folate specific uptake of conjugate PS was enhanced in KB tumours compared to the non-conjugated compound, and no significant difference between KB and HT-29 tumours was observed in case of free mTHPC. Furthermore, the ratio of tumour to normal tissue for conjugated PS showed a selectivity of 5:1 indicating that folate modified mTHPC is a possible approach for better selectivity in PDT of folate receptor positive tumours [16].

2.2.1- Conventional liposomes for photodynamic therapy

Most of the *in vivo* experiences with liposomal formulations have been done with conventional liposomes that were used as carriers for hydrophobic PSs. Conventional or unmodified liposomes are multilamellar or unilamellar vesicles composed of phospholipids and cholesterol. The latter improves the rigidity of the bilayer membrane and so, reduces the permeability for encapsulated molecules and enhances stability of the bilayer in the presence of biological fluids [9], [24].

Various reports comparing the PDT outcome of liposomal versus non-liposomal PSs under identical conditions, give strong evidence that a liposomal formulation can be advantageous because it enhances efficiency of PDT, i.e., it enhances the PS uptake in tumour tissue and the selectivity of tissue damage [9], [37].

Possibly, some interrelated aspects of PSs, liposomes, plasma proteins and tumour cells influence the final PDT outcome. It is verified that most hydrophobic PSs strongly aggregate in aqueous media. This aggregation significantly reduces the PS efficacy, because only monomeric species are considerably photoactive. Thus, it is known that a liposomal formulation can substantially decrease the extent of PS aggregation [9], [37].

Also, the fate and pharmacokinetics of PSs encapsulated in liposomes are affected by the fact that liposomes show a short plasma half-life, in the range of minutes. Two different phenomena contribute to reduce the circulation time of conventional liposomes (Figure 5). Firstly, a lipid exchange between the liposomes and lipoproteins, particularly high density lipoproteins (HDL), leads to an irreversible and rapid disintegration of the liposome, which releases the PS in the bloodstream subsequently associated with lipoproteins and other plasma proteins. The associated molecules then enter the tumour cells mainly by endocytosis mediated by low density lipoproteins (LDL). Secondly and as mentioned before, conventional liposomes easily become opsonised by plasma proteins after which they are rapidly taken up by cells of the MPS. Consequently, they become concentrated in organs and tissues with a rich MPS like in the liver, spleen, bone marrow and blood circulation [9].

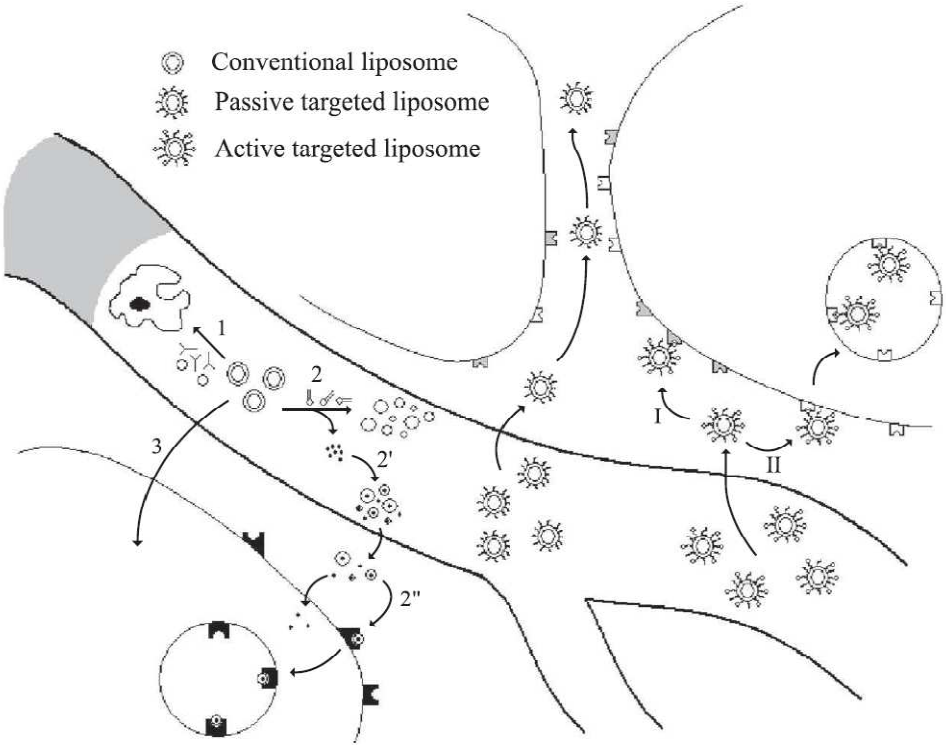

Figure 5: *In vivo* behaviour of the different types of liposomal delivery systems: conventional liposomes have a short plasma half-life either because they become absorbed by protein opsonins, followed by macrophage uptake (1) or because of lipid exchange with plasma proteins, followed by liposome disintegration and consequent PS release (2). The released PS might associate with plasma proteins (2') and then enter the tumour cells mainly by LDL-receptor-mediated endocytosis (2''). A small fraction of the conventional liposomes reaches the tumour tissue in its original formulation for intracellular uptake of the PS by direct binding to cell surface proteins (3). Passively targeted liposomes accumulate in the tumour interstitium without intracellular uptake. Actively targeted liposomes can either be directed to a non-internalising target (I) at the tumour cell or can enter the tumour cell by receptor-mediated endocytosis upon binding to an internalising receptor (II) [9].

In brief, due to a rapid disintegration and unspecific biodistribution, conventional liposomes are less able to establish elevated tumour-to-normal tissue ratios, hampering their generalised use as tumoritropic carriers of PSs [9].

2.2.2- Passively targeted liposomes for photodynamic therapy

Because of angiogenesis in malignant tissue, tumour vessel walls demonstrate an enhanced vascular permeability with fenestrae of a pore size of 100 to 1200 nm. Besides, as tumour tissue lacks a functional lymphatic system, extravasated macromolecules do not go back efficiently to the central circulation. This particular tumour architecture is at the origin of a spontaneous extravasation, accumulation and retention of some macromolecules. Provided that they circulate sufficiently long, the EPR effect allows liposomes to passively accumulate in tumour tissue at high concentrations. Thus, liposomes should be rendered “invisible” for lipoproteins and MPS. Many approaches based on surface modifications were explored to produce long-circulating liposomes with enhanced plasma stability [4], [9], [24], [33], [34].

While conventional liposomes are rapidly cleared from the bloodstream in ten minutes, the presence of glycolipids (e.g. monosialoganglioside) increases the circulation half-life to values up to 12 h. Inclusion of lipids with PEG-headgroups further prolongs the circulation half-life to the order of ten hours. Liposomes with prolonged circulation times due to alteration with glycolipids or PEGylated lipids are named sterically stabilized liposomes or stealth® liposomes [4], [9], [24], [34].

Only one *in vivo* study was performed to explore the PDT efficacy of long-circulating liposomes passively targeting a PS to tumour tissue. In this study, it was demonstrated a significant tumour regression and a high cure rate upon intravenous injection of benzoporphyrin derivative monoacid ring A (BPD-MA) encapsulated in glucuronide-modified liposomes and subsequent tumour illumination [25]. These long-circulating liposomes escaped from being trapped in MPS and accumulated extensively in the tumour (with an extent 3- to 4-fold higher than that of conventional liposomes with DPPG) [9].

It is shown that long-circulating liposomes, with their hydrophilic surface, do not interact efficiently with cells (Figure 5). Hence, one can speculate to what extent these extravasated liposomes accumulating in the tumour interstitium are able to transfer their PS content to tumour cells. This is significant as the cytotoxic singlet oxygen generated by the irradiated PS shows a very short migration radius. In the previous *in vivo* study, it can be supposed that the liposomes were degraded under the huge impact of singlet oxygen, launching the photocytotoxic principle into the restricted extracellular tumoral space to reach high local concentrations and that excited liposomal PSs can collapse the phospholipid barriers releasing themselves. However, in that study, the outcome could have been influenced by the use of a hydrophobic PS

(BPD-MA). It cannot be excluded that after a prolonged stay in the interstitial space, there occurred a limited but essential transfer of PS from the liposomes to the tumour cells [9].

Due to the inexistence of others *in vivo* studies, covering the efficacy of PDT using various PSs incorporated in passively targeted long-circulating liposomes, no general conclusions can be made concerning the general applicability of this type of liposomes as tumoritropic carriers for PSs [9].

2.3- Methods for preparation of liposomal drug formulations

Numerous procedures have been developed to prepare liposomes, but only a few of them are capable of encapsulating large quantities of a molecule with a specific physicochemical nature. Tables 5-8 present the description of the main passive encapsulation methods to prepare liposomal drug formulations. These procedures produce heterogeneous mixture of liposomes that after appropriate extrusion yield LUVs [38].

Table 5: Description of the lipid film hydration method (data was compiled from [28], [29], [39]).

Method of preparation	Lipid film hydration
Chemical nature of the molecule to be loaded	Hydrophilic / Hydrophobic
Principle of the encapsulation	For hydrophilic drugs, the encapsulation results from the hydration of the dry lipid film with an aqueous solution of drug. The spontaneous formation of liposomes passively captures the dissolved drug. For hydrophobic drugs, the compound is dissolved with the lipid constituents in a suitable organic solvent. Afterwards, the solvent is removed and the film is hydrated with an aqueous solution at a temperature above the higher transition temperature of the lipids. For both cases, cycles of warming and mechanical agitation result in the entrapment of the drugs within the lipid bilayer.
Advantages	Easy and fast procedure.
Disadvantages	Low encapsulating efficiency, often less than 10%; unequal distribution of solute.

Table 6: Description of the reverse-phase evaporation method (data was compiled from [32], [38], [40]-[43]).

Method of preparation	Reverse-phase evaporation
Chemical nature of the molecule to be loaded	Hydrophilic
Principle of the encapsulation	The lipid film previously prepared is redissolved in ether (organic phase). The drug solution buffer (aqueous phase) previously prepared is added directly to the organic phase. The system is then sonicated for emulsification of the two phases and the solvents removed under reduced pressure. Removal of the last traces of solvent transforms the gel into LUVs. The ratio of aqueous phase to organic phase is usually 1:3 for ether.
Advantages	High encapsulation efficiency up to 65 % can be obtained in a medium of low ionic strength. The method has been used to encapsulate small, large and biologically active macromolecules such as ribonucleic acid (RNA).
Disadvantages	Exposure of the compounds to be encapsulated to organic solvents and to brief periods of sonication, which can lead to the denaturation of some proteins or breakage of deoxyribonucleic acid (DNA) strands; limited by lipid solubility in organic phase.

Table 7: Description of the film loading method (data was compiled from [44]).

Method of preparation	Film loading
Chemical nature of the molecule to be loaded	Hydrophobic
Principle of the encapsulation	Empty liposomes are added to the drug film and then sonicated and extruded.
Advantages	Allows encapsulation of very hydrophobic drugs.
Disadvantages	Low encapsulation efficiencies for the drugs yet tested.

Table 8: Description of the freeze-thaw method (data was compiled from [41], [43], [45]).

Method of preparation	Freeze-thaw
Chemical nature of the molecule to be loaded	Hydrophilic / Hydrophobic
Principle of the encapsulation	Sonication after film hydration and application of a series of freeze-thaw cycles, which break and re-fuse SUVs formed, replace the cycles of warming and mechanical agitation of the lipid film hydration method. Results in LUVs after extrusion.
Advantages	High encapsulation efficiencies approaching 90%; no detergents or solvents used; fast, simple and mild procedure.
Disadvantages	Process inhibited by increasing the ionic strength of the medium and by increasing the phospholipid concentration.

A method of encapsulation of drugs is acceptable from the pharmaceutical standpoint if it satisfies certain requirements, such as [40]:

- Yield well-defined and reproducible liposomes.
- Be quick, and lead to liposomes that retain the drug for long periods.
- Be applicable to liposomes prepared differently and not be influenced significantly by the liposomal lipid composition.
- Be suitable for various drugs that have similar physicochemical properties.
- Exhibit high encapsulation efficiency and loading capacity.

The three most important factors to be evaluated before selecting the method of preparation are the encapsulation efficiency, the final drug/lipid ratio (loading capacity) and appropriate drug retention properties. Thus, optimal liposomal formulations are those that will exhibit encapsulation efficiencies of 90% or more, employ inexpensive and relatively saturated lipids such as phosphatidylcholine and cholesterol (avoiding oxidation problems), and exhibit the highest possible loading capacity for economical reasons [40].

In this work, the liposomal preparation methods mentioned above were tested for encapsulation of two hydrophobic PSs and compared after physicochemical characterization of the developed formulations.

CHAPTER II - OBJECTIVE

The aim of this work is the development of a liposomal formulation exhibiting an efficient encapsulation of a hydrophobic PS in large unilamellar liposomes (LUVs) with an appropriate size for future intravenous administration. Liposomal formulations show the ability to decrease the tendency of hydrophobic PS to aggregate in aqueous media, prolonging the circulation of the drug in bloodstream and protecting it from premature degradation. Only the monomeric species are considerably photoactive and so liposomes enhance the PDT efficiency [9], [37]. Additionally, liposomes may enhance PS selective uptake and accumulation in tumour tissue, contributing to the selectivity of the treatment [9], [37].

In order to obtain liposomes encapsulating the PS, with the desired characteristics, different methods of preparing liposomes are tested, namely, the lipid film hydration method, the reverse-phase evaporation method, the freeze-thaw method and the film loading method. The liposome lipid composition is also varied by testing different types of phospholipids and different molar ratios of each component in the presence or absence of cholesterol. The more appropriate preparation method and liposome lipid composition will be the one that yields the highest loading capacity and encapsulation efficiency among the different conditions tested.

CHAPTER III – MATERIALS AND METHODS

1.1- Materials

The PS molecules Luz011c and Luz011 were kindly provided by Luzitin, SA (Coimbra, Portugal). Because these two molecules are extremely similar in terms of structure and physicochemical properties (molecular weights of 1136.15 and 1134.15 g/mol, respectively, and logarithm of the octanol/water partition coefficient ($logP_{OW}$) of 2.7), differences in their behaviour when incorporated in a liposome formulation are not expected. Their use in the experiments was determined by their availability at the moment. Phospholipids distearoylphosphatidylcholine (DSPC) and distearoylphosphatidylglycerol (DSPG) were purchased from Lipoid (Ludwigshafen, Germany). Cholesterol, Sephadex G50 and Cremophor EL were supplied by Sigma-Aldrich (St. Louis, USA). Potassium *di*-hydrogen phosphate and perchloric acid 70% were purchased from Panreac (Barcelona, Spain). All others reagents and solvents used were supplied by Merck (Darmstadt, Germany). Polycarbonate membranes used in the extrusion were obtained from Avestin (Mannheim, Germany). The water used was internal ultra-pure water from a Purelab Ultra ultrapure water system (ELGA Process Water, Marlow, United Kingdom).

1.2- Methods

All the experimental steps involving the PS were performed in dim light conditions, because the PS is activated in the presence of sunlight, producing singlet oxygen that will destroy the bilayer of the liposome by peroxidation and will favor the release of the PS from the liposome.

The compound is also sensible to the temperature, which was controlled. The solid PS and its stock solution in chloroform were stored at -18ºC under nitrogen. The preparation of liposomes was performed at room temperature, except in the stages where it was necessary to raise the temperature slightly above the transition temperature (T_m) of the lipids. The T_m corresponds to the temperature required to induce a change in the lipid physical state of a more ordered gel phase (solid phase), when the carbon chains are fully extended and with tilted structure, to a disordered liquid crystal phase, in which the carbon chains of the lipid are less oriented and more

fluid [43]. After the preparation of liposomes encapsulating the PS, these were stored at 4℃.

1.2.1- Encapsulation of a photosensitizer in liposomes

The proportion of lipids DSPC and cholesterol was chosen to be 7:3, because proportion lower or equal to 30% of cholesterol may improve the rigidity of membrane bilayer and enhance the stability of the bilayer in the presence of biological fluids [46].

Alternative lipid compositions with DSPC (neutral phospholipid) and DSPG (negatively charged phospholipid), and with DSPC, DSPG and cholesterol were also tested, in order to evaluate the impact of presence of cholesterol and DSPG in the final encapsulation results.

1.2.1.1– Lipid film hydration method

For the first experience, it was chosen a drug/lipid molar ratio of 5% and 2.5%, according to the literature [47].

After preparation of stock solutions of 100 mM of lipid and 2 mM of Luz011c in chloroform, 0.14 mL of stock solution of DSPC, 0.06 mL stock solution of cholesterol (molar proportion 7:3) and 0.5 mL of Luz011c (drug/lipid molar ratio of 5%) were added in a round-bottom tube previously washed with absolute ethanol, to obtain a total lipid concentration of 20 mM. In another round-bottom tube, to achieve a total lipid concentration of 40 mM, 0.28 mL of stock solution of DSPC, 0.12 mL of stock solution of cholesterol (molar proportion 7:3) and 0.5 mL (drug/lipid ratio of 2.5%) of Luz011c were added. Then, the chloroform was evaporated from the mixture in a rotary evaporator at 65℃ (10ºC above the highest transiti on temperature (T_m) of the lipid with the highest T_m) and at approximately 100 rpm for 30 minutes, resulting in films. The obtained films were placed in an oven at 37 ºC under vacuum overnight to evaporate residual solvent.

The films were hydrated with 1 mL of HEPES-buffered saline (HBS) (10 mM HEPES and 140 mM NaCl, pH = 7.4). This buffer solution was previously warmed to 65ºC for 5-10 minutes. Cycles of heating at 65 ºC for 1 minute and agitation in a vortex

for 30 seconds were repeated 3-4 times, which resulted in the multilamellar liposome vesicles (MLVs).

MLVs suspensions were centrifuged at low speed (200 – 400 rpm) to remove the majority of precipitated non-encapsulated drug.

Two sequential extrusions were made (in a LiposoFast-Basic Extruder - AVESTIN, Inc., Ontario, Canada) at 65ºC with 21 passages through two polycarbonate membranes with pore diameter of 200 nm and 100 nm (respectively), resulting in the desired large unilamellar vesicles (LUVs). Finally, the average diameter of the formulations was measured by dynamic light scattering (DLS) using a N4 Plus Submicron Particle Size Analyzer (Beckman Coulter, Inc., USA).

The DLS technique measures the fluctuations in intensity of scattered light from the nanoparticles Brownian movements that may be analyzed to determine the particle size. In comparison with other techniques, DLS has several advantages: easier sample preparation (only dilution is needed), faster and less expensive method and useful for including a big number of particles obtaining statistically reliable values of size. Some disadvantages of this technique are that the sample has to be dispersed in a liquid, which may alter its characteristics; it is impossible to apply to clusters and does not give information about the surface and morphology (it is primarily used for spherical particles) [40].

In the second experience, this encapsulation method was repeated using a drug/lipid molar ratio of 2% mol Luz011c, in order to evaluate the influence of the drug/lipid molar ratio on the encapsulation efficiency, and verify if the amount of drug could be reduced, avoiding undesired losses during the process. Total lipid concentrations of 20 mM and 40 mM were chosen to confirm if the amount of lipid influences the liposome formation and the drug encapsulation efficiency.

After testing the other liposome preparation methods that will be described in sections 1.2.1.2 to 1.2.1.4, the lipid film hydration method was tested again. Thus, drug/lipid molar ratios of 5% and 2% were used for an initial total lipid concentration of 40 mM and for 1 mL of hydration solution to optimize the experience with the best results. The experimental procedure was the same as described above with the exception that the drug was the Luz011 and the extrusion was made only through polycarbonate membranes with pore diameters of 100 nm. In addition, trying to improve encapsulation efficiency and loading capacity, another test was performed with a drug/lipid molar ratio of 2% for 40 mM of total lipid. The compound Luz011 was not incorporated in the film, but instead was dissolved in the hydration solution, composed

of 1% of Cremophor EL, 2% of absolute ethanol and 97% of HBS. The aqueous phase (1 mL) was added to the lipid film and the rest of the procedure remained the same as in section 1.2.1.1.

Considering the promising results of the preparation method using the Cremophor EL to dissolve Luz011, drug/lipid molar ratios of 2% and 4% for initial total lipid concentrations of 40 mM and 20 mM, respectively, were tested by the same procedure described in the paragraph above. Nevertheless, to help the hydration of the lipid film with the aqueous phase containing Luz011 dissolved, the mixture besides being subjected to cycles of heating and agitation was also sonicated for 1 minute.

To verify the reproducibility of the encapsulation parameters for the formulation DSPC : cholesterol (7:3), the earlier assay with the compound in the lipid film (without Cremophor EL) was repeated for a drug/lipid molar ratio of 2% and initial total lipid concentration of 40 mM.

Finally, a different formulation development approach with Luz011 was selected. Alternative lipid compositions were tested in order to reach better encapsulation parameters than the ones obtained with DSPC : cholesterol (7:3). It was chosen, based on literature [48], a drug/lipid molar ratio of 8.71% and an initial total lipid concentration of 25.3 mM (or of 16.9 mM). Formulations of DSPC : DSPG (9:1), DSPC : DSPG (7:3) and DSPC : DSPG : cholesterol (7:2:1) were prepared in the same way described in section 1.2.1.1, except that the vacuum pressure of rotary evaporation was reduced in steps, after 5 minutes and after 10 minutes intervals. The low pressure was then kept for 45 minutes. Besides, the dry lipid films were dispersed in 1 mL (or in 1.5 mL) of HBS by manual shaking above the phase transition temperature of the lipids for 20 minutes, flushed with nitrogen and let equilibrate overnight at room temperature. Afterwards, cycles of heating at 65ºC for 1 minute and agitation in a vortex for 30 seconds were repeated 3-4 times and the remain procedure was similar as described in section 1.2.1.1.

1.2.1.2- Reverse-phase evaporation method

To accomplish greater encapsulation efficiency and loading capacity, the reverse phase evaporation method was tested. According to literature, for maximum encapsulation the ratio 1 mL aqueous phase to 3 mL organic phase must be maintained, with the possibility of transposition of scale [34], [40].

After preparation of stock solutions of 100 mM of lipid in chloroform, 0.56 mL of stock solution of DSPC and 0.24 mL of cholesterol stock solution were transferred to a round-bottom flask (molar proportion 7:3). The final concentration of lipid in the liposome formulation was 40 mM. The chloroform was removed by rotary evaporation. The lipid film was re-dissolved in 3 mL of chloroform followed by 3 mL of diethyl ether and warmed slightly to facilitate the process. The compound Luz011c was dissolved in an aqueous solution of 2% ethanol, 1% Cremophor EL and 97% phosphate-buffered saline (PBS) pH = 7.4, and 2 mL of this solution was added vigorously through a syringe with a 21-gauge needle into the lipid mixture (corresponding to a drug/lipid molar ratio of 4.4%). The system was kept closed under inert gas (nitrogen).

Afterwards, the mixture was sonicated in an ultrasound bath for 5 minutes to produce a dispersion phase. The sonication temperature was maintained below 10ºC (the bath was cooled with ice).

The organic solvents were then removed by rotary evaporation under reduced pressure at room temperature and at 200 rpm for an hour and a half.

With the help of a vortex agitator and in a closed system, an aqueous suspension was obtained. This was diluted with 1 mL of PBS. Then, the average diameter of liposomes was measured as described before in section 1.2.1.1.

Extrusion was performed at 65℃ through two polycar bonate membranes with pore diameter of 100 nm (21 passages) and after that the average diameter of liposomes was determined again as described in section 1.2.1.1.

1.2.1.3- Film loading method

To achieve greater encapsulation efficiency and loading capacity for a very hydrophobic Luz011, the film loading method was performed [44]. A drug/lipid molar ratio of 4.9% and an initial total lipid concentration of 21 mM were used.

After preparation of stock solutions of 100 mM of lipid in chloroform, 734 µL of DSPC stock solution and 315 µL of cholesterol stock solution were mixed (in the proportion 7:3) with 262 µL of methanol under nitrogen and the mixture was sonicated for 10 minutes. The organic solvents were evaporated in a rotary evaporator at 65℃ and at 100 rpm. Afterwards, 5 mL of phosphate-buffered saline (PBS) pH = 7.4 was added under nitrogen and the hydration occurred for 10 minutes at 65℃ using a vortex agitator. The mixture was sonicated for 20 minutes at 65℃, and then extruded as previously described.

The Luz011 was dissolved in 1.25 mL of chloroform / methanol (4:1 v/v). The organic solvents were evaporated at 40℃, resulting in the drug film.

Next, the empty liposomes obtained earlier were added to the drug film. The mixture was sonicated for 20 minutes at 60℃ and centrifuged at 200 rpm for 30 seconds at room temperature. Finally, the average diameter of liposomes was measured as described in section 1.2.1.1.

1.2.1.4- Freeze-thaw method

Aiming to obtain greater encapsulation efficiency and loading capacity, the freeze-thaw method [45], [49]-[51] was performed for the formulation with the best results. Therefore, a drug/lipid molar ratio of 2.5% of Luz011 was used and an initial total lipid concentration of 40 mM as in the first experience of the lipid film hydration method.

After preparation of stock solutions of 100 mM of lipid in chloroform and 2 mM of Luz011 in chloroform, to achieve a total lipid concentration of 40 mM, 0.28 mL of stock solution of DSPC, 0.12 mL of stock solution of cholesterol (proportion 7:3) and 500 µL of Luz011 were added in a round-bottom flask. Then, the chloroform was evaporated from the mixture in a rotary evaporator at 65℃ and at approximately 100 rpm, resulting in the film drug-lipid. This was placed in an oven at 37ºC under vacuum overnight to evaporate residual solvent.

Afterwards, the film was hydrated with 1 mL of HBS. This buffer solution was previously warmed to 65ºC. The mixture was sonicated at 60ºC during 5 minutes. 5 cycles of freeze and thawing (5 minutes each) were made in dry ice/acetone and at 65ºC, respectively. After each thawing, the mixture was stirred in a vortex agitator for 30 seconds. In the end, MLVs were obtained.

Then, the mixture was centrifuged to remove non-encapsulated drug at a speed of 300 rpm for 30 seconds followed by 500 rpm for 2 minutes twice.

The extrusion was performed at 65ºC and with 21 passages through a two polycarbonate membrane with pore diameter of 200 nm and then 100 nm, resulting in the desired LUVs. Finally, the average diameter of the formulations was measured as described previously in section 1.2.1.1.

1.2.2- Non-encapsulated drug separation method

For the separation of the non-encapsulated drug from the liposomal drug formulation, size exclusion chromatography was performed using a column with Sephadex G-50 and HBS pH 7.4 as the eluent. The aliquot containing the liposomal drug formulation was collected for phospholipid and drug quantification.

1.2.3- Physicochemical characterization of the prepared liposomes

The physicochemical characteristics of liposomes determine their *in vitro* and *in vivo* behavior. Such characteristics can be evaluated by the encapsulation parameters (namely the loading capacity and the encapsulation efficiency) and the mean diameter of the liposomal drug formulations and their size distribution [40].

The loading capacity is defined by the ratio $[drug]_{end}/[total\ lipid]_{end}$ and the encapsulation efficiency by $([drug]/[total\ lipid])_{end} / ([drug]/[total\ lipid])_{initial} \times 100$.

The drug quantification was achieved by ultraviolet-visible (UV/Vis) spectroscopy, using a previously determined drug calibration curve at a stronger absorption peak of the drug's spectrum ranging from 300 to 800 nm that does not suffer interference from other components, such as lipids or solvent.

The total lipid quantification was performed by the colorimetric method of Bartlett for quantification of inorganic phosphate [52]. Afterwards the concentration of lipid was extrapolated from the obtained experimental phospholipid concentration according to the molar proportion cholesterol : total lipid. In this highly sensitive method, inorganic phosphate, resulting from acid hydrolysis of phospholipids, is

converted into phosphomolybdic acid, which, after being reduced, gives a blue complex of phosphomolybdate. The colour intensity was determined by UV/Vis spectroscopy and is proportional to the quantity of inorganic phosphate [52].

1.2.3.1- Phospholipid quantification

Aliquots of the samples resulting from the successful extrusion (or from centrifugation in the case of film loading method) were collected for phospholipid quantification.

The quantification of inorganic phosphate was made using the colorimetric method of Barlett [52]. Different volumes (0, 25, 50, 100, 200, 300 µL) of KH_2PO_4 0.65 mM standard solution, for the calibration curve, and the samples were added to tubes previously washed with absolute ethanol, followed by addition of 0.5 mL of perchloric acid 70% to each tube. The tubes were then stirred in a vortex agitator. The acid digestion was conducted in a sand bath for 30 minutes at about 200ºC. Afterwards, the tubes were cooled in water, and 5 mL of ammonium molybdate reagent plus 0.2 mL of standard Fiske and Subbarow reagent were added to the tubes. The tubes were stirred in a vortex agitator, and were placed in a boiling water bath for 15 minutes. After cooling the tubes in water, the absorbance at 830 nm was read, and the phospholipid concentration was determined against the calibration curve.

1.2.3.2- Drug quantification

For drug quantification by UV/Vis spectrophotometry (in a UV-160 A spectrophotometer, SHIMADZU, Japan), a drug calibration curve was previously determined.

In general, 100 µL of the samples were dispersed in 2400 µL of methanol and the absorbance was read at the appropriate wavelength without interference from other components, as shown in Table 9. Drug concentration was quantified by means of the calibration curve.

Table 9: Absorption wavelengths for drug quantification of the PSs used (Luz011c and Luz011).

Drug / PS	λ / nm
Luz011c	408
Luz011	343

1.2.3.3- Validation of the method for non-encapsulated drug separation by size exclusion chromatography

For the validation of the non-encapsulated drug separation method by size exclusion chromatography, performed in the same conditions as described in section 1.2.2, successive aliquots of 500 µL were collected in 14 microtubes for the DSPC : DSPG (9:1) formulation and then quantified in terms of phospholipid and drug as described in sections 1.2.3.1 and 1.2.3.2.

CHAPTER IV – RESULTS AND DISCUSSION

1.1- Physicochemical characterization of the developed liposomal drug formulations

1.1.1- Mean size diameter and polydispersity index

The present thesis describes the development and characterization of liposomes composed of DSPC : cholesterol (7:3), of DSPC : DSPG (9:1) or (7:3) or of DSPC : DSPG : cholesterol (7:2:1) encapsulating a hydrophobic PS. It was already mentioned that hydrophobic PSs tend to aggregate in aqueous media; therefore the use of liposomes is extremely important since they will prolong the circulation of these compounds, protecting them from premature degradation and avoiding their precipitation in the bloodstream.

Some preliminary experiences involving liposomes made of DSPC and cholesterol were performed in the presence of Cremophor EL, which is a non-ionic poloxyethylated castor oil used as a emulsifying agent to resolve the solubility problems of hydrophobic PSs [53]. PS was dissolved in 1% Cremophor EL.

Table 10 presents the mean size diameter and polydispersity index (PI) obtained by DLS for all the liposomal drug formulations after the different preparation methods followed by the extrusion procedure.

Ideally, the most appropriate mean size diameter of liposomes intended for intravenous administration in cancer therapy should be less than 200 nm. By exhibiting that size, liposomes do not present an embolism risk and additionally they can exit the leaky vasculature in tumour tissue and reach tumour cells, but are kept in circulation by the endothelial wall in healthy tissue vasculature. As it can be observed in Table 10, all the preparation methods tested allowed to obtain liposomes with a mean diameter close to 100 nm or even inferior. Nevertheless, it is important to evaluate the polydispersity index and not only the mean size diameter.

Table 10: Experimental results of mean size diameter and PI obtained by DLS for all the liposomal drug formulations after the different preparation methods followed by the extrusion procedure.

Preparation method	**Liposome composition**	**Drug/lipid molar ratio (%)**	**$[lipid]_{initial}$ (mM)**	**Mean size diameter (nm)**	**PI**
Lipid film hydration	DSPC : cholesterol (7:3)	5.0	20.0	129.2	0.058
		2.0		105.3	0.029
			40.0	106.3	0.280
				106.1	0.262
				103.3	0.277
		2.5		110.3	0.077
		5.0		104.8	0.138
	DSPC : DSPG (9:1)	8.7	25.3	116.2	0.606
				79.5	0.292
				94.8	0.160
	DSPC : DSPG (7:3)			73.9	0.366
				75,8	-0.547
				58.7	1.618
	DSPG : DSPC : cholesterol (7:2:1)			77.8	0.159
				80.3	0.252
Freeze-thaw	DSPC : cholesterol (7:3)	2.5	40.0	87.0	0.123
Film loading		4.9	21.0	86.5	0.098

The PI value is a measure of the distribution of liposome population, which refers to the level of homogeneity in terms of size diameter. A high PI value indicates the existence of different particles sizes or aggregates [40]. The ideal PI value should be inferior to 0.2 in module and indicates a homogenous vesicle population. Hence, the obtained experimental values for PI suggest that the formulations prepared by the lipid film hydration method, the freeze-thaw method and the film loading method have a relatively narrow size distribution although in some case the PI value exceeded 0.2. For that reason a further analysis was performed in the equipment software that allowed to determine the percentage of each population present in the sample. It was observed for the referred methods one narrow peak at approximately 100 nm (or less) corresponding to one population of liposomes (>95%) and another peak at

approximately 5 nm corresponding to a small population (<5%) that was assumed to be free drug aggregates in solution. It can be assumed that in some cases an overestimated PI value was obtained when a small population of free drug was present.

Another liposomal drug preparation method tested was the reverse-phase evaporation method. For this method, worst values of size (361.6 nm) and PI (1.348) (data not shown) were obtained, which indicate that probably the nanoparticles prepared with DSPC : cholesterol (7:3) are not appropriate liposome structures since large aggregates are formed. Additionally, a visual inspection of the suspension before the extrusion showed that it had not the normal appearance as the ones obtained after the other liposome preparation methods.

Another attempt to improve the encapsulation parameters of the lipid film hydration method was made. For that purpose 1% cremophor EL was used to allow the dissolution of the hydrophobic PS in the aqueous buffer, instead of its incorporation in the lipid film. After hydration of the lipid film and subsequent extrusion through two polycarbonate membranes of 100 nm, the obtained mean size and PI results were high (192.6 nm and 284.7 nm for drug/lipid molar ratios of 2% and 4%, respectively and 0.340 and 0.481 for drug/lipid molar ratios of 2% and 4%, respectively), suggesting that liposomes may not be formed. The following intensity analysis performed in the software showed that for this case there were two populations with similar percentage corresponding to two large peaks and not only a predominate population. Once again, a visual inspection of the suspension suggested that liposomes may not have been formed.

It should be noted that the phospholipid DSPG is negatively charged and tends to prevent aggregation following the formation of MLVs [40]. As it is shown in Table 10, the obtained mean size values are bellow 100 nm but the PI of most DSPG liposomal formulations prepared by the lipid film hydration method was relatively high. Nevertheless, the intensity analysis performed in the equipment software revealed a similar situation as reported previously when a small population (<5%) probably of free drug aggregates was present.

1.1.2- Loading capacity and encapsulation efficiency

1.1.2.1- Phospholipid quantification

The quantification of inorganic phosphate was performed using the colorimetric method of Barlett, being the total lipid concentration extrapolated from the experimentally obtained phospholipid concentration. This method is characterized by a high sensitivity and reproducibility [52]. In Annex I it is presented a typical calibration curve obtained in the Bartlett assay and an example of how lipid concentration was determined from the phospholipid concentration.

1.1.2.2- Drug quantification

The quantification of the two PSs was made at the appropriate wavelength (Table 9). It was confirmed that at the wavelength where the PS presents the higher absorption peak the lipids and solvent do not interfere with the measure. At this adequate wavelength, it was obtained a calibration curve with a good coefficient of determination (R^2) for each molecule as shown in Figure II and III (Annex II).

An example of how the PS concentration was determined from the calibration curve is also presented in Table III in Annex II.

1.1.2.3- Encapsulation parameters

The encapsulation parameters were determined from the lipid and PS concentrations as following:

$$\textbf{Loading capacity} = [PS]_{end} / [Lipid]_{end}$$

$$\textbf{Encapsulation efficiency} = (\text{Loading capacity} / ([PS]_{initial} / [Lipid]_{initial})) \times 100$$

Initial and end terms refer to before and after size exclusion chromatography.

The loading capacity and the encapsulation efficiency results obtained for all liposomal formulations prepared by different methods are given in Table 11, except those obtained with the reverse-phase evaporation method and the lipid film hydration method with the use of Cremophor EL, since they showed to be inadequate for the proposed objectives.

In all preparation methods where the drug was incorporated in the lipid film, large aggregates composed of non-encapsulated drug and lipid were formed after hydration with buffer. These aggregates were removed from liposomal formulations by centrifugation, which explained the significant losses of PS and lipid in this step. After the removal of the drug/lipid aggregates, the supernatant, composed of liposomes encapsulating PS, was used to subsequent extrusion.

Table 11: Loading capacities and encapsulation efficiencies for all the liposomal formulations prepared by different procedures.

Method	**Formulation**	**Drug/lipid molar ratio (%)**	**$[Lipid]_{initial}$ (mM)**	**Loading capacity[a]**	**Encapsulation efficiency[b]**
Lipid film hydration	DSPC : cholesterol (7:3)	5.0	20.0	0.001	**2.3**
		2.0		0.002	**3.2**
			40.0	0.001	**3.9**
				0.001	**4.6**
				0.001	**4.5**
		5.0		0.001	**5.4**
	DSPC : DSPG (9:1)	8.7	25.3	0.021	**24.2**
				0.016	**18.8**
				0.013	**14.8**
	DSPC : DSPG (7:3)			0.013	**14.4**
				0.014	**15.8**
				0.013	**14.6**
	DSPC : DSPG : cholesterol (7:2:1)			0.006	**6.5**
				0.009	**10.8**
Freeze-thaw	DSPC : cholesterol (7:3)	2.5	40.0	< 0.001	**0.9**
Film loading		4.9	21.0	0.002	**4.5**

[a] Loading capacity = $[PS]_{end}$ / $[Lipid]_{end}$

[b] Encapsulation efficiency = (Loading capacity / ($[PS]_{initial}$ / $[Lipid]_{initial}$)) × 100

Analyzing the results presented in Table 11 and Figure 6, it is possible to conclude that the freeze-thaw method exhibited the lowest loading capacity (<0.001) and an encapsulation efficiency of less than 1%, which is not in accordance with the literature [51].

The lipid film hydration method was the one that resulted in the best encapsulation parameters among all tested methods (Table 11). In addition, this preparation method turned to be the easiest and the fastest of all tested procedures.

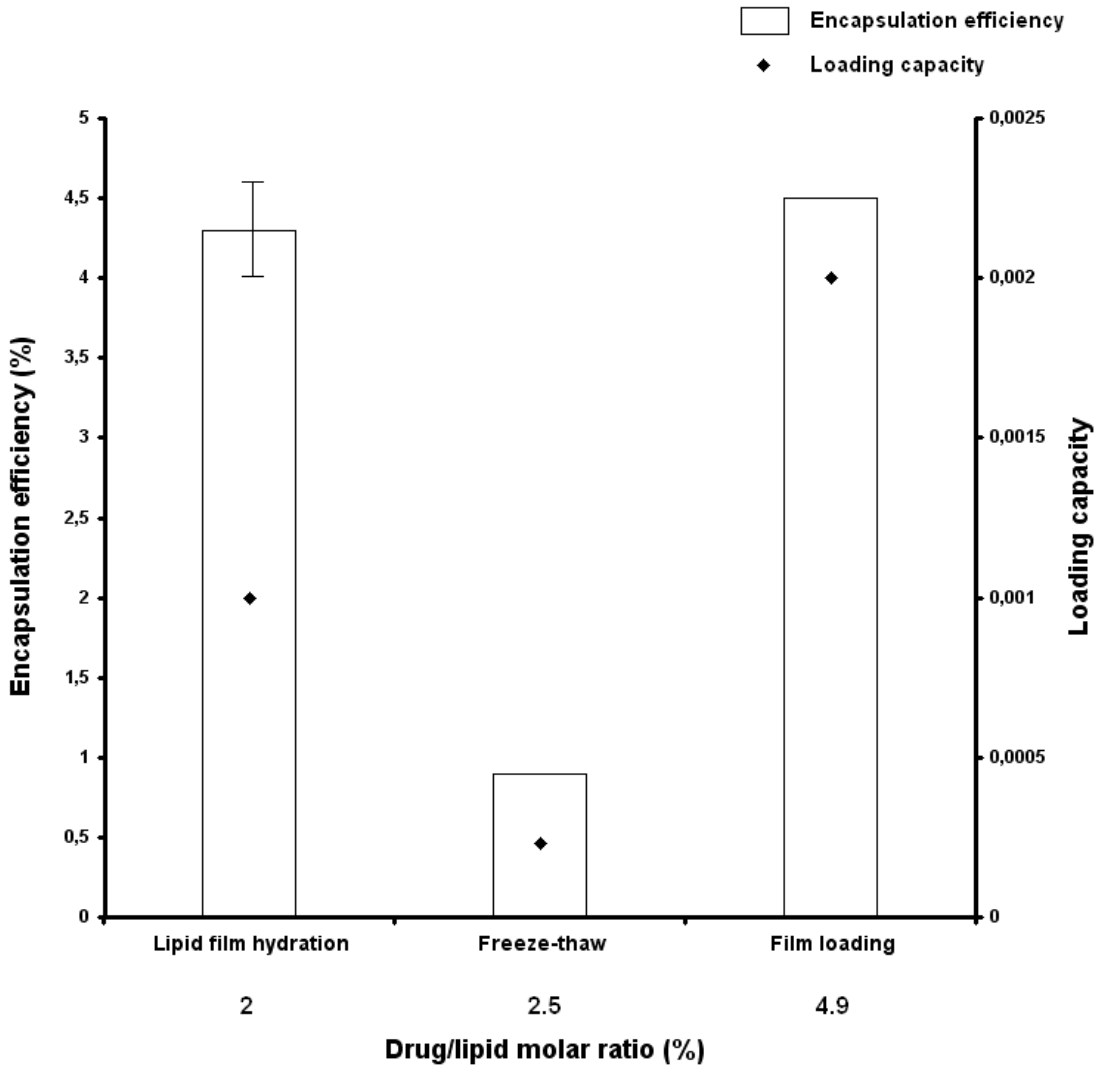

Figure 6: Comparison of the encapsulation efficiencies and loading capacities for DSPC : cholesterol (7:3) liposomes encapsulating PS molecule and prepared by different methods: lipid film hydration, freeze-thaw and film loading. For the last two methods only one experience was performed for the referred drug and lipid concentrations due to inferior results obtained as compared with the lipid film hydration method.

In the lipid film hydration method, for DSPC : cholesterol (7:3) liposomal formulations, an increased concentration of lipid and of PS did not resulted in significant changes in efficiency encapsulation or loading capacity values, as it can be seen in Figure 7.

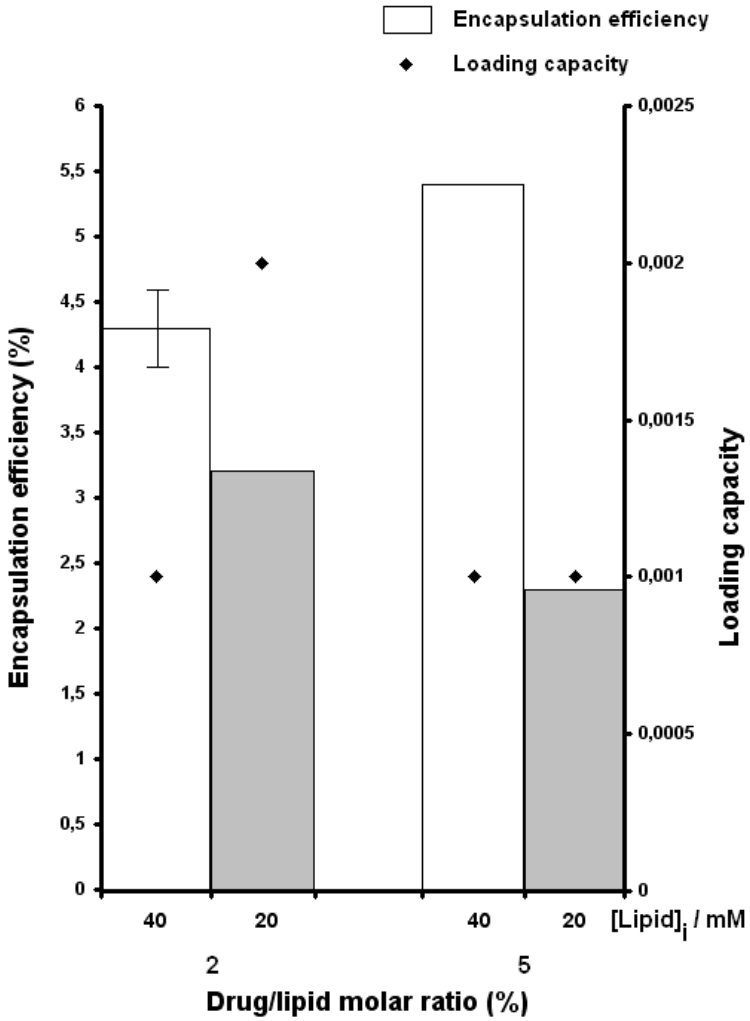

Figure 7: Comparison of the encapsulation efficiencies and loading capacities of DSPC : cholesterol (7:3) liposomes prepared by the lipid film hydration method.

Since changes in lipid and in PS concentration did not produce significant increases in encapsulation parameters for the DSPC : cholesterol (7:3), it was decided to test different approaches concerning the liposome lipid composition:

i) Decrease cholesterol content in the formulation and introduce a new phospholipid – DSPC : DSPG : cholesterol (7:2:1) as described in literature [54].

ii) Develop two formulations lacking cholesterol - DSPC : DSPG in two different molar proportions (9:1) as described in literature [48] and (7:3). With these two

quantitative compositions it would be possible to compare the influence of DSPG content.

As it can be seen from the results of Table 12 and Figure 8, the highest loading capacity and encapsulation efficiency values were obtained for DSPC : DSPG (9:1) liposomal formulation followed by DSPC : DSPG (7:3) formulation, both prepared by the lipid film hydration method and for an initial lipid concentration of 25.3 mM.

Table 12: Encapsulation parameters (encapsulation efficiency and loading capacity) obtained for four liposomal drug formulations with different lipid compositions prepared by the lipid film hydration method.

Method	**Composition of formulation**	**Drug/lipid molar ratio (%)**	**Loading capacity ± SD**	**Encapsulation efficiency ± SD (%)**
Lipid film hydration	DSPC : cholesterol (7:3)	2.0	0.001 ± 0.000	4.3 ± 0.3
	DSPC : DSPG (9:1)	8.7	0.017 ± 0.004	19.3 ± 4.7
	DSPC : DSPG (7:3)		0.013 ± 0.001	14.9 ± 0.8
	DSPC : DSPG : cholesterol (7:2:1)		0.007 ± 0.003	8.6 ± 3.1

Data represent the mean ± standard deviation (SD) of 2-3 independent experiments.

The DSPC is a known membrane stabilizer due to its cylindrical shape favoring bilayer formation [40].

Initially, a neutral lipid composition including a saturated phospholipid (DSPC) with high transition temperature and cholesterol content of 30 mol% was chosen because of the known chemical stability, enhanced drug retention properties and decreased interaction with serum proteins [46].

The formulation DSPC : DSPG (9:1) was recently tested by another group [48] that developed liposomal formulations for a PS molecule with hydrophobic characteristics similar to our PS molecule. For that reason, in this work this lipid composition was also evaluated. Additionally, the DSPG phospholipid, due to its negative charge, contributes to liposome stability since the electrostatic repulsions reduce the occurrence of aggregation [40].

Another formulation with increased DSPG content (DSPC : DSPG 7:3) was also tested in order to assess the influence of DSPG on the encapsulation parameters.

The presence of cholesterol in the DSPC : DSPG : cholesterol (7:2:1) formulation appears to influence negatively the outcome, since higher encapsulation parameters were obtained with the formulations lacking cholesterol (DSPC : DSPG (7:3) and DSPC : DSPG (9:1) (Table 12 and Figure 8). Because of its hydrophobicity, PS molecule is encapsulated in the bilayer, but the presence of cholesterol might difficult the incorporation of PS during the liposome formation.

The results obtained by increasing the proportion of DSPG in the formulation from 1/10 to 3/10 do not improve the encapsulation parameters (Table 12 and Figure 8).

The presence of cholesterol seems to have a negative impact on the obtained encapsulation parameters and the presence of DSPG may have a favourable contribution. For that reason, the formulations DSPC : DSPG (9:1) and DSPC : DSPG (7:3) yielded the highest encapsulation efficiency and loading capacity values (Table 12 and Figure 8).

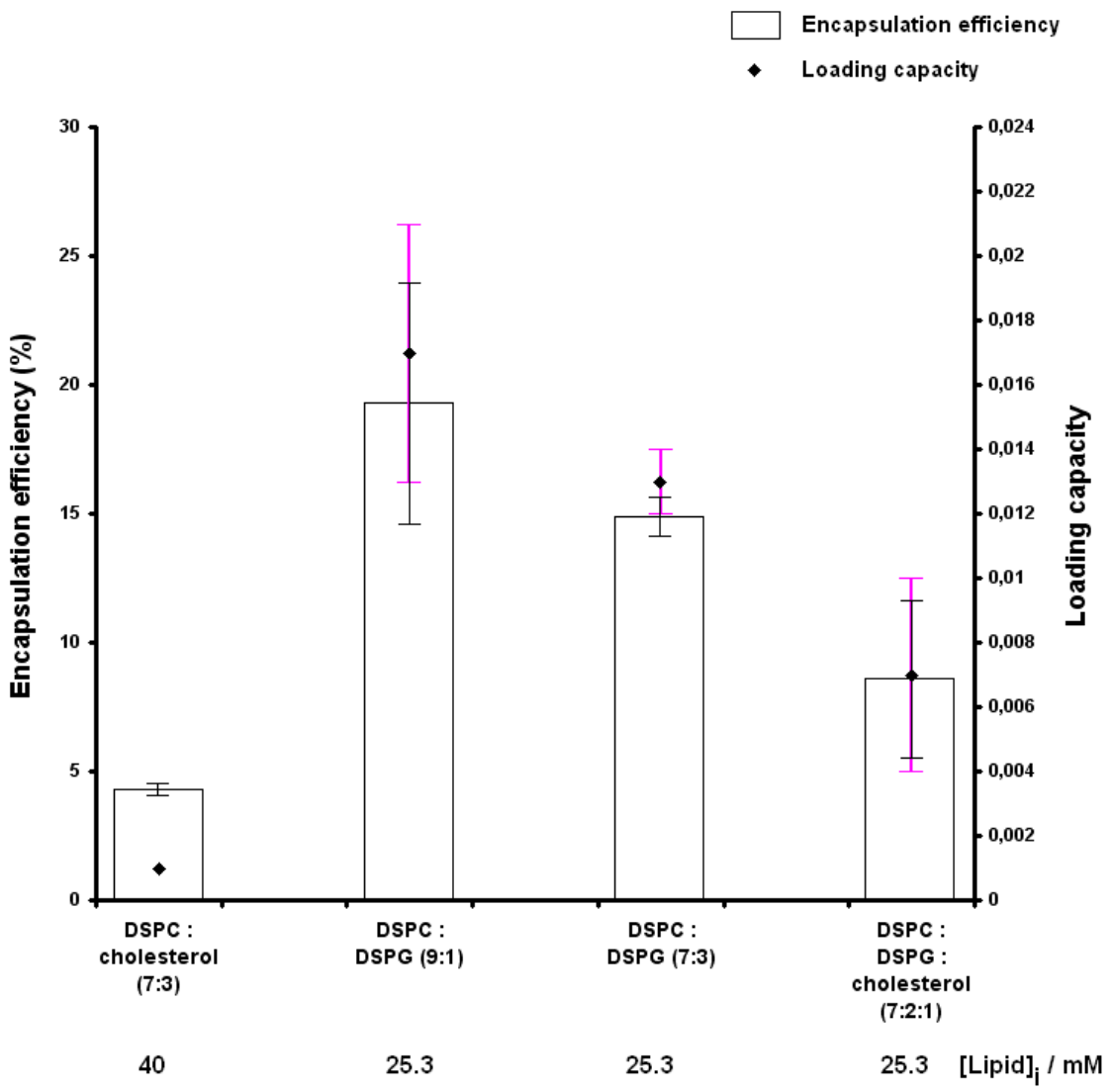

Figure 8: Encapsulation parameters (encapsulation efficiency and loading capacity) for the liposomal formulations with different lipid compositions prepared by the lipid film hydration method. Data represent the mean ± standard deviation (SD) of 2-3 independent experiments.

Although most of the non-encapsulated drug was separated during the centrifugation, since a large aggregate of drug was formed at the bottom of the tube, it was thought to be important to assure that any non-encapsulated drug that remained after centrifugation would be separated in the size exclusion chromatography that followed the extrusion.

The validation of the free drug separation method by size exclusion chromatography is graphically represented in Figure 9 for DSPC : DSPG (9:1) liposomes.

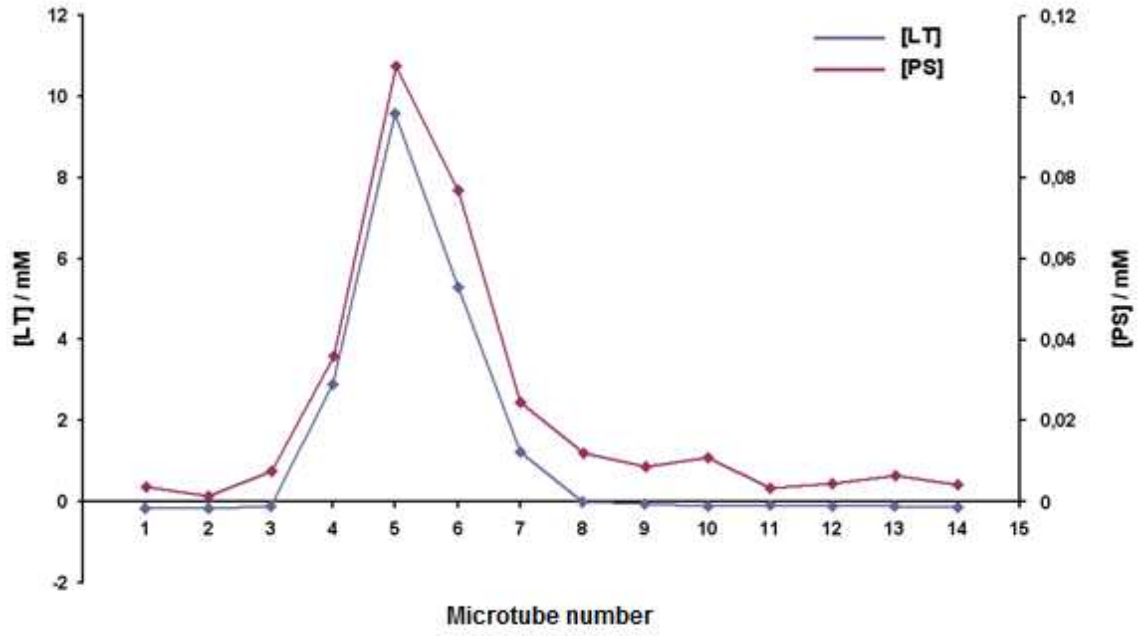

Figure 9: Graphical representation of the validation of free drug separation by size exclusion chromatography for DSPC : DSPG (9:1) liposomes. [LT] corresponds to the total lipid concentration and [PS] to the concentration of drug.

As it can be observed in Figure 9 the maximum concentration value for lipid and drug was obtained at the fifth microtube, which was the one subsequently used to determine the encapsulation parameters. It is clear that the liposomes encapsulating the PS molecule are collected from microtube 4 to 7. From the 8th microtube until the last microtube collected the [LT] is zero but there is a small proportion of non-encapsulated drug eluted in the column. For this reason it can be concluded that the size exclusion chromatography should be performed since it is able to successfully separate liposomal drug from the free drug that remains in solution after the centrifugation.

CHAPTER V – GENERAL CONCLUSIONS AND FUTURE WORK

In this work it was possible to develop and characterize several liposomal formulations encapsulating a hydrophobic PS molecule with different lipid compositions and prepared by several passive encapsulation methods.

Among the different preparation methods, the lipid film hydration method resulted in the best encapsulation parameters (encapsulation efficiency and loading capacity).

The presence of cholesterol seems to have a negative impact on the obtained encapsulation parameters and the presence of DSPG may have a favourable contribution. For that reason, the formulations DSPC : DSPG (9:1) and DSPC : DSPG (7:3) yielded the highest encapsulation efficiency and loading capacity values. Additionally, these formulations also exhibited appropriate average liposomal size for potential intravenous administration.

In future work it would be interesting to evaluate the stability of DSPC : DSPG liposomes upon storage at 4ºC and in human plasma at 37ºC in terms of mean diameter maintenance and drug retention. In addition, it should be considered the incorporation of polyethylene glycol (PEG) at the liposome surface aiming to prolong its blood circulation half-live and eventually evaluate the influence of its presence in the encapsulation parameters. The presence of PEG could also be used as an anchor to attach a ligand molecule in order to actively target the liposomal drug formulation to tumour cells. This strategy would allow the increase of treatment specificity and efficacy in a future *in vivo* application.

It is expected that the results obtained in the present work will prove useful in developing new and efficient methodologies for the preparation of liposomal formulations incorporating photosensitizing molecules for photodynamic therapy of cancer.

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ANNEX I

As an example, in Table I it is shown the absorbance (Abs) values at the specified wavelength for standard solutions S1-S5 made from KH_2PO_4 0.65 mM stock solution.

Table I: Absorbance values measured at 830 nm for standard solutions S1-S5 made from KH_2PO_4 0.65 mM stock solution.

	Phosphate stock solution	0.65 mM	
	V (L)	**$n_{phosphate}$ (mol)**	**Abs**
Blank	0	0	0
S1	2.50E-05	1.63E-08	0.085
S2	5.00E-05	3.25E-08	0.178
S3	1.00E-04	6.50E-08	0.368
S4	2.00E-04	1.30E-07	0.679
S5	3.00E-04	1.95E-07	1.05

From Table I it is possible to plot the graph of Abs = f ($n_{phosphate}$) shown in Figure I.

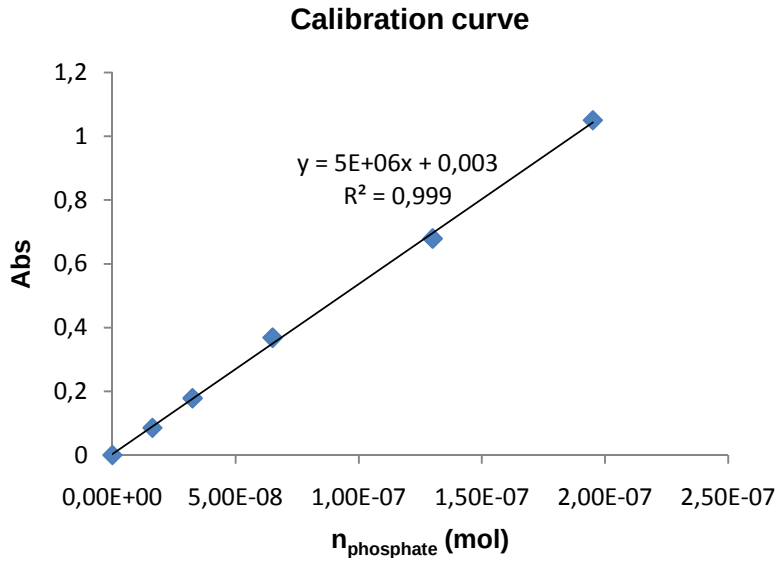

Figure I: Calibration curve of standards S1-S5 prepared from KH_2PO_4 0.65 mM stock solution.

For each liposome sample prepared in triplicate, the absorbance was measured at 830 nm. Then, from the slope and the Y-intercept of the calibration curve, it was possible to calculate the phospholipid concentration ([PL]) and after that extrapolate the total lipid concentration ([LT]) in a given formulation, according to the phospholipid:total lipid molar ratio, as it is shown in Table II.

Table II: Exemplificative data for DSPC : DSPG (9:1) formulation – phospholipid concentration and total lipid concentration.

Liposomal formulation	**Stage**	**V (µL)**	**n(PL)[a] / mol**	**Abs**	**Average Abs**	**[PL][b] / M**	**[LT][c] / mM**
DSPC : DSPG (9:1)	After chromatography	20	1.23E-07	0.292	0.310	6.15E-03	6.147
				0.315			
				0.324			

[a] n(PL) = ((Average Abs – intercept) / slope) × dilution factor
[b] [PL] = n(PL) / V
[c] [LT] = ([PL] / 10 × 10) × 1000

ANNEX II

As an example, in Figures II and III it is shown the calculations for the drugs quantification.

	[PS] / mM	Abs
Blank	0	0
S1	0.0004	0.035
S2	0.0040	0.275
S3	0.0100	0.674
S4	0.0160	1.059

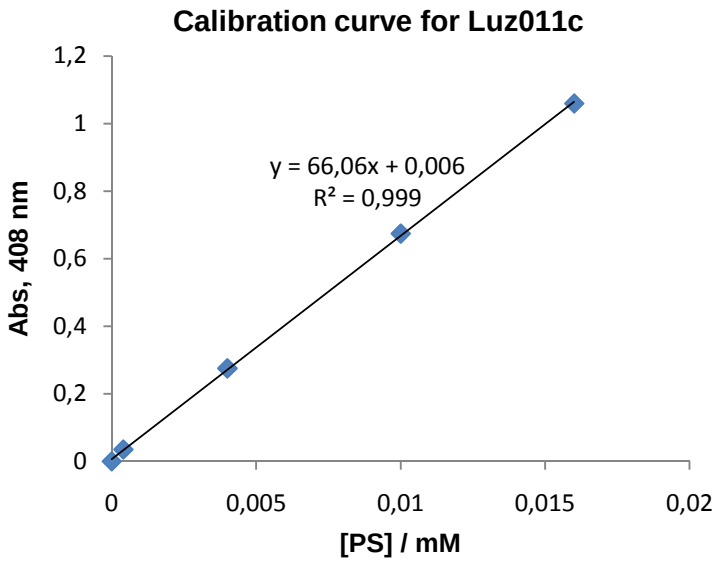

Figure II: Calibration curve for Luz011c at 408 nm.

	[PS] / mM	Abs
Blank	0	0
S1	0.0001	0.009
S2	0.0004	0.024
S3	0.0040	0.276
S4	0.0100	0.682

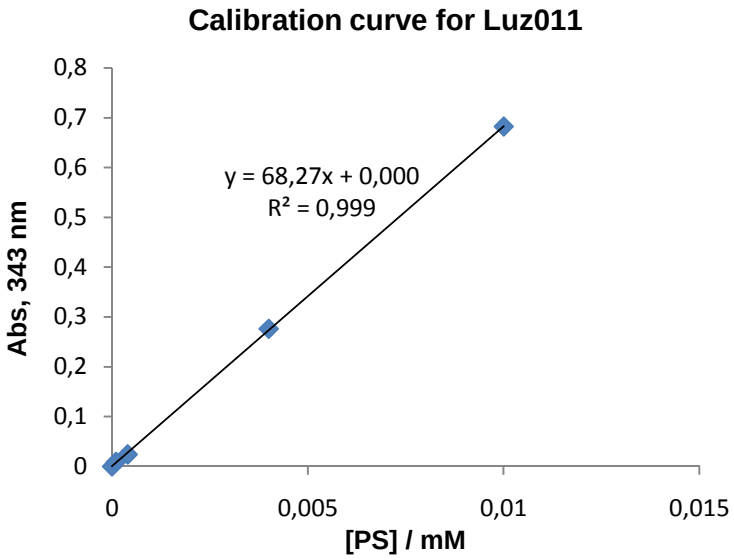

Figure III: Calibration curve for Luz011 at 343 nm.

For each sample the absorbance was measured at the adequate wavelength. From the slope and Y-intercept of the corresponding calibration curve the PS

concentration was determined. For example, Table III presents the quantification of Luz011 in liposomes of DSPC : DSPG (9:1).

Table III: Data for quantification of Luz011 in DSPC : DSPG (9:1) liposomes.

Sample	Stage	Abs	[PS][a] / mM
DSPC : DSPG (9:1)	After chromatography	0.275	0.101

[a][PS] = ((Abs – intercept) / slope) × dilution factor