DEPARTMENT OF LIFE SCIENCES

FACULTY OF SCIENCE AND TECHNOLOGY
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Development of liposomal formulations for drug delivery to breast cancer cells.

Catarina Araújo Gomes Rebelo

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Dissertation submitted to the University of Coimbra in partial fulfilment of the requirements for the Master Degree in Biochemistry, carried out under the supervision of Dr. Henrique Faneca (University of Coimbra) and Prof. Dr. M. Conceição Pedroso de Lima (University of Coimbra) .

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ABREVIATIONS

ABC	Adenosine triphosphate-binding cassette
ATP	Adenosine triphosphate
Chol	Cholesterol
D/L	Drug to lipid (molar ratio)
DNA	Deoxyribonucleic acid
DSPG	1,2-distearoyl-3-sn- phosphoglycerol
DSPE-PEG	1,2-distearoyl-3-sn-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000]
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EPR	Enhanced permeability and retention (effect)
ER	Estrogen receptor
Fab'/F(ab')$_2$	Antigen-binding fragment
FBS	Fetal bovine serum
FDA	Food and drug administration
GM1	monosialotetrahexosylganglioside
HBS	HEPES buffered saline
HDL	High-density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	Human epidermal growth factor receptor 2
HPMA	N-(2-Hydroxypropyl)methacrylamide
HR	Hormone receptor
HSPC	Hydrogenated soy phosphatidylcholine
IC_{50}	concentration required to inhibit cell growth by 50%
i.v.	intra venous
LUV	Large unilamellar vesicle
MDR	Multidrug resistance
MLV	Multilamellar Vesicle
MMP	Matrix metalloproteinases
MPS	Mononuclear phagocytic system
PA	Phosphatidic acid
PAMAM	Poly(amidoamine)
PBS	Phosphate buffered saline
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
PGA	Polyglutamic acid
P-gp	Permeability glycoprotein
PI	Phosphatidylinositol
PLA	Poly(lactic acid)
PLA_2	Phospholipase A_2
PLGA	poly(lactic-co-glycolic acid)
PR	Progesterone receptor
PS	Phosphatidylserine
RNA	Ribonucleic acid
scFV	single-chain variable fragment
S.D.	Standard deviation
$sPLA_2$	Secreted Phospholipase A_2
VEGF	Vascular endothelial growth factor
VCAM-1	Vascular cell adhesion molecule-1
WHO	World health organization

ABSTRACT

Breast Cancer is a heterogeneous disease and a leading cause of death in women. Chemotherapy is a crucial treatment to improve survival and quality of life of breast cancer patients. However, chemotherapy is limited by severe and harmful toxic effects to normal cells and its effect is not long lasting. Moreover, patients can develop resistance to many different types of chemotherapeutic agents. Regarding this, drug delivery systems have been attempted to overcome these problems. By using both passive and active targeting strategies, nanocarriers can enhance the intracellular concentration of chemotherapeutic agents in cancer cells while avoiding toxic effects on healthy tissues. Moreover, they have the potential to overcome drug resistance. In this context, liposome-based technology has been widely studied and evolved to overcome chemotherapeutic needs.

The purpose of this work was to develop a novel liposomal-based drug delivery approach that had the ability to target and release epirubicin specifically to breast tumors, by using an anti-EGFR Affibody® as a targeting moiety, in order to improve the current breast cancer chemotherapeutic strategies. Regarding this goal, several liposomal formulations were developed. A conventional liposome formulation of HSPC and cholesterol was enriched with a steric stabilizer, DSPE-PEG, and an anionic lipid, DSPG. Formulations were characterized according to their size, entrapment efficiency and drug release. The obtained results showed that the incorporation of DSPG into liposomes improved the epirubicin entrapment efficiency. On the other hand, inclusion of DSPE-PEG in liposomes resulted in an increased release of epirubicin during storage. However, this effect was abolished in the presence of 10% FBS. The HSPC:Chol:DSPG:DSPE-PEG (6:3:0.6:0.4 molar ratio) liposome formulation combined high epirubicin encapsulation efficiencies with the surface characteristics and the size (near 150nm) favorable to passively target solid tumors. The *in vitro* antitumoral activity studies, performed in a triple-negative breast cancer cell line (MDA-MB-231 cells), showed only slight differences, in terms of cell death, between the developed formulations. It was also observed that free epirubicin presented a higher cytotoxicity than the developed liposomal-epirubicin formulations. This fact is most probably due to the reduced cell association registered with these formulations. Therefore, an active targeting strategy would be a promising approach to enhance

cellular association and, consequently, the therapeutic efficacy of the epirubicin-containing liposomes. On the other hand, epirubicin could be released from liposomes specifically in the tumor microenvironment in response to a $sPLA_2$ (an enzyme overexpressed in breast tumors) stimulus. The obtained results demonstrated that $sPLA_2$ has the ability to induce the leakage of epirubicin, this $sPLA_2$-mediated drug release being dependent on the liposomes composition. The incorporation of DSPG in the liposomes resulted in a significant increase in the $sPLA_2$ activity.

Overall, the obtained results suggest that HSPC:Chol:DSPG:DSPE-PEG (6:3:0.6:0.4 molar ratio) liposomes present a great potential to be used in the development of a drug delivery system that has the ability to specifically and efficiently release epirubicin in breast cancer cells.

Key words: Breast cancer; Drug delivery; Liposomes; Epirubicin

RESUMO

O cancro da mama é uma doença heterogénea e é a maior causa de morte por cancro entre as mulheres. A quimioterapia é um tipo de tratamento essencial à sobrevivência e à qualidade de vida dos pacientes com cancro da mama. Contudo, a quimioterapia pode estar associada a uma toxicidade em células não cancerígenas sendo, deste modo, limitada por efeitos secundários severos. Por outro lado, o seu efeito terapêutico pode ser temporário e, muitas vezes, os pacientes acabam por desenvolver resistência a muitos agentes de quimioterapia. Os sistemas de transporte e entrega de fármacos foram propostos como uma estratégia para combater estas limitações. Os nanosistemas, quando desenvolvidos com base em estratégias de direcionamento passivo e activo, têm o potencial de aumentar a concentração intracelular dos fármacos no tumor e evitar a toxicidade nos outros tecidos. Neste contexto, vários estudos têm sido realizados por forma a desenvolver formulações de lipossomas que possam ser utilizadas como sistemas de transporte e entrega de agentes de quimioterapia.

O objectivo deste trabalho consistiu no desenvolvimento de uma nova formulação de lipossomas, para transporte e entrega de fármacos, que tivesse a capacidade de direcionar e libertar a epirrubicina especificamente nas células do cancro da mama, através da utilização de um Affibody® contra o EGFR. Desta forma, esta estratégia poderia aumentar substancialmente a eficiência dos tratamentos do cancro da mama disponíveis actualmente. Para isso, foram desenvolvidas novas formulações de lipossomas, a partir de lipossomas convencionais compostos por HSPC e colesterol, aos quais se acrescentou DSPG (lípido aniónico) e DSPE-PEG (lípido modificado com um polímero hidrofílico). Após a encapsulação do fármaco, realizou-se a caracterização das formulações tendo em conta a eficiência de encapsulação do fármaco, o tamanho e estabilidade dos lipossomas. Os resultados obtidos mostraram que a incorporação de DSPG nos lipossomas aumentou a eficiência de encapsulação da epirrubicina. Por sua vez, a inclusão de DSPE-PEG nos lipossomas resultou num aumento de libertação do fármaco durante o armazenamento. Contudo, este efeito foi anulado na presença de 10% de FBS. A formulação de lipossomas composta por HSPC:Chol:DSPG:DSPE-PEG (6:3:0,6:0,4 razão molar) combina a elevada capacidade de encapsulação de epirrubicina com características de superfície e tamanho (perto de 150nm) favoráveis ao seu direcionamento passivo e acumulação nos tumores sólidos.

Os estudos de actividade antitumoral *in vitro*, realizados numa linha celular de cancro da mama do tipo triplo-negativa (células MDA-MB-231), mostraram que existiam apenas pequenas diferenças, em termos de morte celular, entre as diferentes formulações de lipossomas desenvolvidas. Verificou-se também que a epirrubicina livre apresentou uma citotoxicidade muito superior à epirrubicina encapsulada nas formulações de lipossomas. Este facto deve-se, muito provavelmente, à associação celular reduzida das formulações de lipossomas desenvolvidas. Neste sentido, uma estratégia de vectorização específica para o cancro da mama poderá ser solução para aumentar a internalização celular dos liposomas contendo epirrubicina e, consequentemente, a sua eficácia terapêutica. Por outro lado, o conteúdo do lipossoma pode ser libertado no microambiente tumoral em resposta a um estímulo específico, nomeadamente em resposta à degradação causada pela enzima $sPLA_2$ (sobrexpressa no cancro da mama). Os resultados obtidos demostraram que a $sPLA_2$ é capaz de induzir a libertação da epirrubicina contida nos lipossomas, sendo a sua actividade dependente da composição dos lipossomas. A incorporação de DSPG nos lipossomas resultou num aumento significativo da actividade da $sPLA_2$.

Os resultados obtidos sugerem que os lipossomas HSPC:Chol:DSPG:DSPE-PEG (6:3:0,6:0,4) apresentam um grande potencial para serem utilizados no desenvolvimento de um sistema de transporte e entrega de fármacos que tenha a capacidade de libertar a epirrubicina, nas células de cancro da mama, de forma específica e eficiente.

Palavras-chave: Cancro da mama; *Drug delivery*; Lipossomas; Epirrubicina

I- INTRODUCTION

1- BREAST CANCER

1.1 - Worldwide prevalence

Cancer is a leading cause of death worldwide. The World Health Organization (WHO) estimated that cancer accounted for 7.6 million deaths in 2008 (around 13% of all deaths). Lung, stomach, liver, colon and breast cancer cause the most cancer deaths each year. The most frequent types of cancer differ between men and women, and breast cancer is the most common cancer in women (Siegel et al., 2012; DeSantis et al., 2011). Breast cancer is about 100 times more common in women than in men; however men tend to have poorer outcomes due to delays in diagnosis.

According to GLOBOCAN 2008[1], breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in women worldwide. It was responsible for 23% (1.38 million) of the total new cancer cases and 14% (458,400) of the total cancer deaths in 2008. The GLOBOCAN 2008 online tool projects that deaths from cancer worldwide will continue to rise, with an estimated 13.1 million deaths in 2030. It is believed that a significant proportion of the worldwide burden of cancer could be prevented through the application of existing cancer control knowledge (Jemal et al., 2010).

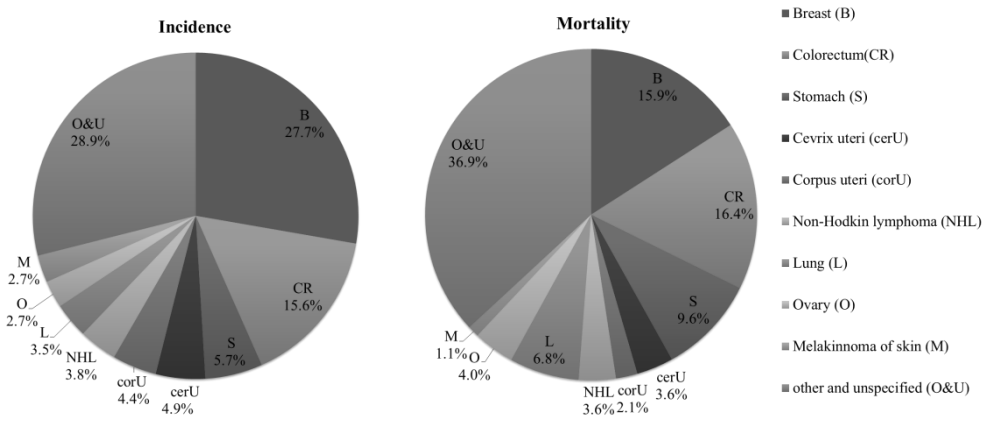

Figure I.1 – Graphical representation of the estimated incidence and mortality of cancer for Portuguese women. Source: GLOBOCAN 2008 [1]

[1] GLOBOCAN 2008 (Cancer Incidence, Mortality and Prevalence Worldwide in 2008): project produced by the International Agency for Research on Cancer (IARC) for 2008.

1.2 - Etiology and risk factors

When breast cells lose their ability to stop growing and dividing, the abnormal tissue forms a tumor. Some tumors are benign (not cancerous) and other tumors are malignant (cancerous). These last ones have the ability to invade the tissue around the breast, spread to others parts of the body and disrupt normal functions in those areas. This process is called metastasis.

Cancer arises from a change in one single cell. The change may be started by external agents and genetic factors. Risk factors include family and personal health history, gynecologic history, mutations in certain genes, race, age and diet habits (Tot, 2011).

A family history of breast cancer is known to increase the risk of presenting the disease by a factor of two or three. Mutations in genes like BRCA1, BRCA2 and p53 are associated with an increased risk of presenting breast cancer (Dunning et al., 1999; Lacroix & Leclercq, 2005). However, these mutations are rare and hereditary breast cancer accounts only for 5-10% of the total cases.

Prolonged exposure to hormones (estrogen or even exogenous hormones) was also indicated as a common risk factor (Martin & Weber, 2000) . Therefore, factors that increase the number of menstrual cycles are associated with an increased susceptibility for breast cancer, such as early age at menarche and late menopause. Similarly, a decrease in the number of these cycles appears to have a protective role, which can be achieved by moderate levels of exercise and a longer lactation period.

Danaei et al. (2005) concluded that an astonishing total of 21% of all breast cancer worldwide deaths could be attributed to alcohol use, overweight and obesity, and physical inactivity.

1.3 - Classification

One of the key challenges of cancer study and treatment is the detected great heterogeneity. Although the breast cancer morphological taxonomy has been well defined by the WHO (Tavassoli & Devilee, 2003) and usefully applied to patient management, it has become clear that tumors classified under the same subtype may be distinct in their clinical behavior. It is believed that these limitations are associated with the incapacity of morphological classifications to consider the underlying biological features of these tumors (Reis-Filho et al., 2006).

Microarray technologies, applied to the study of DNA, RNA and protein profiles, can be used to define a tumors detailed phenotype. This detailed tumor characterization greatly enhance our understanding of the causes and progression of cancer. It has opened a way to discover new molecular markers and new possibilities for therapeutic intervention, indicating a more personal treatment for breast cancer patients (C M Perou et al., 2000; Reis-Filho et al., 2006; Sørlie, 2004; Yerushalmi, Hayes, & Gelmon, 2009; van ’t Veer et al., 2002).

The overall expression patterns of different breast cancer cells showed a clear distinction between the expression levels of the genes of estrogen and progesterone receptors (ER and PR) and the human epidermal growth factor receptor 2 (HER2) (Reis-Filho et al., 2006; Sørlie, 2004; Yerushalmi et al., 2009).

Estrogen and progesterone hormones are normally produced in a woman’s body and stimulate the growth of normal breast tissue. However, the presence of estrogen and progesterone receptors in the breast cancer cells also gives these hormones the ability to stimulate the growth of the tumor. Defining if a tumor is positive or negative for these hormone receptors (HR) is important when a treatment is chosen because there are drugs that can block the effects of these hormones on the cancer cells to help stop tumor growth. These HR–positive tumors tend to grow more slowly and are less likely to invade the lymph nodes (Reis-Filho et al., 2006; Sørlie, 2004).

Breast cancer cells can also be categorized as being HER2-positive or HER2-negative. HER2 is a receptor on the cell surface that sends messages to the cell to grow and divide more frequently. An increased number of gene copies in this type of cancer leads to the overexpression of the HER2 protein on the surface cells and sends a message for the cell to grow and divide. In this way HER2-positive breast cancer tends

to grown faster and is considered more aggressive, presenting a poor survival rate (Reis-Filho et al., 2006; Sørlie, 2004).

Perou et al. (2000) used the microarray methods to find four "intrinsic subtypes" of breast cancer tumors, based upon the similarity in gene expression to normal breast-like group. Tumors were classified into luminal cell-like (subdivided into A and B), basal cell-like and HER2 group (Reis-Filho et al., 2006; Sørlie, 2004; Yerushalmi et al., 2009). Recent studies have identified a new and intriguing subtype called the "claudin-low" group (Herschkowitz et al., 2007), but for the time being, these five tumor subtypes mentioned above are the ones commonly accepted (Figure II.2).

Luminal tumors generally have high expression of hormone receptors and HR-regulated genes and low expression of the HER2. Luminal A shows low expression of proliferation-associated genes. The HER2-enriched subtype present elevated expression of HER2, low expression of the luminal cluster and basal-like genes and high expression of the proliferation cluster. The basal-like subtype is characterized by low expression of ER, PR and HER2 (triple negative), high expression of the proliferation cluster and high expression of a unique cluster of genes called the basal cluster, which includes epidermal growth factor receptor (EGFR) (Perou & Borresen-Dale, 2010; Sørlie, 2004; Yerushalmi et al., 2009)

The analysis of survival rates showed that there are considerably different outcomes for patients from the diverse subtypes (Perou & Borresen-Dale, 2010). Each group presented different overall survival rates and different times to distant metastasis (Sørlie, 2004), and so, they define the clinical courses taken when treating breast cancer. With this understanding, that breast cancer is a heterogeneous disease, a 'one-size-fits-all' therapy approach is becoming yesterday news. The gained knowledge of cellular molecular targets in those different types of breast cancer is being translated into the clinic and has led to the development of exciting new therapies for both triple negative and HER2-positive relapsed disease.

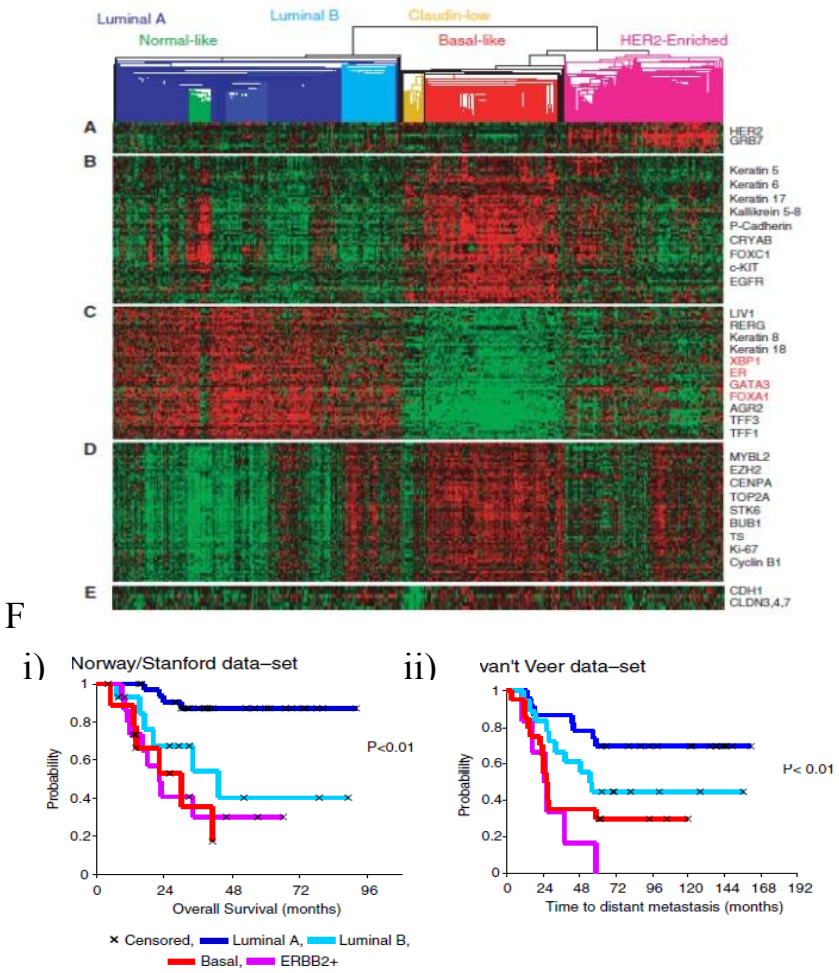

Figure I.2 - Hierarchical clustering of 677 breast tumour tissues using the ‘‘intrinsic’’ gene-set. The clustering analysis identified the 5 major intrinsic subtypes of luminal A, luminal B, normal-like, basal-like, and HER2-enriched, and also identified the newest subtype in the center called the “claudin-low” group. The gene sets most definitive of each subtype are shown and are (A) HER2-amplicon gene set, (B) basal epithelial gene set, (C) luminal epithelial gene set containing ER, and (D) proliferation gene set. (E) Claudin-low gene set including E-cadherin and claudin 3, 4, and 7. Adapted from C. M. Perou & Borresen-Dale, 2010. **(F) Kaplan–Meier analysis of disease outcome in two patient cohorts.** (i) Overall survival for 72 patients with locally advanced breast cancer in the Norway cohort. The normal-like tumour subgroups were omitted from both data-sets in this analysis. (ii) Time to development of distant metastasis in the 97 sporadic cases from van’t Veer and colleagues. Adapted from Sørlie, 2004.

1.4 - Treatment

Breast cancer treatment includes local and systemic therapy (Guarneri & Conte, 2004; Tanaka et al., 2009). Surgery alone or in combination with radiation therapy is used to treat cancers that are contained to the breast. On the other hand, systemic therapy not only has an important role in reducing the size of locally contained tumors, but also presents a more crucial impact when dealing with cancers with a high risk to be invasive or that have already metastasized. Examples of systemic treatment include chemotherapy, hormone therapy and targeted therapy.

2 - CHEMOTHERAPY

Chemotherapy uses drugs that are harmful to cancer cells. Chemotherapeutic agents are designed to suppress cell division and inhibit the proliferation of cancer cells (ElHazzat & El-sayed, 2010). This "same" strategy to kill cancer cells can be used for distinct purposes and, in this way, different types of chemotherapy treatments are recognized(Guarneri & Conte, 2004).

When chemotherapy is given before surgery to decrease the tumor size, achieving operability and improving the rate of breast-conserving surgery, it is mentioned as neoadjuvant or primary systemic chemotherapy. Chemotherapy can also be given after surgery for early stage breast cancer and, in these cases, is called adjuvant chemotherapy. Patients presenting hormone-negative tumors are at average risk of relapse, and so, adjuvant chemotherapy is considered a good option. The last type of chemotherapy is used in patients with metastatic breast cancers, for example HER2-positive cancers. They have poor survival rates and chemotherapy represents their treatment of choice(Guarneri & Conte, 2004; Tanaka et al., 2009).

Between the drugs given in breast cancer chemotherapy we can mention some groups. Breast cancer chemotherapy agents include anthracyclines, antitubulins (mitotic inhibitors), fluoropyrimidines, between others drugs (Hamilton & Hortobagyi, 2005).

Anthracyclines are compounds used to inhibit DNA and RNA synthesis, as they intercalate base pairs strands of genetic materials (Cortés-Funes & Coronado, 2007). This prevents DNA replication and induces cell death. They can also inhibit topoisomerase II function of relaxing the supercoiled DNA and create free oxygen's radicals that damage lipids membranes and DNA. Doxorubicin and epirubicin, two main examples of this class of compounds, are the foundation of most breast cancer chemotherapies (Takimoto & Calvo, 2008).

Also very widely used is the group of mitotic inhibitors, which includes the taxanes and vinca alkaloids. These agents have the ability to stabilize the microtubules or prevent the polymerization of tubulin (respectively), inhibiting, in both ways, mitotic division of the cells. Breast chemotherapy uses very common taxanes, like docetaxel and paclitaxel, and vinca alkaloids, like vinorelbine and vimblastine (Hamilton & Hortobagyi, 2005; Takimoto & Calvo, 2008).

Fluoropyrimidine class contains fluorouracil and its prodrug capecitabine. Capecitabine requires three enzymatic steps for activation. The last step involves thymidine phosphorylase, an enzyme that is overexpressed in cancer cells, ideally protecting normal tissues from exposure to the active drug. These agents act as a thymidylate synthase inhibitor, interrupting the synthesis of the thymidine, which is a nucleotide required for DNA replication (Hamilton & Hortobagyi, 2005; Takimoto & Calvo, 2008).

Alkylating agents have the ability to alkylate nucleophilic functional groups in cells. Platinum-based drugs, like carboplatin and cisplatin, bind to the DNA and trigger apoptosis. Cyclophosphamide is a prodrug, activated in the liver, which adds alkyl groups to bases of DNA (Takimoto & Calvo, 2008).

If sometimes these drugs are used alone (single agent therapy), other times they are used in combination(Tanaka et al., 2009). In Table I.1 we can see some of the most usual agents used in chemotherapy to treat breast cancer patients with metastatic breast cancer.

Table I.1 - Chemotherapy options for patients with metastatic breast cancer. Modified from Perez, 2009.

Single agents	Combination chemotherapy
Anthracyclines (doxorubicin, epirubicin, or pegylated liposomal doxorubicin)	FAC/CAF—fluorouracil/doxorubicin/cyclophosphamide
Taxanes (paclitaxel, docetaxel, or albumin-bound paclitaxel)	FEC—fluorouracil/epirubicin/cyclophosphamide
Capecitabine	AC—doxorubicin/cyclophosphamide
Ixabepilone	EC—epirubicin/cyclophosphamide
Other active drugs	AT—doxorubicin/docetaxel or doxorubicin/paclitaxel
Platinum drugs (cisplatin, carboplatin)	CMF—cyclophosphamide/methotrexate/fluorouracil
Cyclophosphamide	XT-Docetaxel/capecitabine
Etoposide	GT—gemcitabine/paclitaxel
Vinorelbine	Ixabepilone plus capecitabine
Gemcitabine	

2.1 - Side effects to chemotherapy

Although there are many approved drugs that are effective in treating breast cancer, it is important to know that, even the most effective drugs, do not always work or can present some serious side effects (Tanaka et al., 2009).

Cancer cells share many common features with the normal host cells from which they derive. Chemotherapy is given to kill cancer cells, but it also can damage normal tissues. Most cancer chemotherapeutics owe their small selectivity to the high proliferation rates of cancer cells. They present, in this way, increased toxicities against normal tissues that also show enhanced proliferative rates, such as the bone marrow, gastrointestinal tract and hair follicles (Tanaka et al., 2009; Allen, 2002).

This is a problem especially because these drugs travel through the bloodstream to the entire body. For example, anthracyclines, which are among the most effective anticancer drugs for treating breast cancer, present a notorious toxicity to healthy tissues in the liver and bone marrow. Within the adverse reactions to these agents is the notorious cardiotoxicity. This cardiotoxicity, which is manifested clinically as an irreversible congestive heart failure and/or cardiomyopathy, is the most important chronic cumulative dose-limiting toxicity of anthracyclines (Cortés-Funes & Coronado, 2007; Leonard et al., 2009; Lorusso et al., 2007; Palmieri et al., 2010).

Efforts were made to identify molecular markers able to discriminate low- and high-risk patients in order to avoid unnecessary treatments or underestimation of probability of relapse.

2.2 - Resistance to chemotherapy

Chemotherapy alters tumor growth and is important for improving the survival and quality of life of cancer patients. Nevertheless, this effect is not long lasting and chemotherapy fails in most of the cases (Liu, 2009; Perez, 2009; Rivera & Gomez, 2010). Chemotherapy drug resistance is a major obstacle to survival of cancer patients.

The incidence of breast cancer at an advanced stage is decreasing nowadays in developed countries due to screening programs and usage of novel chemotherapy agents at an adjuvant setting. Recurrence is the major problem seen in > 50% of breast cancer patients diagnosed at an early stage (Arslan et al., 2011).

About 10% of breast cancer patients have metastatic disease and another 10 to 20% have locally advanced breast cancer (Perez, 2009). Almost one in three of the

patients diagnosed with early stage breast cancer will progress to metastatic breast cancer (O'Shaughnessy, 2005), for which therapeutic options are limited. The prognosis for these patients remains poor. Median survival from manifestation of metastases is 2–3 years (Perez, 2009).

Response rates for first-line chemotherapies in metastatic breast cancer are approximately 30–70%. However, following this first treatment, the disease progresses in only 7–10 months (Perez, 2009; Rivera & Gomez, 2010). In subsequent chemotherapies the response rate falls to approximately 20–30%.

There are many reasons responsible for failure of cancer chemotherapy. Cancer has the ability to become resistant to many different types of drugs, including compounds characterized by different chemical structures and by different mechanisms of intracellular activity. Multidrug resistance (MDR) is a protection system of the cell against these numerous drugs. Chemotherapy resistance can be primary (tumor insensitivity to initial treatment) or acquired (occurs after initial response to therapy). In this way, exposure to one specific drug can result in the cross-resistance to other structurally and functionally unrelated anticancer drugs (Perez, 2009; Rivera & Gomez, 2010; Liu, 2009).

MDR can arise through a number of different mechanisms. One of the most widely studied mechanisms for tumor resistance is the alteration in the expression of transporters of the ABC family (Perez, 2009; Rivera & Gomez, 2010; Liu, 2009). These transporters can use the energy from ATP hydrolysis and act as efflux pumps to remove various intracellular chemotherapeutic drugs.

Modification of drug target expression or function is considered other resistance mechanisms (Perez, 2009; Rivera & Gomez, 2010). For example, altered β-tubulin isotype expression, β-tubulin mutations (affecting the drug-binding sites) and modifications in microtubule regulatory proteins were associated with resistance to microtubule-targeting drugs, such as taxanes and vinca alkaloids (He, Yang, & Horwitz, 2001; Kamath, Wilson, Cabral, & Jordan, 2005).

Enzymes responsible for regulating drug inactivation or detoxification may also affect chemotherapy efficiency (Rivera & Gomez, 2010). Overexpression of aldehyde dehydrogenase isoforms can catalyze the oxidation of cyclophosphamide and reduce the cell sensitivity to this agent (detoxification). The cellular thiol-containing proteins,

glutathione (GSH) and glutathione S-transferase are involved in the detoxification of alkylating agents and Pt-based antitumor agents, so the modulation of their activity might affect the resistance to these compounds (Rivera & Gomez, 2010; Liu, 2009).

These mechanisms, together with drug compartmentalization in cellular organelles, altered repair of drug-induced DNA damage (Fedier et al., 2001) and changes in apoptotic signaling pathways (Rivera & Gomez, 2010), are responsible for the failure of chemotherapy.

Overcoming resistance represents an important therapeutic goal. There are progress being made in finding new strategies to manage patients with progressing or relapsing disease resistant to anthracyclines and taxanes, but additional work is needed. Many studies have already focused in finding inhibitors of the ABC transporters but poor results were obtained. P-gp inhibitors such as tariquidar, zosuquidar, and laniquidar are currently under development (Perez, 2009).

Another logical option has been to continue investigation of novel chemotherapies with distinct mechanisms of action or different structures, which might present low susceptibility to develop resistance and promising activity in MDR cancer cells (Rivera & Gomez, 2010). These chemotherapeutic agents, under investigation, include the antifolate pemetrexed, the topo-I inhibitor irinotecan, and the epothilones, a novel class of antineoplastic agents, and their analogs (Perez, 2009).

2.3 - Overcoming chemotherapy challenges

Oncology dreams with providing a more effective treatment with a lower toxicity. At the beginning of the 21st century, an outbreak of important advances in anticancer therapeutics occurred. Much of this progress was driven by the explosion of knowledge in the field of molecular oncology (Takimoto & Calvo, 2008). The increasing knowledge of molecular mechanism for survival and proliferation of cancer cells has dramatically increased the number of promising molecular targets for novel anticancer treatment strategies(Higgins & Baselga, 2011). These advances hold great promise for developing a new generation of agents with high specificity for tumor cells.

Target therapy was developed towards certain markers or processes occurring in tumor cells (Ross et al., 2004). Targeted treatments can include monoclonal antibodies

or tyrosine kinase inhibitors. Inactivation of only one oncogene, as well as the inhibition of only one pathway with one or more inhibitors, can lead to the regression of tumors. However, even these targeted therapies caused serious side effects (Tanaka et al., 2009) and are susceptible of some resistance mechanisms.

For example, trastuzumab is the first genomic research-based, targeted anti-kinase therapy approved by the Food and Drug Administration (FDA) for the treatment of patients with HER2 positive breast cancer (Tanaka et al., 2009). This anti-HER2 monoclonal antibody has changed the prognosis of the disease improving survival in the metastatic breast cancer (Fang et al., 2011). As a single agent, it produces response rates similar to those of many single-agent chemotherapeutic agents active in metastatic breast cancer and has limited toxicity. Combining trastuzumab with chemotherapy can result in synergistic antitumor activity. Unfortunately, the clinical efficacy of trastuzumab is limited. In fact, a significant number of patients with HER2 overexpressing tumors are, or will eventually become, resistant to trastuzumab (Fang et al., 2011; Fiszman & Jasnis, 2011). Trastuzumab alone or in combination with chemotherapy may cause serious heart problems (Leonard et al., 2009).

Therefore, the development of a novel treatment strategy including selective delivery of cytotoxic agents to tumor mass for the treatment of advanced breast cancer is critical to improve the therapeutic index and the efficacy/toxicity balance.

Advances in nanotechnology were able to revolutionize cancer diagnosis and therapy (Tanaka et al., 2009). Several nanotechnological approaches have been used to deliver chemotherapeutic agents to cancer cells and to improve the therapeutic efficacy. By using both passive and active targeting, drug delivery strategies can enhance the intracellular concentration of chemotherapeutic agents in cancer cells while avoiding toxic effects on healthy tissues and presenting some potential to overcome the drug resistance (Cho et al., 2008; Malam et al., 2009; Wong et al., 2007)

3 - DRUG DELIVERY

When a patient is submitted to systemic chemotherapy, the drugs are distributed in the entire body, in a non-specific way. In consequence, they affect not only cancers cells but also normal tissues. This is the reason for the previously mentioned high toxicity effects of chemotherapeutic agents. For this reason, doses that can be administered are limited and, to avoid excessive toxicities, a suboptimal treatment is applied. It is possible to improve the patient rate of survival and quality of life by increasing the intracellular concentration of drugs and reducing dose-limiting toxicities simultaneously (Cho et al., 2008; Malam et al., 2009).

Effective strategies for chemotherapy in cancer treatment should, after administration, be able to reach and accumulate in the tumor tissues, penetrating barriers in the body with minimal loss of their volume or activity in the blood circulation. After this, drugs should have the ability to selectively kill cancer cells without affecting normal tissues with a controlled release mechanism of the active molecule. Nanotechnology seems to have the potential to please all these requirements (Andresen et al., 2005; Cho et al., 2008; Tanaka et al., 2009).

‘‘Nanotechnology’’ is a multidisciplinary field, derived from engineering, biology, physics and chemistry, which involves creation and utilization of materials, devices or systems on the nanometer scale (Sahoo & Labhasetwar, 2003). These materials present unique features, otherwise absent at a macroscopic level, displaying functions that have exceptional benefits when applied to medical science (Morigi et al., 2012). They give the opportunity to work at the same scale of several biological processes, cellular mechanisms, and organic molecules (Morigi et al., 2012).

Nanotechnological applications in the field of medical science have expanded rapidly towards multiple directions in the past years, with a great emphasis in medical diagnosis and drug delivery (Sahoo & Labhasetwar, 2003; Tanaka et al., 2009). Many different types of nano-sized systems (or nanocarriers), with different materials and physio-chemical properties, have been used as drug delivery systems and applied to deliver chemotherapeutic agents to cancer cells.

For this purpose, nanocarriers must have the ability to remain in the blood stream for a considerable time without being eliminated. Nanocarriers are, most of the times, susceptible to be caught in the circulation by the reticuloendothelial system (like liver and spleen). Their vulnerability depends on their size and surface characteristics. So, by adjusting the size and modifying the surface, it is possible to manipulate the fate of the nanocarriers (Andresen et al., 2005; Cho et al., 2008).

The size of nanocarriers used as drug delivery system should be large enough to prevent their fast leakage into blood capillaries but small enough to escape capture in the reticuloendothelial system (Andresen et al., 2005; Cho et al., 2008). If small particles (0–30 nm) are rapidly cleared by renal excretion, when they are over 30 nm they are cleared by the mononuclear phagocytic system (MPS), consisting of macrophages located in the liver and the spleen (Malam et al., 2009). In solid tumors, the gap junctions between endothelium cells of the vasculature vary from 100 to 780 nm (Andresen et al., 2005). Nanocarriers size should allow them to pass through the gap junctions between endothelium cells of the tumor vasculature and reach the tumor tissue.

Another important factor that determinates the life time and fate of nanocarriers in the blood-stream is the characteristics of their surface. Ideally, they should have a hydrophilic surface, which allows them to escape macrophage capture (Cho et al., 2008). To achieve this goal, nanocarriers can be covered with a hydrophilic polymer. Hydrophilic polymers, possessing a flexible chain that occupies the periliposomal layer, tend to exclude other macromolecules like opsonins from this space and, therefore, reduce the MPS recognition (Drummond et al., 1999). Among the different polymers investigated, poly-(ethiylene glycol) (PEG) is the most used because of its solubility in aqueous and organic media, high biocompatibility, nontoxicity, low immunogenicity and antigenicity (Immordino et al., 2006). PEGylation protects nanocarriers from absorsion of plasma protein and reduces opsonization (Andresen et al., 2005; Cho et al., 2008; Malam et al., 2009; Tanaka et al., 2009).

If the nanocarriers satisfy the size and surface characteristics requirements to escape the reticuloendothelial system, they circulate for longer time in the bloodstream and have a better chance of reaching the tumor tissues.

3.1 - Passive targeting cancer cells.

Most solid tumors possess unique pathophysiological characteristics that differ from normal tissues (Figure I.3) (Danhier et al., 2010). Fast-growing cancer cells demand an extensive angiogenesis near the tumor mass to support their need of oxygen and nutrients. In cancers, there is an imbalance in factors that regulate angiogenesis, such as overexpression of vascular endothelial growth factor (VEGF) (Malam et al., 2009). This results in a neovasculature that differs from the one of normal tissues in anatomical architecture and impaired lymphatic drainage. The blood vessels in the tumor are irregular in shape, dilated, leaky or defective and the endothelial cells are poorly aligned or disorganized with numerous pores showing enlarged gap junctions between them. Also, the perivascular cells and the basement membrane, or the smooth-muscle layer, are frequently absent or abnormal in the vascular wall.

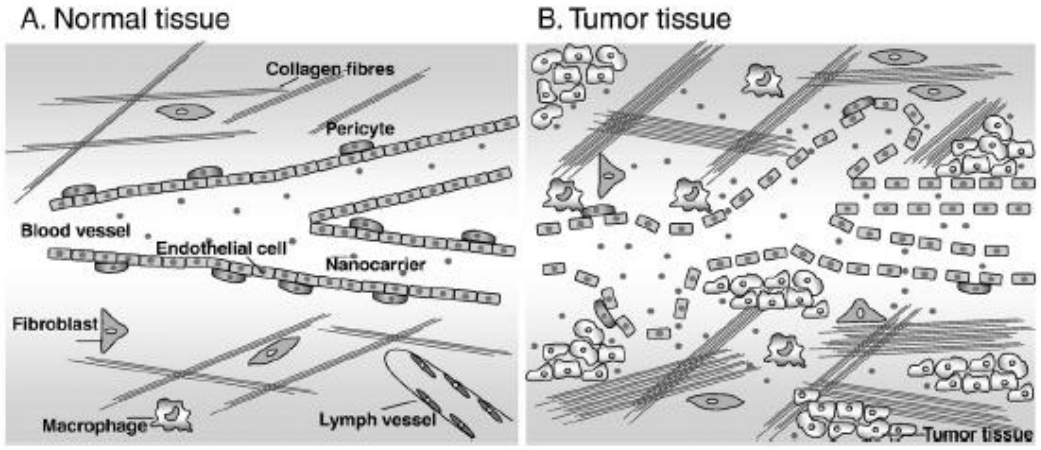

Figure I.3 – Illustration of the differences between normal and tumor tissues. Normal tissues (A) contain linear blood vessels maintained by pericytes. Collagen fibers, fibroblasts and macrophages are in the extracellular matrix and lymph vessels are present. On the other hand, in the tumor tissues (B) blood vessels are defective, irregular in shape, dilated and leaky. Together with the lack of lymph vessels, this differences result in the retention of macromolecules in the tumor (Enhanced Permeability and Retention effect). Also, the extracellular matrix contains more collagen fibers, fibroblasts and macrophages than in normal tissue. Adapted from Danhier et al., 2010.

This results in extensive leakage of blood plasma components which, together with the slow venous return in tumor tissue and the poor lymphatic clearance, means that macromolecules are retained in the tumor. These features, named the enhanced permeability and retention (EPR) effect, are the basis for the passive targeting of anti-cancer agents to the solid tumors (Iyer et al., 2006; Danhier et al., 2010; Malam et al., 2009; Cho et al., 2008; Tanaka et al., 2009).

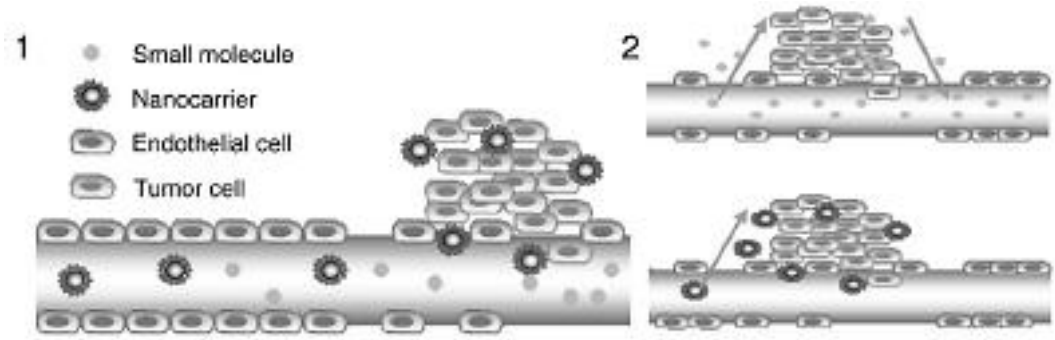

Figure I.4 - Passive targeting of nanocarriers. (1) Nanocarriers reach tumors selectively through the leaky vasculature surrounding the tumors. (2) Schematic representation of the influence of the size for retention in the tumor tissue. Drugs alone diffuse freely in and out the tumor blood vessels because of their small size and thus their effective concentrations in the tumor decrease rapidly. By contrast, drug-loaded nanocarriers cannot diffuse back into the blood stream because of their large size, resulting in progressive accumulation. Adapted from Danhier et al., 2010.

The resulting high interstitial fluid pressure in solid tumors avoids successful uptake and homogenous distribution of drugs in the tumor. Small molecules (free drugs), mainly transported by diffusion, move freely in and out of the tumor blood vessels. On the other and, larger and long-circulating nanocarriers are more retained in the tumor (Figure I.4) (Danhier et al., 2010).

3.2 - Active targeting cancer cells

Passive targeting mechanisms inevitably face intrinsic limitations to its specificity (Cho et al., 2008). Overcoming these limitations can be done by the inclusion of a targeting ligand or antibody in nanocarrier-drug conjugates. The recent advances in developing drug delivery carriers and the great number of potential drugs to conjugate with them have allowed the development of many active targeting drug-carrier conjugates. However, when constructing targeting nanocarriers some factors must be considered to efficiently deliver drugs to the target tissues (Cho et al., 2008). Cell-surface antigens and receptors should be expressed exclusively on tumor cells and not expressed on normal cells. Additionally, their expression should be homogeneous on all targeted cancer cells. The targeted nanocarrier should be stable in the bloodstream to reach their target without being eliminated. After reaching the tumor and binding to the surface of the cells, they should be capable of being internalized. This internalization usually occurs via receptor-mediated endocytosis (Cho et al., 2008). Targeting ligands are monoclonal antibodies, antibody fragments or non-antibody ligands (peptidic or not).

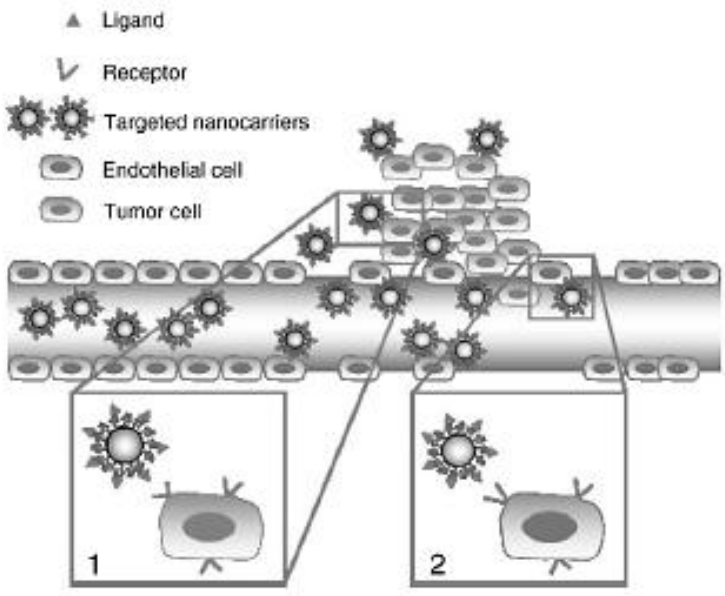

Figure I.5 - Active targeting strategies. Ligands grafted at the surface of nanocarriers bind to receptors (over)expressed by (1) cancer cells or (2) angiogenic endothelial cells. Adapted from Danhier et al., 2010.

A very common example of this type of delivery is the folate receptor targeting. Folate receptor binds folic acid with a high affinity and carries it into the cells via receptor-mediated endocytosis. Folic acid is essential for the synthesis of nucleotide bases (Andresen et al., 2005; Cho et al., 2008; Danhier et al., 2010). Folate receptor is overexpressed on the surface of multiple human cancers, possibly due to increased requirements for DNA synthesis (Andresen et al., 2005). The folate receptor is one example of target that directs the drug to cancer cells. Studies with folate-targeted liposomes loaded with doxorubicin showed a greater *in vitro* cytotoxic when compared to the non-targeted liposome formulation (Goren et al., 2000).

Transferrin, a serum glycoprotein, transports iron in the bloodstream. It delivers the iron into cells through binding to the transferrin receptor and is internalized via receptor-mediated endocytosis (Cho et al., 2008; Tian et al., 2010). Transferrin has been investigated as a target ligand for cancer specific drug delivery since its receptor is overexpressed in tumor tissues, when compared to normal tissues. Transferrin was conjugated to liposomes and nanoparticles to enhance the drug efficacy of anticancer-agents, like doxorubicin (Li et al., 2009).

Cancer cells often express different glycoproteins compared with their normal tissues (Danhier et al., 2010). Lectins are proteins that recognize and bind to carbohydrate moieties attached to glycoproteins on the extracellular side of the plasma membrane (Cho et al., 2008). This interaction could be used as a targeting strategy to

desired cells and tissues. This interaction could also be used in the reverse directions (glycans incorporated nanocarriers).

In 1971, Judah Folkman suggested that the blood supply, the size and metastatic capabilities of tumors could be regulated by preventing angiogenesis. This observation is the base of the design of nanocarriers that actively target tumor endothelial cells (Danhier et al., 2010), therefore killing angiogenic blood vessels and (indirectly) the tumor cells that these vessels support. The main targets in this strategy include: vascular endothelial growth factors (VEGF) receptors, αvβ3 integrin, vascular cell adhesion molecule-1 (VCAM-1) and matrix metalloproteinases (MMPs) (all overexpressed in the surface of endothelial tumor cells) (Danhier et al., 2010).

Integrins are specific receptors expressed in the neovasculature during the angiogenic process of tumor growth (Andresen et al., 2005). Targeting of small peptides towards integrins was investigated. Integrins are highly expressed on neo-endothelial cells, but poorly expressed in the tumor cells themselves, and seems to be a promising approach in cancer treatment (Danhier et al., 2010).

Monoclonal antibodies, because of their high affinity, specificity and wide range of available targets, have been the support of molecular targeting (Hicke et al., 2006). Antibody coated nanocarriers can target surface molecules expressed either in the vascular system (vascular endothelial surface of growing tumors are the most accessible) or in the extravascular system on tumor cell membranes (Andresen et al., 2005). The epidermal growth factor receptor (EGFR) and the human epidermal receptor 2 (HER2) are examples of antigens overexpressed in some types of breast cancer cells that have already shown to allow recognition of the specific receptor and enhancement of the therapeutic effect of encapsulated chemotherapeutic agent (Danhier et al., 2010).

Although successes have been documented, antibody-conjugated nanocarrier are larger in size, presenting decreased tumor penetration and higher clearance rates (Allen, 2002).. When the binding affinity is high, there is some evidence that the targeted-nanocarriers have a decreased penetration of solid tumors because of the ‘binding-site barrier’: they bind strongly to the first targets encountered but fail to diffuse further into the tumor(Danhier et al., 2010). In addition, antibodies can show problematic immune responses (Allen, 2002). Therefore, new targeting agents and clinical protocols were studied. Antibodies fragments, which lack the Fc domain and the complement-activating region, present lower immunogenicity. Recent applications focused on the use of antibody fragments. Fragments can be made by enzymatic

cleavage of the whole monoclonal antibody (F(ab′)2 or Fab′) or by molecular biological techniques (scFV, bivalent or recombinant fragments).

Nucleic acid aptamers are small oligonucleic acids such as DNA or RNA(Cho et al., 2008). They are comparable to antibodies in specificity and affinity for their target molecule and accept unique three-dimensional conformations capable of binding to target antigens. At 8–15 kDa, aptamers are smaller in size when compared to antibodies (150 kDa) (Hicke et al., 2006). They have been applied to drug delivery systems as a ligand to enhance selectivity and *in vivo* efficacy of the chemotherapeutic agent (Cho et al., 2008).

3.3 - Tumor microenvironment and triggered release

Another contributor to passive targeting is the characteristic of tumor microenvironment (Figure I.3). It comprises cancer cells and stromal cells (fibroblasts and inflammatory cells) that are embedded in an extracellular matrix and nourished by a vascular network (Trédan et al., 2007). The tumor microenvironment of a solid tumor is a heterogeneous structure as their components may vary from one location to another in the same tumor and has several characteristics that distinguish it from the corresponding normal tissue (Danhier et al., 2010; Cukierman & Khan, 2010). As mentioned, tumors present a disorganized vasculature with multiple structural and functional abnormalities and a complete lack of lymphatic system. One direct result is the high interstitial fluid pressure and the low ability of the tumor vasculature to deliver nutrients and remove waste products. Because cancer cells show a high metabolic rate, the supply of oxygen and nutrients is not sufficient (hypoxia). The over need of oxygen forces cancer cells to use glycolysis to obtain extra energy, therefore resulting in an acidic environment (Cairns, Papandreou, & Denko, 2006; Danhier et al., 2010; Ganta, Devalapally, Shahiwala, & Amiji, 2008; Trédan et al., 2007). Additionally, cancer cells express and release unique enzymes which are implicated in their movement and survival mechanisms (Cho et al., 2008).

The use of site-specific targeting has not yet been sufficient to obtain a significantly increased efficacy in the treatment of cancer when compared to passive targeting (Andresen et al., 2005). Targeting strategy directs nanocarriers to the surface of the tumor cells and leads to their internalization by endocytosis. The endosomes

transport their cargo to lysosomes, which may result in degradation of the carried drugs (if they cannot escape the harsh endosomal/lysosomal environment) (Andresen et al., 2005). Additionally, drugs usually escape from the nanocarriers by passive diffusion and this often leads to a suboptimal drug concentration in the tumor (Arouri & Mouritsen, 2011a). Consequently, nanocarriers have to be designed either to escape the endosomes after cell internalization or to release the drugs outside the cell. Several strategies have been proposed to accomplish site-specific triggered drug release in tumor tissue(Andresen et al., 2005). Drug release from nanocarriers can be triggered by external (light, temperature and ultrasound) and internal (pH and enzymes) stimuli (Ganta et al., 2008; Danhier et al., 2010; Gao et al., 2012).

In photo-responsive lipids and polymers, light (of the appropriate wavelength) can induce structural transformations like isomerization, fragmentation and polymerization (Andresen et al., 2005; Danhier et al., 2010). In this way, they can be applied to drug delivery systems and cause leakage upon photoexcitation. However, UV and visible light is easily absorbed by the skin and these systems may present some limitations (Danhier et al., 2010). Together with the fact that UV-light is not very suitable for biological applications, due to the potential damage of healthy tissues (Andresen et al., 2005), nanocarriers sensitive to infrared or near-infrared lights are recently being studied (Volodkin et al., 2009).

In 1978, Yatvin et al. proposed the use of mild local hyperthermia for tumor-specific drug release. Using nanocarriers unstable at temperatures just above 37ºC (physiological temperature), drugs would be kept encapsulated in the systemic circulation or upon contact with normal tissues (Ganta et al., 2008). By application of a hyperthermic stimulus in the tumor, the system would release the cargo. Site-specific heating may be achieved by various physical means, among which the least invasive, easiest and cheapest is ultrasound (Danhier et al., 2010). The use of hyperthermia apparently has some advantages over other triggering concepts: hyperthermia itself presents some cytotoxicity and tumor accumulation of the nanocarrires is increased as a consequence of increased tumor blood flow and increased microvascular permeability (Andresen et al., 2005).

During the last decade, ultrasound has attracted growing attention in drug delivery (Ganta et al., 2008). Ultrasound has been used to achieve triggered and targeted delivery to the tumor by local application of ultrasound. Sonication could not only

induce drug leakage in sonosensitive nanocarrires but also increase extravasation of nanocarrires to the tumor and enhance cell membrane permeability (Evjen et al., 2011).

The pH-sensitive nanocarriers are designed to be stable at a physiologic pH but degrade when the pH is lower. They can exploit the biochemical properties of the tumor microenvironment to release the active drug in target tissues (Ganta et al., 2008; Danhier et al., 2010). However, the pH of the microenvironment rarely declines below pH 6.5, making it technically difficult to design liposomes that are stable in the blood but become disrupted in the tumor tissue (Andresen et al., 2005). Thus, in a more viable strategy, nanocarrires exploit the acidic environment in endosomes and lysosomes. When liposomes are internalized to endosomes, they enter a very acidic environment where the pH is below 5.0. To prevent lysosomal degradation of the carried drug, the drug has to escape the endosomes once internalized (Andresen et al., 2005).

Other emerging field in drug delivery able to specifically kill tumor cells is based on tumor site-specific enzymatically destabilization of nanocarriers (Danhier et al., 2010). Enzymes play a role in cell regulation and a specific enzymatic activity can be associated to a particular tissue. In the tumor microenvironment it is possible to find specific enzymes (proteases, lipases and glycosidases) at higher concentrations when compared to normal tissues (de la Rica et al., 2012; Meers, 2001). For example, phospholipases, such as phospholipase A_2 (PLA_2), are known to be abnormally up-regulated in infectious and inflammatory diseases. As PLA_2 is only found in high levels next to the tumor and not in the blood stream, nanocarriers whose physical properties are responsive to PLA_2 activity can be used to specifically deliver drugs at the tumor (Zhu et al., 2011). This powerful concept has been successfully applied to the fabrication of drug delivery systems and is gaining much interest in therapeutics. Moreover, enzymatic transformations induced in the nanocarrier can also be used to generate therapeutic molecules, therefore allowing the design of drug delivery systems with synergistic effects (de la Rica et al., 2012). Phospholipid-like prodrugs are phospholipids with a therapeutic agent attached to acyl chain at position sn2. Arouri & Mouritsen (2011) incorporated these prodrugs in PLA_2-sensitive liposomes. PLA_2 allowed the degradation of the liposomes facilitating not only the release of the cargo but also of the release of the therapeutic molecule from C6-RAR prodrug (Arouri & Mouritsen, 2011b).

3.4 - Multifunctional nanocarriers

If nanocarrires evolved to present a wide variety of useful properties for drug delivery, the next logic step was to engineer nanocarriers, which, depending on their purpose, demonstrated a combination of several of these properties. An increasing number of publications are now proposing the combination of functions such as: (i) prolonged circulation in the blood; (ii) ability to accumulate specifically or non-specifically in the tumor microenvironment; (iii) responsiveness to local stimuli resulting, for example, in accelerated drug release, (iv) allow an effective intracellular drug delivery, and (v) bear a contrast/reporter moiety allowing the real-time observation of its accumulation inside the target (Danhier et al., 2010; Torchilin, 2006). Therefore, multifunctional nanocarriers may combine, with high efficiency and specificity, different therapeutic approaches (transporting drugs, DNA, etc.) and diagnosis strategies (Sanvicens & Marco, 2008).

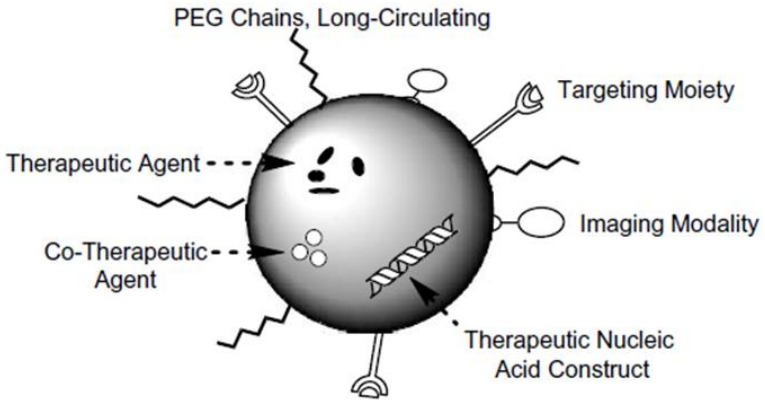

Figure I.6- Schematic illustration of a multifunctional nanocarrier. Adapted from Jabr-Milane et al., 2008.

3.5. - Potential to overcome drug resistance

The multi-drug resistance (MDR) phenotype usually results from a synergistic combination of mechanisms and the inhibition of only one contributor to cellular resistance is most likely insufficient to overcome MDR. Therefore, designing an advanced multifunctional delivery system should be priority to reverse MDR in cancer chemotherapy (Gao et al., 2012). Nanotechnology has the ability to specifically target drugs to tumors or tumor cells, enhance drug internalization, avoid drug

clearance/degradation and release drug in response to a specific stimulus. These characteristics endow nanocarriers with the ability to address and solve MDR (Gao et al., 2012).

It has been suggested that drug delivery systems may be able to eliminate the ABC-transporters mediated resistance (Cho et al., 2008; Gao et al., 2012; Jabr-Milane et al., 2008). Nanocarriers may avoid the recognition of the drug by efflux pumps because they are enveloped in the endosome when they enter the cell (in an "invisible" way) and release their cargo deep inside the cytoplasm (away from the membrane ABC transporters) (Gao et al., 2012; Kunjachan et al., 2012). This was said to be responsible for a higher intracellular concentration in the cancer cells(Cho et al., 2008).

Wong et al. (2006) discovered the potential of a polymer-lipid hybrid nanoparticle to enhance cellular accumulation and retention of doxorubicin, known to be a P-gp substrate, in P-gp-overexpressing cancer cell lines. Guo et al. (2010) indicated that stealth liposomal daunorubicin plus tamoxifen could have the potential to eliminate both breast cancer cells and cancer stem cells, which overexpresses the ABC transporters.

Additionally, ligand-targeted strategies may have a special potential for overcoming drug resistance because these ligands are usually internalized via receptor-mediated endocytosis (Cho et al., 2008). Lee et al. (2005) and Parveen et al. (2010) verified the benefice effect of receptor-mediated endocytosis on overcoming MDR, using doxorubicin loaded pH-sensitive polymeric micelles (a folate-receptor target) and transferrin-conjugated paclitaxel-loaded nanoparticles respectively.

4 - DRUG DELIVERY SYSTEMS

Nanocarriers are submicron-sized particles (<1µm) that can be made by using a variety of materials including polymers (polymeric nanoparticles, micelles, or dendrimers), lipids (liposomes or solid lipid nanoparticles), viruses (viral nanoparticles) and even organometallic compound (Cho et al., 2008; Rawat et al., 2006). This nanoscale drug delivery systems exhibit unique physical and chemical properties that can be exploited for drug delivery by conjugation with drugs (physically entrapped or covalently bound) (Malam et al., 2009).

Figure III.7 represents the structure of some of these different nanocarriers.

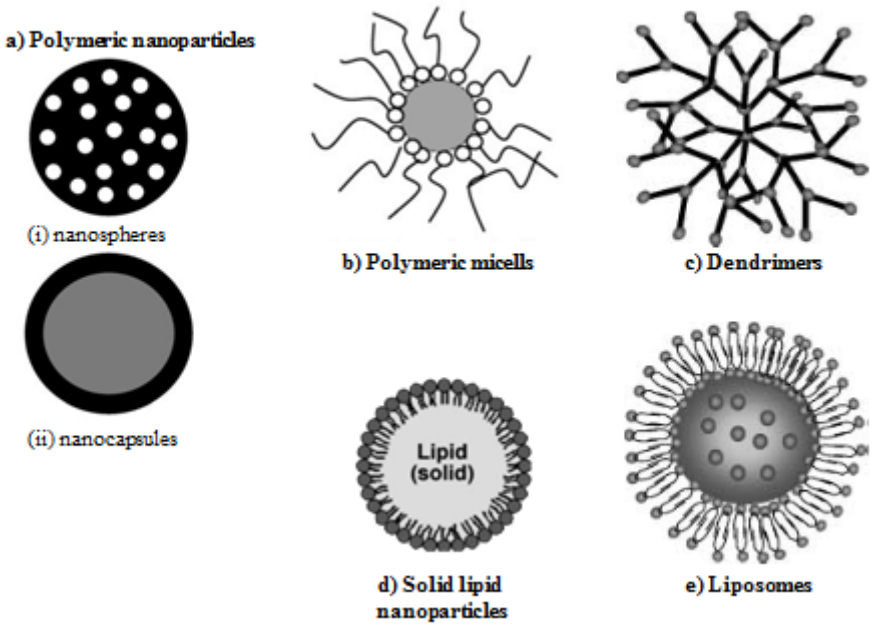

Figure III.7 - Types of nanocarriers for drug delivery. (a) Polymeric nanoparticles. (i) Nanospheres (drug is dispersed throughout the polymeric matrix). (ii) Nanocapsules (drug is encapsulated within polymeric membrane). (b) Polymeric micelles (amphiphilic polymers). (c) Dendrimers (branched). (d) Solid lipid nanoparticles. (e) Liposomes: lipid bilayer. Modified from Cho et al., 2008 and from Rawat et al., 2006.

4.1 - Polymer-based drug carriers

Polymers are widely used in delivery systems. These carriers include either natural polymers like albumin, chitosan, and heparin or synthetic polymers like polylactide (PLA), poly(D,L-lactide-co-glycolide) (PLGA), polyethylene glycol (PEG),

poly-L-glutamic acid (PGA), polyamidoamine (PAMAM) and [N-(2-hydroxypropyl)-methacrylamide copolymer (HPMA) (Cho et al., 2008; Malam et al., 2009; Rawat et al., 2006). Depending on the method of preparation, the resulting compounds may have the structure of capsules (polymeric nanoparticles), amphiphilic core/shell (polymeric micelles) or hyperbranched macromolecules (dendrimers) and the drug is either physically entrapped in or covalently bound to the polymer matrix. Polymeric materials exhibit several desirable properties including biocompatibility, biodegradability and surface modification (Rawat et al., 2006).

In polymeric nanoparticles a polymeric backbone (i) forms a nanoscale capsule able to load the drugs by entrapment of an aqueous drug phase inside the structure or (ii) ,by means of an ester or amide bound hydrolyzed *in vivo*, links directly the drug molecules (Malam et al., 2009). A nanoparticle formulation of paclitaxel (Abraxane), in which serum albumin is conjugated to the drug, has been applied in the clinic for the treatment of metastatic breast cancer (Cho et al., 2008; Rawat et al., 2006). Albumin nanoparticles have also been conjugated with others drugs (like noscapine) in potential drug delivery systems for breast cancer (Satya Prakash, 2010). Regarding synthetic polymers, PGA was the first biodegradable polymer to be used for drug delivery. Among their uses are their PGA-paclitaxel formulations (Cho et al., 2008). HPMA conjugations with doxorubicin have been evaluated in clinical trials as an anticancer agent (Seymour et al., 2009).

Polymeric micelles are based on amphiphilic block co-polymers, which self-assemble to form a shell in aqueous media. The hydrophobic core works as a pool for hydrophobic drug molecules whereas the hydrophilic shell stabilizes the micelles and renders the polymers water-solubility. These systems have been used to conjugate some poorly soluble anticancer drugs like paclitaxel, making them suitable for intravenous administration (Cho et al., 2008; ElHazzat & El-sayed, 2010; Rawat et al., 2006). Loading of hydrophobic drug molecules occurs in two ways: physical entrapment or chemical covalent attachment to the hydrophobic block (to achieve more control over the rate of drug release) (ElHazzat & El-sayed, 2010). Lee et al. (2005) developed a pH-sensitive polymeric micelle to deliver doxorubicin in drug resistant tumors, showing that this formulation was effective when suppressing both sensitive and MDR tumors.

Dendrimers are characterized by a tree-like branching architecture (ElHazzat & El-sayed, 2010) and represent a unique class of polymers that are fabricated from monomers using either convergent or divergent step growth polymerization (Rawat et al., 2006). Drug loading is possible in two different ways. Non-covalent encapsulation of the therapeutic drugs occurs in the available internal cavities. Alternatively, direct coupling of the drug molecules to the large number of surface groups present in the dendrimers may occur via customized chemical linkages that hydrolyze in response to specific intracellular signals (ElHazzat & El-sayed, 2010; Rawat et al., 2006). The polyamidoamine (PAMAM) dendrimer, the most widely used, has been conjugated with cisplatin, 5-fluorouracil, methotrexate or doxorubicin (Cho et al., 2008; ElHazzat & El-sayed, 2010). Dendrimers can easily modify their surface characteristic, which allows them to conjugate with several molecules simultaneously (can act as multifunctional drug delivery system) (Cho et al., 2008).

4.2 - Solid lipid nanoparticles

Lipids can also be used to form other nanocarriers. Solid lipid nanoparticles, also referred as lipospheres or solid lipid nanospheres (Malam et al., 2009), are made from solid lipids at human physiological temperature and consist of submicron colloidal carriers (50—1000 nm) dispersed either in water or in an aqueous surfactant solution (Rawat et al., 2006). They are used to transport hydrophobic drugs as the solid core contains them, dissolved or dispersed in the solid high melting fat matrix (Rawat et al., 2006). They have been used to encapsulate placlitaxel (Yuan et al., 2008), among others drugs (Malam et al., 2009). Variations of solid lipid nanoparticles were developed to expand their functions so that they could transport hydrophilic and ionic compounds (Wong et al., 2007). For example, Wong et al. (2006) investigated the potential of a polymer-lipid hybrid nanoparticle system to enhance cellular accumulation and retention of doxorubicin, especially in multidrug resistant breast cancer cells.

4.3 - Liposomes

Liposomes are microscopic vesicles formed by a phospholipid bilayer that can successfully encapsulate an active drug (ElHazzat & El-sayed, 2010). They can be composed by either synthetic or natural phospholipids (Rawat et al., 2006). The physical and chemical properties of a liposome (including permeability, net charge, density and steric hindrance) are based on the net properties of the constituent phospholipids (ElHazzat & El-sayed, 2010; Malam et al., 2009).

Liposomes have a spherical shape in which an outer lipid bilayer surrounds a central aqueous space(Cho et al., 2008). They are formed, in a spontaneous way, as a result of the hydrophilic–hydrophobic interaction between phospholipid molecules and the surrounding aqueous environment (ElHazzat & El-sayed, 2010; Malam et al., 2009). These interactions originate thermodynamically stable vesicles. Liposomes have been formulated using sonication, homogenization, shaking or heating strategies to obtain the necessary energy to disperse the phospholipid molecules into the surrounding aqueous medium (ElHazzat & El-sayed, 2010).

Liposomes have been used as carriers for anticancer drugs to increase their aqueous solubility, minimize their toxicity, increase their plasma residence time, and provide a controlled-release profile (ElHazzat & El-sayed, 2010). They can be tailored by modification of their size, layers (unilamellar or multilamellar), composition and surface charge to provide a controlled and sustained drug release system (Hung, 2006). Liposomes can be used to entrap both hydrophilic and lipophilic drug molecules. Hydrophilic drugs are typically loaded in the center aqueous environment whereas hydrophobic ones are loaded into the lipid bilayer (ElHazzat & El-sayed, 2010).

Among the drug delivery systems, liposomes have been the most studied (Immordino et al., 2006). Their widespread attention comes from unique characteristics. Liposomes are biocompatible, biodegradable, biologically inert, weakly immunogenic and present low intrinsic toxicity (Drummond et al., 1999; Immordino et al., 2006; Mufamadi et al., 2011).

Since they were first developed by Bangham around 1961(Bangham, 1961), liposome-based technology has evolved from generation to generation to meet chemotherapeutics' needs (ElHazzat & El-sayed, 2010; Mufamadi et al., 2011). Conventional liposomes are the first generation composed of phospholipids such as

phosphatidyl choline (most commonly used) (Drummond et al., 1999). Since conventional liposomes formulations were made up of phospholipids exclusively, liposomes have faced major challenges - namely the instability in plasma, which results in short blood circulation half-life (Mufamadi et al., 2011). One of the first attempts to overcome this problem was focused on manipulation of lipid membrane in order to modify bilayer fluidity (Immordino et al., 2006; Mufamadi et al., 2011). Several studies demonstrated that liposomes composed of phospholipids with saturated acyl chains are more stable in blood (Immordino et al., 2006). Liposomes containing lipids with a phase transition temperature above 37ºC were considerably more rigid, resisting to the binding of serum opsonins and recognition from phagocytes (Drummond et al., 1999). Addition of cholesterol to liposome's formulations resulted in a better maintenance of membrane bilayer stability and higher circulations times *in vivo*. Cholesterol reduces lipids transfer to HDL (high density lipoproteins) by increasing packing of phospholipids (Immordino et al., 2006).

Liposome size and surface characteristics were correlated to phagocytosis process *in vivo.* In liposomes of similar composition, the larger ones are eliminated from the blood circulation more rapidly than smaller ones (Immordino et al., 2006). Liposome surface charge has also been studied for its part on macrophage capture. If positively charged liposomes are toxic and quickly removed from circulation (Immordino et al., 2006), early studies have shown that the presence of negatively charged lipids, including phosphatidylserine (PS), and phosphatidic acid (PA), also results in rapid uptake by the MPS (Drummond et al., 1999). However, the inclusion of other anionic lipids such as the ganglioside GM1 or phosphatidylinositol (PI) resulted in longer half-life in blood than neutral liposomes. This relationship is extremely complex and each lipid must be analyzed independently(Drummond et al., 1999). Indeed the replacement of small amounts of the hydrogenated soy phosphatidylcholine (HSPC) with distearoylphosphatidylglycerol (DSPG; Tm=55ºC) shows longer circulation lifetimes (Drummond et al., 1999). Additionally, some authors suggest that charged liposomes, that were larger in size, showed better drug entrapment and were often used to prevent aggregation during storage (Drummond et al., 1999).

Although the success of cholesterol and other lipids, they did not completely overcome the major challenge of escaping the MPS (Immordino et al., 2006). The following strategies were developed based on the idea of coating the surface of the liposomes with inert molecules, attempting to mimic the surface of red blood cells or

increasing the hydrophilicity with addition of polymers (like PEG) (Drummond et al., 1999). Stealth liposomes came to be the next generation in liposome-based technology. PEGylated liposomes, due to long circulations lifetimes and the EPR effect, could passively accumulate in solid tumors.

As stealth liposomes failed to avoid uptake of active molecules by normal cells, the use of targeted liposomes has been suggested (Mufamadi et al., 2011). Coupling of targeting moieties increases co-localization between the liposomes and cancer cells and improve cellular internalization via receptor-mediated endocytosis (Immordino et al., 2006). Targeted liposomes could therefore enhance drug accumulation in tumor with a more selective therapeutic approach.

Ideally, liposomes should accumulate at the tumor site in an intact state and subsequently release their drug load. However, in some cases the drug leaks too slowly from the liposome. With the idea that triggered destabilization of the liposome within the tumor area could substantially increase drug availability and improve therapeutic efficiency, the next liposome generation emerged. Stimuli-sensitive liposomes depend on different environmental factors in order to release drug content. The environmental factors include pH, light, magnetism, temperature and ultrasonic waves(Andresen et al., 2005).

Because of their versatility, simplicity in fabrication and well-documented behavior in preclinical and clinical settings, liposomal formulations will continue to function as a carrier for a wide range of chemotherapeutic agents.

5 - CLINICAL APPLICATIONS OF LIPOSOMES AS DRUG DELIVERY SYSTEMS

Late-stage clinical failures due to lack of efficacy or toxicity continues to be a challenge. The optimization of drug absorption, distribution, metabolism and elimination has improved their selection and reduced early clinical failure (Ho & Chien, 2009). The integration of drug delivery strategies in the clinical trials, in order to reduce exposure of the drugs to off-target tissues, may hold the key to increase the success rates in drug development (Ho & Chien, 2009).

The clinical advantage of drug delivery was established with the approval of Doxil® by USA Food and Drug Administration (FDA) in 1995. Doxil® (Caelyx® in Europe) is a PEGylated liposome formulation of doxorubicin hydrochloride and was first approved for acquired immune deficiency syndrome (AIDS)-related Kaposi's sarcoma (Jiang et al., 2011; Slingerland et al., 2012). This formulation consists in a rigid bilayer composed of HSPC, CHOL, and DSPE-PEG (molecular weight 2000) (Immordino et al., 2006). Resulting liposomes present slow pharmacokinetics: plasma half-lives of 1.5 and 45 hours (median values) (Immordino et al., 2006) in comparison with 0.2 hours for the free drug (Danhier et al., 2010). Due to the high stability in plasma and slow clearance from circultion, Doxil® allowed a significant decrease in the toxic effects when compared with an equi-effective dose of conventional doxorubicin.

Since then, outstanding progress has been made in the use of liposomes in drug delivery approaches. Other liposomal formulations have reached the market: liposomal doxorubicin (Myocet®), liposomal daunorubicin (DaunoXome®), and liposomal vincristine (Onco-TCS®) (Danhier et al., 2010). Among them, Doxil® Myocet® and DaunoXome® are currently used in the clinic to treat metastatic breast cancer (Cho et al., 2008; Tanaka et al., 2009).

Besides these approved agents, many liposomal chemotherapeutics are currently being evaluated and some are even in advanced stages of clinical development. Although many authors reported the efficacy of targeted liposomes in preclinical models, until now only two clinical trials have been conducted: the GAH-targeted doxorubicin-containing immunoliposomes and the transferrin-targeted oxaliplatin containing liposomes (Danhier et al., 2010).

Seventeen years after the approval of Doxil® we can conclude that liposomal anticancer drugs, using their theoretical advantages, have grown to maturity in several points and are in wide development to fulfill the high expectations. In either way, challenges still exist in delivering clinically optimal levels of chemotherapeutic agent. Further studies with liposome-encapsulated anticancer drugs, including the development of novel liposomal formulations, seem the right path to provide increased efficacy and tolerability.

6 - OBJECTIVES

The purpose of this work was to generate a new liposomal-based drug delivery system that had the ability to target and release epirubicin specifically to breast cancer cells, to enhance the amount of drug within the tumor, to improve the pharmacokinetics and biodistribution of the drug and to avoid toxicity to normal cells.

Regarding this, several specific aims were defined for the liposome formulation:

- It should allow high and stable epirubicin entrapment.
- It should have good stability, both in serum and in storage conditions.
- It should display a reduced mean diameter in order to allow long blood circulation time.
- After reaching the tumor, liposomes should efficiently release their drug load on tumor microenvironment.
- Finally, liposomes should allow coupling of targeting moieties capable of recognizing the epidermal growth factor receptor (EGFR) overexpressed in the target breast cancer cells.

III- MATERIALS AND METHODS

1 - Cell culture

MDA-MB-231 cells, an immortalized breast cancer cell line, were obtained from a human epithelial mammary adenocarcinoma. The cell line was maintained as subconfluent cultures in RPMI 1640 media (Sigma, St Louis, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum FBS, 100 U/ml penicillin and 100 g/ml streptomycin. Cells were grown at 37ºC in a humidified incubator with 5% CO_2. To maintain a continuous proliferation (within their exponential growth phase), cells were subcultivated two times per week. For this purpose, media was removed by aspiration, cells were washed with phosphate buffered saline (PBS) and harvested with 0.25% (w/v) Trypsin- EDTA solution for a few minutes. Cells were detached by gently tapping the flask and suspended in the media to obtain the desired dilution (subcultivation ratio between 1:2 and 1:8). For the *in vitro* studies, MDA-MB-231 cells were prepared 24 hours before the experiments. Cells were submitted to the same process of trypsinization described above. However, in this case, the detached cells were counted, in a hemacytometer, using trypan blue and were diluted in the culture medium to obtain the desired cellular density. Cells were seeded on different multi-well plates, according to the different experiments, at densities from 1.2 to 6 $x10^4$ cells per well. All cell work was carried out in aseptic conditions, using a laminar flow cabinet and only sterile material. Material was sterilized through autoclave and solutions were filtered through 0.22µm pore-diameter filters.

2 - Preparation of Liposomes

Large unilamellar liposomes (LUV) were prepared by thin-film hydration technique followed by extrusion of multilamellar liposomes (MLV). Liposomes were composed of different molar ratios mixtures of hydrogenated soy phosphatidylcholine (HSPC), cholesterol (Chol), 1,2-distearoyl-3-sn- phosphoglycerol (DSPG) and 1,2-distearoyl-3-sn-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-PEG). For binding and uptake studies, liposomes were labeled with 1% rhodamine-dioleoylphosphatidylethanolamine (rhodamine-PE). Lipids, purchased from Avanti Polar Lipids (Alabaster, AL, USA), were dissolved in chloroform or in a chloroform/methanol/water mixture (65:35:8) for DSPG. Lipids were mixed at the

desired molar ratio in glass round-bottom tubes (previously washed with chloroform to remove possible impurities). Subsequently, chloroform was evaporated using a dry nitrogen stream. The dried lipid films were hydrated with 300mM $(NH_4)_2SO_4$ at 65ºC to obtain a final lipid concentration of 10 mM. The resulting multilamellar vesicles (MLVs) were then vortexed and sonicated for 5 min. These suspensions were extruded sequentially through two stacked polycarbonate membranes of 200 and 100 nm pore size using a LiposoFast mini extruder (Avestin, Toronto, Canada).

3 - Drug encapsulation

Epirubicin was encapsulated into the liposomes using the transmembrane ammonium sulfate gradient method (Bolotin et al., 1994; Haran et al., 1993) (Figure II.1). This method can actively load weak bases into liposomes at very high efficiency. Additionally, sulfate can stabilize anthracyclines for prolonged storage periods (> 6 months) due to precipitation of the anthracycline sulfate salt. When ammonium sulfate concentration in the liposomal interior is higher than its concentration in the external medium, a small amount originates neutral ammonia which diffuses out of the liposome. This creates an acidic interior and epirubicin (weak base) diffuses into the liposomal interior. The incoming epirubicin is protonated, thereby elevating the pH, renewing the dissociation of NH_4^+ to NH_3 and H^+ enabling the continuation of the loading cycle.

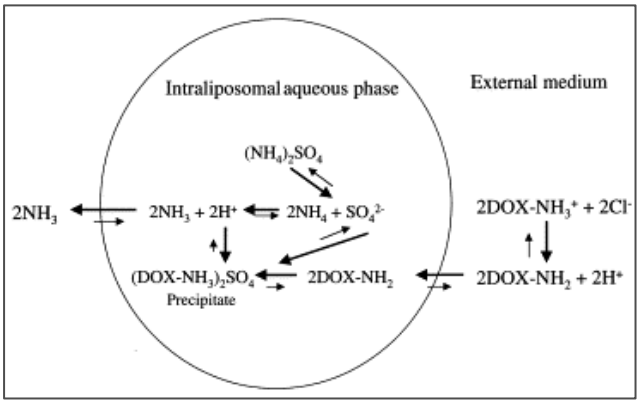

Figure II.1 – Intraliposomal remote loading of amphipathic weak bases by creating an ammonium sulfate gradient between the intraliposomal aqueous phase and the external medium. Doxorubicin (DXR) was used as a model. Adapted from Bolotin et al., 1994.

Unilamellar liposomes were initially formed in buffer containing ammonium sulfate as described above. Liposomes were then subjected to a size exclusion

chromatography on Sephadex G-50 columns to replace the extravesicular medium with HEPES-buffered saline (HBS) (10mM HEPES/140mM NaCl, pH 7.4) and establish a transmembrane ammonium sulfate and pH gradient for active loading. Epirubicin hydrochloride (Sigma, St Louis, USA) was added to the liposome suspension at a drug/ lipid molar ratio of 1:6. The liposome-epirubicin mixture was incubated in a water bath for 1h at 65ºC. After encapsulation, the resulting liposome dispersion passed, once more, through the Sephadex G-50 column with HBS (pH 7.4) as the eluent to separate free epirubicin from the epirubicin-loaded liposomes.

4 - Cholesterol quantification

Liposomal concentration was obtained through the quantification of the cholesterol content and according to cholesterol/total lipid ratio. Cholesterol quantification was determined by our modification of the Liebermann-Burchard methood or acetic anhydride reaction. This test was described initially by Liebermann in 1885 and applied to cholesterol analysis shortly after by Burchard (1889). Lieberman–Burchard method is a colorimetric reaction to detect cholesterol, which consists in adding acetic anhydride and sulfuric acid to the cholesterol (Huang et al., 1961). This procedure gives the solution a deep green-blue color that can be followed spectrophotometrically and is directly proportional to the cholesterol concentration in the sample.

With this purpose, 1.5ml of the reagent (35ml Glacial Acid Acetic; 55ml Acetic Anhydrid; 10ml Concentrated Sulfuric Acid; 1g sodium sulfate) was added per 100µL of sample. A mild agitation was performed, followed by an incubation period of 20 minutes at 37ºC. Since this test uses acetic anhydride, acetic acid and sulfuric acid as reagents, the reagent was handled with caution in a fume hood. The absorbance was measured at 625nm. A calibration curve, built from a set of standard cholesterol samples with known concentrations (0-1mg/mL), was used to assess the cholesterol concentration in the liposomes sample.

5 - Drug quantification

Epirubicin is intensely orange-red and is sensitive to light, absorbing light both in the UV range (232 nm, 254 nm and 291 nm) and in the visible range (around 480 nm- Figure II.2).

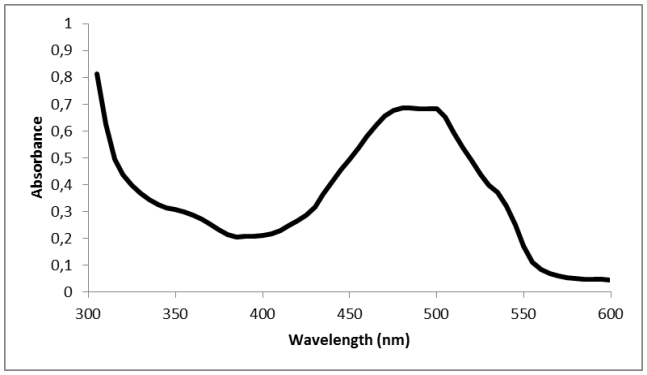

Figure II.2 - UV and visible spectrum of epirubicin at 25ºC.

In this regard, epirubicin concentrations were determined by spectrophotometric assay at 480nm, after liposome disruption with methanol. A calibration curve, built from a set of standard epirubicin samples, with known concentrations, was used to assess the concentration of epirubicin in the final liposomes suspension.

6 - Drug/lipid molar ratio and encapsulation efficiency

Epirubicin and cholesterol concentrations were determined as mentioned above. Total lipid concentration was calculated according to cholesterol/total lipid proportion and drug-lipid (D/L) molar ratios were determined. The final drug-lipid ratio (after the encapsulation and free drug removal) is mentioned as loading.

Total lipid concentration was determined before incubation of the liposomes with the drug. After the encapsulation period, free drug was separated on Sephadex G-50 columns and the final drug-lipid ratio was measured. To determine the encapsulation efficiency the loading parameter was compared to the initial drug-lipid ratio as shown in Equation (1):

$$EE(\%) = \frac{drug/lipid\ molar\ ratio\ after\ free\ drug\ removal}{drug/lipid\ molar\ ratio\ at\ drug\ incubation} \times 100 \quad (1)$$

7 - Particle size distribution

Liposomes were characterized with respect to their size using a photon correlation spectroscopy (PCS)-based technique, which measures scattered laser light resulting from particles in suspension. Scattered laser light intensity differs in time due to the Brownian motion of particles in suspension. These variations are detected by a photo-multiplier whose output current is passed to an autocorrelator. The autocorrelator analyses time dependent fluctuations in scattered laser light, determining the size of the particles. The diffusion coefficient (D) is related to the medium diameter (d) of the particles through the Stokes–Einstein equation:

$$D = \frac{kT}{3\pi\eta d} \qquad (2)$$

In this equation "k" is the Boltzmann constant, "T" is the temperature and "η" is the solvent viscosity. Small liposomes present a higher mobility in solution comparing to larger liposomes, leading to more frequent fluctuations of scattered laser light.

Liposomes were prepared as mentioned above and filtered through 0,22µm pore-diameter filters to remove any impurities. Measurements were performed in a PCS spectrophotometer, the Coulter N4 Plus (Coulter Corporation, Miami, FL), at a scattering angle of 90 degrees, a wavelength of 633 nm, and a temperature of 20°C.

8 - Stability of epirubicin-containing liposomes

In vitro stability studies were performed in order to quantitatively compare drug leakage between epirubicin-loaded formulations, intended to be used for *in vivo* applications on an ultimate goal.

The release of epirubicin from the liposomes was monitored using fuorescence-dequenching assay. In solutions at concentrations higher than 10 µM the drug fluorescence is partially quenched. Inside the loaded liposomes, the achieved epirubicin concentration far exceeds this limit and, in fact, fluorescence is almost 100% quenched. Therefore, this high self-quenching of epirubicin fluorescence within the liposome gives a direct and easy method to determined epirubicin leakage from liposomes.

Equal volumes of epirubicin-loaded liposomal suspension were diluted in HBS to obtain a 5μM solution and incubated for 0.5, 1, 2, 4, 8, 12, 24, 48 and 72h at 4ºC and 37ºC. Due to the release of epirubicin in the external liposomal phase there is an increase in fluorescence intensity (λ_{ex}=470 nm and λ_{em}=585nm). At the end of each measurement, total epirubicin fluorescence (100% dequenched) was determined by lysing the liposomes at a final Triton X-100 concentration of 0.5% (v/v). Percentage of epirubicin release was calculated according to the following equation:

$$\%\, Drug\ release\ = \frac{Ft - F0}{Fmax - F0} \times 100 \qquad (3)$$

In this equation "Ft" is the intensity of the fluorescence at a specific time (t), "F0" is the intensity of the fluorescence at t=0, "Fmax" is the intensity of the fluorescence after the addition of Triton X-100.

To better understand the *in vivo* behavior of these formulations, the release of epirubicin from the liposomes in HBS solution containing 10% fetal bovine serum (FBS) was accessed as described above.

9 - In vitro studies

9.1 - Cytotoxicity assays

The *in vitro* anticancer effect of drug-loaded liposomes (cytotoxicity) were evaluated using the Alamar Blue assay. The assay measures the metabolic activity without the detachment of adherent cells. Redox capacity of the cells, resulting from the production of metabolites in cell growth, allows the reduction of resazurin (blue) to the fluorescent molecule resorufin (pink).

MDA-MB-231 cells were seeded into 96-well plates at a density of $1.2x10^4$ cells per well. After 24h, the growth medium was replaced by 200 μl of a sterile filtered liposomal epirubicin suspension in different concentrations that were obtained by diluting in the RPMI growth medium the prepared liposomes formulations. Cells were incubated for 48h at 37ºC in a humidified chamber with 5% CO_2. After the incubation period, growth medium was replaced and cells were again incubated, this time for 72 h period. In order to measure the cell viability, the medium was removed and cells were

incubated with 150 µl of RPMI 1640 medium containing 10% (v/v) Alamar Blue dye (resazurin reagent at 0.1% in PBS) per well. After 2 hours, 120 µl were removed to another 96-well plate and the absorbance at 570 and 600 nm was measured in a SPECTRAmax PLUS 384 spectrophotometer (Molecular Devices, Union City, CA). Cell viability was calculated, as a percentage of the non-treated control cells, according:

$$Cell\ viability\ (\%\ control) = \frac{(A570-A600)\ of\ treated\ cells}{(A570-A600)\ of\ control\ cells} \times 100 \qquad (4)$$

IC_{50} was defined as the epirubicin concentration, encapsulated in the liposomes or in a free form, which inhibits cell growth by 50% compared to the non-treated control cells (cells incubated with medium). IC_{50} values were determined for the different formulations with the GraphPad Prism 5.01 software (GraphPad Inc.), trough the nonlinear regression analysis of the survival curve.

9.2 - Cellular association

For cellular association studies, liposomes were labeled with a fluorescent dye, rhodamine. Liposomes were prepared, as previously indicated in section 2.2, with 1 mol% of lissamine rhodamine B-phosphatidylethanolamine (egg) (Rhodamine-PE) (Faneca et al., 2008).

Twenty-four hours before transfection, 6×10^4 MDA-MB-231 cells/well were seeded onto 48-well culture plates. After this period, the growth medium was replaced and liposomes were added to the cells at a final lipid concentration of 120µM. Cells were incubated for 3h at 37ºC(uptake) or 4ºC(binding) and then washed twice with (PBS). In order to detach and lyse cells, 100 µL of 1% Triton X-100 were added to the cells. Fluorescence was monitored in a SPECTRAmax GEMINIEM fluorometer (Molecular Devices, Union City, CA) by measuring the fluorescence at excitation and emission wavelengths of 550 and 590 nm, respectively.

10 - PLA_2-triggered drug release

The effect of $sPLA_2$ on lipid-based drug carriers was studied using a snake venom $sPLA_2$ as model of the human Group IIa $sPLA_2$. For this purpose, the release of the encapsulated epirubicin was used as an indicator of the functional activity of $sPLA_2$.

Release of epirubicin from liposome samples (diluted to a final drug concentration of 5 µM) was determined in the absence and presence of different concentrations (0.5 – 2.5 µg/mL) of $sPLA_2$ from *Naja mossambica mossambica* at 37ºC. The experiments were performed in triplicates, in a 96-well microplate (200 µl final volume), and fluorescence was monitored in a SPECTRAmax GEMINIEM fluorometer (Molecular Devices, Union City, CA). After equilibrating the sample at the required temperature, the enzymatic reaction was started by the addition of the enzyme to a mixture containing the liposomal epirubicin diluted in a HBS buffer enriched with $CaCl_2$ (1mM).

Epirubicin's fluorescence intensity was observed every 10 minutes at 585 nm (excitation at 470 nm). The observed increase in the fluorescence can be explained by the epirubicin-dequenching effect and is an indicator of liposomal degradation. After 180 min of measurement, Triton X-100 was added to the wells, at a final Triton X-100 concentration of 0.5% (v/v), and the percentage of epirubicin release was calculated using the Equation 3 (section 2.8).

11 - Statistical analysis

Data were analyzed using the GraphPad Prism software (version 5.0). Statistical significance of differences between data was evaluated by one-way ANOVA using the Tukey and the Dunnett tests. A value of $p<0.05$ was considered significant. The viability curves were fitted according to the sigmoidal dose–response model.

III- RESULTS AND DISCUSSION

Chapter 1 - Preparation and characterization of a liposomal epirubicin formulation.

1.1 - INTRUDUCTION

Epirubicin is an epimer of doxorubicin, belonging to the anthracyclines class (Cortés-Funes & Coronado, 2007). Anthracyclines are between the most effective anticancer drugs for treating solid cancers (Leonard et al. , 2009a; Palmieri et al., 2010). Epirubicin acts by intercalating with DNA strands and triggering DNA cleavage via topoisomerase II, resulting in death of cancer cells (Cortés-Funes & Coronado, 2007). In addition, it generates free radicals that cause cell and DNA damage (Cortés-Funes & Coronado, 2007; Leonard et al., 2009; Lorusso et al., 2007). Epirubicin is widely used to treat breast cancer patients, alone or even in combination with other chemotherapeutic agents. EC (epirubicin, and cyclophosphamide) and FEC (fluorouracil, epirubicin, and cyclophosphamide) regimens are commonly used to treat breast cancer patients (Perez, 2009). Clinical trials have demonstrated that regimens containing epirubicin achieved equivalent response rates and overall median survival when compared to similar doxorubicin-containing regimens in the treatment of advanced and early breast cancer. However, despite having similar antitumor activity, epirubicin has an advantage over doxorubicin since it presents reduced side effects. In fact, *in vitro* and clinical studies have shown that, at equimolar doses, epirubicin is less myelotoxic than doxorubicin (Zagotto et al., 2001). Aditionally, epirubicin has a lower propensity to produce cardiotoxic effects than doxorubicin. These characteristics have allowed dose-intensification of epirubicin-containing regimens (Leonard et al., 2009). Anthracycline liposomal formulations are currently the best known alternative to improve the index and spectrum of activity of conventional anthracyclines (Lorusso et al., 2007). Although epirubicin has an advantage over doxorubicin toxicity profile, the encapsulation of the drug in liposomes would further reduce the toxicity and improve the pharmacokinetics and biodistribution of the drug.

For this work, we proposed a liposomal formulation composed of different amounts of hydrogenated soy phosphatidylcholine (HSPC), cholesterol (Chol), 1,2-distearoyl-3-sn-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG_{2000}) and 1,2-distearoyl-3-sn- phosphoglycerol (DSPG). HSPC is a saturated

phospholipid with a high phase transition temperature (52ºC) (Drummond et al., 1999). HSPC might has a slightly advantage over the commonly used 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), since it presents higher entrapment efficiency, low drug release and longer circulations times (Le & Cui, 2006). In any case, these phospholipids with high phase transition temperatures are important in drug delivery systems as they form liposomes stable in blood (Immordino et al., 2006; Le & Cui, 2006; Mufamadi et al., 2011). Cholesterol has one of the most important roles in the stabilization of the lipid bilayer, since it increases membrane packing (Immordino et al., 2006; Le & Cui, 2006; Mufamadi et al., 2011). In this regard, cholesterol is considered a helper lipid and is part of most of the liposomal formulations. The incorporation of DSPE-PEG is important to reduce MPS recognition. PEG, a highly hydrophilic and flexible polymer, tends to spatially inhibit interactions with macrophages and increase blood circulation times (Immordino et al., 2006; Le & Cui, 2006; Mufamadi et al., 2011). Moreover, DSPG (Tm = 55ºC) is an anionic phospholipid and the addition of small amounts of negatively charged lipids is associated to better entrapment efficiencies and storage stabilities (Drummond et al., 1999; Samad, Sultana, & Aqil, 2007).

Liposomes were prepared by thin-film hydration technique followed by extrusion of multilamellar liposomes (MLV). Epirubicin was encapsulated through a transmembrane ammonium sulfate gradient (Haran et al., 1993). After preparation of liposomes and encapsulation of the drug, loaded liposomes were characterized in terms of their physicochemical properties: size, drug-lipid ratio, entrapment efficiency and drug release. These are important parameters for the development of liposomal formulations. One of the main goals of this study was to establish a correlation between these physicochemical properties and the therapeutic potential of different formulations. Liposomal characterization is an essential step to understand the *in vivo* behavior these systems. Therefore, the effect of each component and of their molar ratios in these parameters was analyzed.

1.2 - RESULTS AND DISCUSSION

The successful application of drug delivery strategies depends on the development of nanocarriers, like liposomes, to efficiently deliver drugs to the targeted tumor. Differences in lipid content, size and surface characteristics are able to modulate both technological and biopharmaceutical parameters of liposomes (Drummond et al., 1999). For this reason, several liposomal formulations were prepared and investigated as potential nanocarriers for epirubicin (Table III.1).

Table III.1 - Liposome Formulations.

Formulation	Lipid Molar Ratio
HSPC:Chol	6:2
HSPC:Chol	6:3
HSPC:Chol:DSPG	6:3:0.6
HSPC:Chol:DSPG:DSPE-PEG	6:3:0.6:0.4
HSPC:Chol:DSPE-PEG	6:3:0.4

1.2.1 - Encapsulation efficiency

We evaluated the epirubicin loading ability of five liposomal formulations (Table III.1). Epirubicin was loaded through a transmembrane ammonium sulfate gradient which is known to efficiently load weak bases in lipid vesicles (Bolotin et al., 1994). After encapsulation, the drug and lipid content was evaluated to determine the loading and encapsulation efficiency.

In Figure III.1, it is illustrated the percentage of encapsulation obtained for different formulations. HSPC:Chol (6:2) showed an encapsulation efficiency of approximately 100% (105.6 ± 0.395%). Despite this great loading ability, this formulation presented a very rigid membrane making the extrusion process very difficult and inefficient. In this context, a higher cholesterol amount was used. With 33.3% of cholesterol (HSPC:Chol(6:3)) the extrusion was more efficient and the encapsulation efficiency wasn´t drastically affected. HSPC:Chol (6:3) presented an loading efficiency of 85.8 ± 5.845 %.

Stealth liposomes have been widely studied and some of these formulations were approved for clinical use (Immordino et al., 2006). HSPC:Chol:DSPE-PEG (6:3:0.4), a long-circulating sterically-stabilized liposome formulation, presented a reduced ability to encapsulate epirubicin (76.4 ± 6.015%). This encapsulation efficiency was slightly lower than that observed with the corresponding formulation prepared without PEG (HSPC:Chol (6:3)). However, the utility of liposomes as drug delivery systems has been connected to their ability to avoid recognition and removal from blood by the cells of the mononuclear phagocyte system, particularly the macrophages in liver and spleen (Cho et al., 2008), and, therefore, the incorporation of a hydrophobic polymer, like PEG, is very important in formulations which the final goal is their in vivo application.

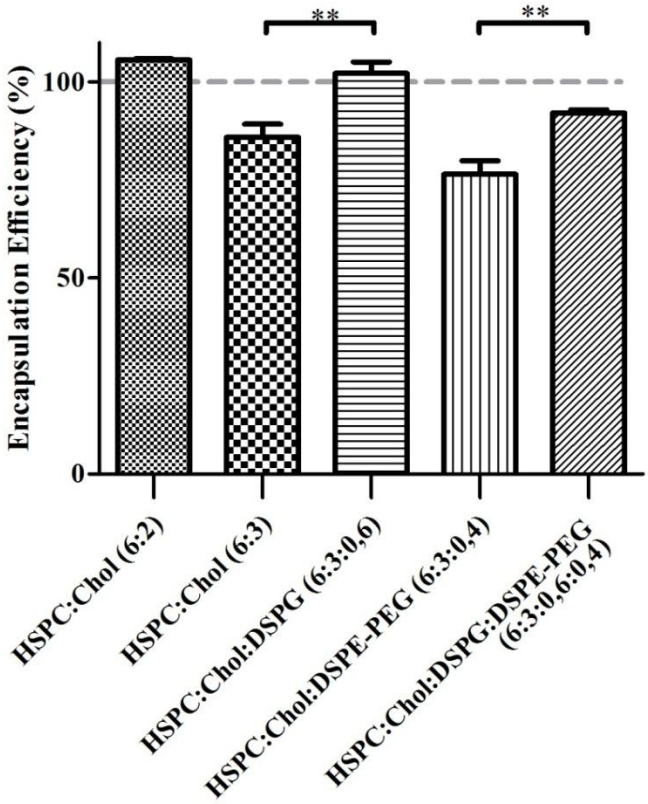

Figure III.1 - Encapsulation of liposomal epirubicin formulations. Percentage of encapsulation efficiency was calculated according to the formula: drug-lipid molar ratio after free drug removal ×100/ drug-lipid molar ratio at drug incubation. Lipid content and epirubicin concentrations were determined prepared as described in 'Materials and methods'. The results correspond to the mean±S.D. obtained from at least three independent experiments. P<0.05 (*), P<0.01 (**).

In this work, we also tested the addition of small amounts of an anionic phospholipid (DSPG) to the formulation. As shown in Figure III.1 HSPC:Chol:DSPG (6:3:0.6) and HSPC:Chol:DSPG:DSPE-PEG (6:3:0.6:0.4) presented higher encapsulation efficiencies (of 102.1 ± 5.052% and 91.8 ± 2.035% respectively) than the equivalent formulations prepared without the anionic lipid, showing that the incorporation of DSPG into the liposomes significantly increases the drug encapsulation efficiency. These results were in agreement with previously published data (Drummond et al., 1999).

Despite all the differences, these results demonstrate that ammonium sulfate gradients can be used to efficiently encapsulate epirubicin into different liposomal formulations. From the tested formulations, HSPC:Chol:DSPG:DSPE-PEG (6:3:0.6:0.4) combines the characteristics of PEGylated liposomes with an encapsulation efficiency over 90%.

1.2.2 - Mean Size

The extensive angiogenesis near the tumor results in blood vessels with disorganized endothelial cells and pores showing enlarged gap junctions from 100 to 780 nm (Andresen et al., 2005). Nanocarrier's size should allow them to pass through these gap junctions and reach the tumor tissue. Therefore, liposomes size is a crucial factor for passive targeting. Having this in consideration, liposomes were extruded trough a 100nm pore size polycarbonate membranes. The liposomes size was measured using a photon correlation spectroscopy (PCS)-based technique as described in "Material and methods". The tested liposomes formulations presented mean diameters of approximately 150 nm, as showed in Table III.2, which allows them to move from the blood compartment into the extravascular space surrounding the tumor cells (Abraham et al., 2005).

Table III.2 – Physical and chemical characterization of the epirubicin-loaded liposomes. Liposomal epirucin was prepared as described in 'Material and methods'. Mean size and Encapsulation efficiency results represent the mean±S.D. of at least two independent experiments. Loading in defined as the drug-lipid (D/L) molar ratio.(ND) was not determined.

Formulation	Size (nm±SD)	Loading (D/L molar ratio)	Encapsulation Efficiency (%±SD)
HSPC:Chol (6:2)	ND	0.176	105.6 ± 0.396
HSPC:Chol (6:3)	146.9 ± 4.491	0.151	85.8 ± 5.,845
HSPC:Chol:DSPE-PEG (6:3:0.4)	152.4 ± 0.495	0.137	76.4 ± 6.015
HSPC:Chol:DSPG (6:3:0.6)	158.6 ± 2. 722	0.183	102.1 ± 5.052
HSPC:Chol:DSPG:DSPE-PEG (6:3:0.6:0.4)	142.7± 12.06	0.154	91.8 ± 2.035

1.3.3 - Stability of epirubicin–containing liposomes

Stability can be associated to several different aspects of a liposomal drug formulation: chemical stability of both drug and lipid components, colloidal stability, and drug retention (Drummond et al., 1999). Despite these distinct aspects, destabilization of the liposomes mainly results in drug leakage. In order to evaluate the stability of the developed liposome formulations under different experiment conditions, drug release studies were performed. Epirubicin concentration inside the liposomes is very high and, consequently, there is a quenching of its intrinsic fluorescence (Haran et al., 1993). The escape of epirubicin from liposomes results in an increase of its fluorescence, since the drug becomes diluted to concentrations under the quenching point. In Figure III.2 the profiles of drug release in HEPES-buffered saline (HBS) were obtained for different temperatures (4ºC and 37ºC).

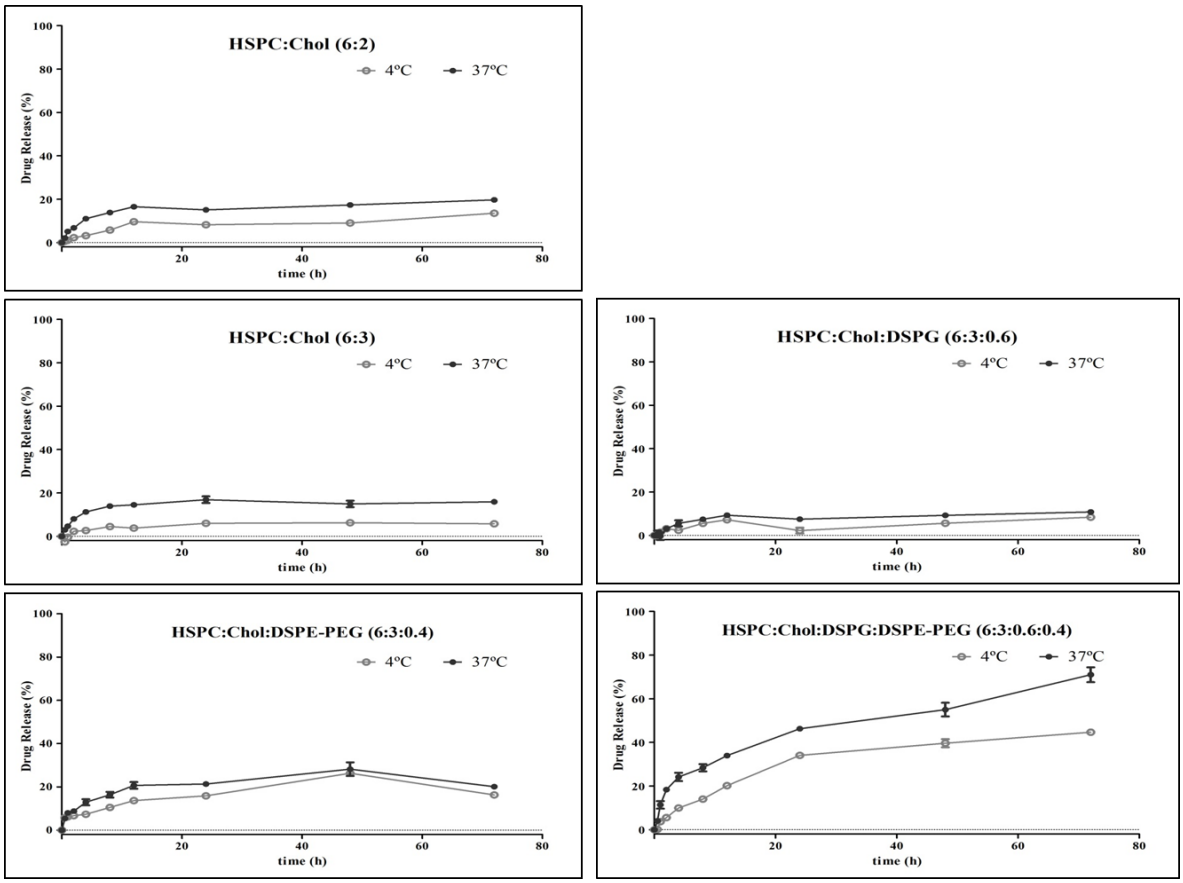

Figure III.2 - Comparative results of *in vitro* drug release from different liposome formulations. Liposomal epirucin was prepared as described in 'Material and methods'. The effect of lipid composition on epirubicin release was determined by dequenching of the epirubicin fluorescence in HBS buffer (ph7,4; final epirubicin= 5µM) for 0–72h at 4º and 37◦C. Percentage of release was obtained by measuring the fluorescence at excitation and emission wavelengths of 470 and 585 nm, respectively. The results correspond to the mean±S.D. obtained from triplicates and are representative of at least two independent experiments.

The lipid composition as well as the nature of the drug are the main factors which determine the stability of the drug inside the liposomes. Differences in leakage rates have been related to the chemical and structural properties of the drug and to its location in liposomes (Maurer et al., 2001). Ammonium sulfate gradient method loads anthracyclines in a very stable way as they tend to precipitate in the liposome interior (Bolotin et al., 1994). However, in some cases, the rapid leakage of drug is related to its lack of precipitation, since this precipitation varies with concentration and liposomal membrane charge.

HSPC and cholesterol formed liposomes that are stable under different conditions of storage. As illustrated in Figure III.2, after 72h, epirubicin release was under the 20%

mark at 37ºC and up to 15% at 4ºC. These results support the idea that the combination of cholesterol and saturated phospholipids do not allow a considerable drug leakage from the liposome. The incorporation of small amounts of anionic phospholipids should further stabilize the loaded liposomes. These phospholipids inhibit liposomal interactions due to the repulsive forces, that occur between their negative charges (Drummond et al., 1999), and retard the leakage of oppositely charged drugs (like the precipitates of anthracyclines) through their stable interaction with the liposome membrane. However, in our case the addition of DSPG didn't significantly affect the drug release (Figure III.2). In fact, 72h after the beginning of the experiment, HSPC:Chol:DSPG (6:3:0.6) presented less than 17% and 10% of release at 37ºC and 4ºC, respectively, which were similar to that observed with HSPC:Chol (6:3). The high stability presented by HSPC:Chol (6:3) formulation, by itself, most probably disguise a potential enhancement in stability of liposomes with the incorporation of DSPG.

Also described in literature is the ability of PEG to prevent liposomal aggregation during storage, particularly in the presence of serum (Immordino et al., 2006; Ulrich, 2002). Having this in consideration, it was expected that PEGylated liposomes had small drug leakage ratios. Despite these indications, higher epirubicin release rates were observed in the absence of the serum. After 72h at 37ºC, HSPC:Chol:DSPE-PEG (6:3:0.4) and HSPC:Chol:DSPG:DSPE-PEG (6:3:0.6:0.4) had already released 30% and 60 % of the epirubicin, respectively.

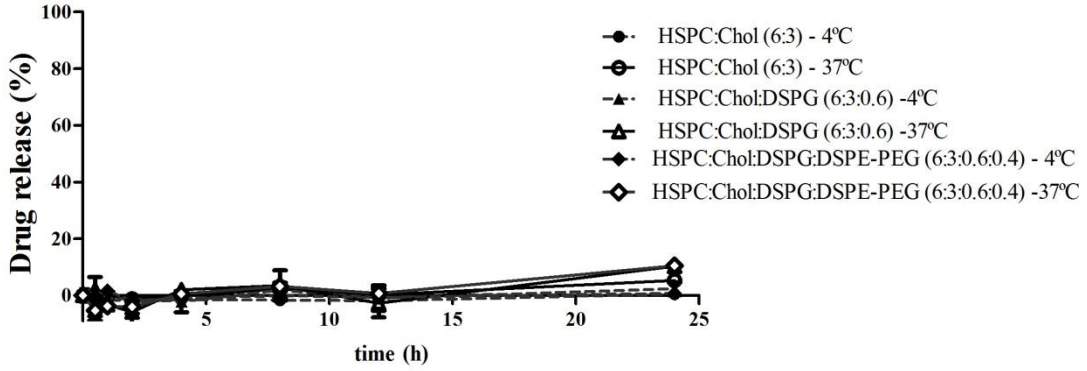

Figure III.3 - Comparative results of *in vitro* drug release from different liposome formulations in the presence of fetal bovine serum (FBS). Liposomal epirucin was prepared as described in 'Materials and methods'. The effect of lipid composition on epirubicin release was determined by dequenching of epirubicin fluorescence in HBS buffer containing 10%FBS (ph7,4; final epirubicin= 5µM) for 0–24h at 4º and 37◦C. Percentage of release was obtained by measuring the fluorescence at excitation and emission wavelengths of 470 and 585 nm, respectively. The results correspond to the mean±S.D. obtained from triplicates and are representative of two independent experiments.

Liposomes applied as drug delivery systems to the tumors must substantially retain their contents during the circulation time in blood. Drug release rates in serum are a more accurate model for evaluating the *in vivo* behavior of drug delivery systems, since these rates might present potential implications in the therapeutic activity and in the side effects of the formulations. Figure III.3 shows the epirubicin release profiles of different loaded liposomes in the presence 10 % of FBS. As illustrated in the figure, HSPC:Chol:DSPG:DSPE-PEG (6:3:0,6:0,4) formulation was highly stable in the presence of FBS contrary to that observed in the absence of the serum, retaining approximately 90% of the loaded epirubicin after 24h. This result indicates that this formulation presents characteristics of stealth liposomes, which is a positive indicator for clinical applications. Nevertheless, these comparatives studies demonstrated no differences between formulations in terms of drug release in the presence of serum. However, these kind of studies don´t take in consideration other advantages associated to the incorporation of a hydrophilic polymer in the liposomes, such as the reduction of the uptake by phagocytic cells.

Chapter 2 – *In Vitro* studies: cellular association and cytotoxicity

2.1 - INTRUDUCTION

Breast cancer, a leading cause of cancer death in women (Jemal et al., 2011), is recognized to be a molecularly heterogeneous disease (Sørlie, 2004). Breast cancer cell lines have been widely used to investigate breast cancer and to screen and characterize new therapeutics (Kao et al., 2009). The recognition of the five molecular subtypes in breast cancer points to the need for additional consideration in cell line selection (Perou & Borresen-Dale, 2010).

In order to evaluate the therapeutic potential of the liposomal epirubicin, MDA-MB-231 cell line was used as an *in vitro* model. MDA-MB-231 breast cancer cell line presents an epithelial-like morphology (Lacroix & Leclercq, 2004). cDNA micro-array studies and gene expression profiles revealed low expressions levels of ER , PR and HER2 (triple negative) and high expression levels of EGFR (Kao et al., 2009; Lacroix & Leclercq, 2004; Neve et al., 2006). Taking in consideration the set of "intrinsic genes" used originally to define the tumor subtypes, MDA-MB-231cells were identified as basal-like breast cancer. Basal-like tumors present high proliferation rates and poor outcomes and so they are good subjects for therapeutic studies. Nevertheless, breast cancer cell lines are divided in three different subtypes, according to theirs transcriptional profile: luminal, basal-A and basal-B (Kao et al., 2009; Neve et al., 2006). MDA-MD-231 cell line was classified as a basal-B subtype, which resembles either basal-like or HER2 tumors. This group presents a $CD44^{+}/CD24^{-}$ phenotype associated with invasive properties(Sheridan et al., 2006). Regarding this, MDA-MB-231 cell line is a good *in vitro* model for the aggressive metastatic breast cancer.

The first occurrence in liposome-cell interaction *in vitro* is the binding of liposomes to the cell membrane. Binding is followed by uptake, where liposomes are internalized by the cells. Inside the cells, the payload is able to reach the target and, consequently, lead to the cell death. In this chapter, the results which elucidate these interactions between the cell and the liposomal epirubicin are presented. Incubations of several liposomal formulations with cells at different temperatures (4ºC and 37ºC to

represent the binding and uptake, respectively) together with cytotoxic studies were used to evaluate the *in vitro* therapeutic potential of our liposomal epirubicin.

2.2 - RESULTS AND DISCUSSION

2.2.1 - Cytotoxicity

Liposomes are able to transport the drugs and accumulate in the tumor, and, in this way, they may improve the drugs therapeutic efficacy. However, within the tumor, it is important to understand how they can execute their activity. To understand how liposomal epirubicin can exercise their function as a chemotherapeutic agent, the effect of free and liposomal drug on the cellular viability was evaluated. MDA-MB-231 cells were incubated with the therapeutic agents for 48h and then for more 72h after medium renewal. Cytotoxicity was evaluated, as a function of concentration, using the Alamar Blue assay, as described in "Materials and Methods". These experiments were used to determine the epirubicin concentration, encapsulated in the liposomes or in a free form, which inhibits cell growth by 50% as compared to the non-treated control cells (IC_{50} values). Table III.3 summarizes the obtained results.

Table III.3 - Cytotoxicity of various formulations of epirubicin against MDA-MB-231 cells. The effect of epirubicin concentration on cell viability was assessed by the Alamar Blue assay for liposomal epirubicin and for the free drug. Cells were incubated with different concentrations of liposomal epirubicin (diluted in the RPMI growth medium). After 48 h incubation, the medium was replaced with RPMI-1640 and the cells were further incubated for 72h. Cell viability (as a percentage of control cells) was calculated according to the formula (A570−A600) of treated cells×100/ (A570−A600) of control cells. IC50 values, epirubicin concentration required to inhibit cell growth by 50%, were determined with GraphPad Prism 5.01 software (GraphPad Inc.) by a nonlinear regression analysis of the survival curve. The results correspond to the mean±S.D. obtained from at least three independent experiments, each done in triplicate.

Formulation	IC50 (µM±SD)
Free epirubicin	0.242 ± 0.07
HSPC:Chol (6:3)	9.397 ± 1.19
HSPC:Chol:DSPG (6:3:0.6)	10.534 ± 1.33
HSPC:Chol:DSPE-PEG (6:3:0.4)	7.322 ± 2.16
HSPC:Chol:DSPG:DSPE-PEG (6:3:0.6:0.4)	8.124 ± 2.42

Free epirubicin, due to its physicochemical properties is able to surpass the MDA-MB-231 cell membrane and enter in intracellular compartments (Cortés-Funes & Coronado, 2007), resulting in high levels of cytotoxicity *in vitro*. This observation is in agreement with the results of our cytotoxicity experiments where epirubicin showed a great *in vitro* antitumoral efficacy with the lowest value of IC_{50} (Table III.3). However, these *in vitro* experiments do not take into account the unfavorable pharmacokinetics and biodistribution presented by the free drug *in vivo* applications**.**

On the other side, for drugs encapsulated in the liposomes, was obtained a much lower cytotoxicity than that observed with the free drug. Lipid composition had only a slight effect on IC_{50} values. However the small differences observed between formulations are in agreement with theirs physic-chemical characteristics. By instance, HSPC:Chol:DSPG (6:3:0.6) liposomes presented the lowest cytotoxicity most probably due to their high stability and negative charge, which, consequently, can reduce the drug release and the liposome-cell interactions. On the other hand, incorporation of DSPE-PEG in the liposomes resulted in a decrease in the IC_{50} values, which can be attributed to the higher drug release of this formulation and to the absence of superficial negative charges, due to the presence of the hydrophilic polymer.

These results were reinforced by the non-cytotoxic nature of empty liposomes as illustrated in Figure III.4. Our liposomes formulations were prepared with nontoxic and biodegradable lipid contents, consequently resulting in a non-toxic effect themselves. Regarding this, the cytotoxicity of liposomal epirubicin was exclusively due to the effect of drug.

There are several factors that affect the antitumoral activity of a liposomal drug such as: binding, internalization and drug release. Liposomes constitute an additional barrier to the drug diffusion and, therefore, targeting and triggering strategies may hold the key for a dramatic improvement on the therapeutic activity (Andresen et al., 2005). These results confirmed the limitation of liposomes and the need of using strategies to enhance the drug delivery in the target cells, in order to increase their therapeutic efficacy.

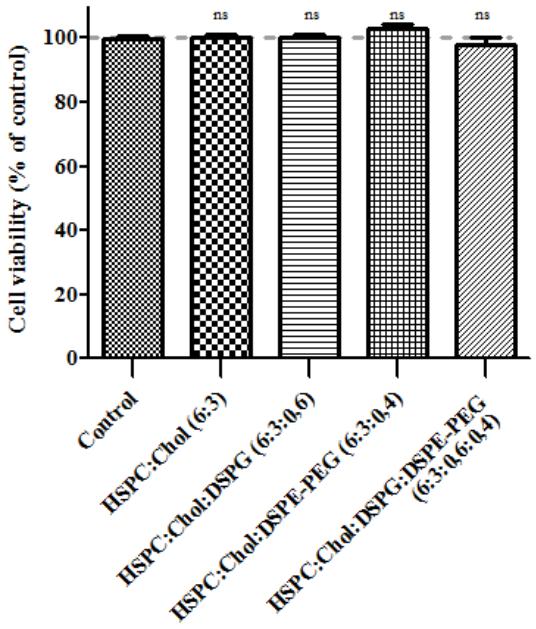

Figure III.4 - Cytotoxicity of various liposomal formulations against MDA-MB-231 cells. The effect of empty liposomes c on cell viability was assessed by the Alamar Blue assay. Cells were incubated with liposomes (diluted in the RPMI growth medium to a final total lipid concentration of 120µM). After 48 h incubation, the medium was replaced with RPMI-1640 and the cells were further incubated for 72h. Cell viability (as a percentage of control cells) was calculated according to the formula (A570−A600) of treated cells×100/ (A570−A600) of control cells. The results correspond to the mean±S.D. obtained from three independent experiments, each done in triplicate. P>0.05 (ns).

2.2.2 - Cellular association

In order to clarify the liposome-cell interactions of our formulations, we evaluated the cell binding and uptake in MD-MB-231 cell line, by using liposomes labeled with rhodamine-PE. Binding, performed at 4ºC (at this temperature endocytosis does not occur), and intracellular uptake, performed at 37ºC, of four liposomal formulation were investigated and the obtained results are presented in Figure III.5.

Differences in cellular association can be attributed to the distinct bilayer composition of formulations. Lipid content is able to modulate the interaction between liposomes and biological membranes (Miller et al., 1998). Anionic liposomes, due to charge repulsion, should not easily enter the cells showing a relatively low level of uptake. In addition, the presence of large molecules such as PEG on the liposomal surface was associated with reduced liposome-cell interactions (Immordino et al., 2006). However, this effect is dependent on the amount of PEG (Peeters et al., 2007), being particularly evident for high concentrations, higher than that used in these

formulations. On the other hand, the effect of PEG incorporation on the liposome-cell interactions also depends on the lipid composition of liposomes, being more obvious for cationic lipid compositions (Dan, 2002), which was not the case.

Anyway, the incorporation of DSPG and PEG in our liposomes formulations did not substantially affect the cellular association when compared to that obtained with the neutral formulation of HSPC:Chol (6:3). In fact, addition of DSPE-PEG seems to slightly increase liposomal cellular association. Despite this profile, it is obvious that all the formulations present low internalization levels and a targeting strategy might be a good option to increase the therapeutic efficacy.

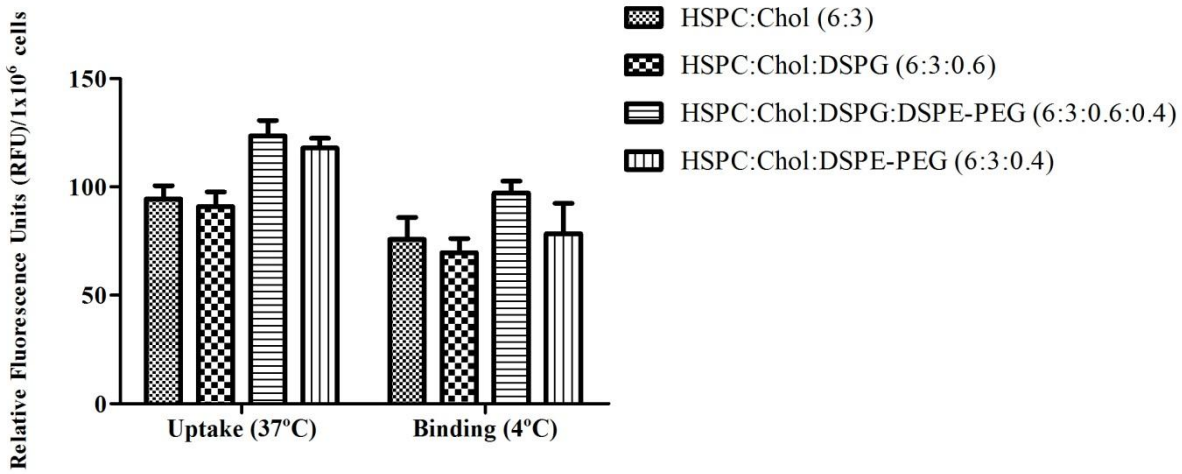

Figure III.5 - Cellular association of several formulations of liposomes with MDA-MB-231 cell line. Liposomes were labeled with rhodamine-PE. Cells were incubated with labeled liposomes for 3h , at 4ºC (binding) and 37ºC (uptake).The extent of cellular association was assessed by measuring the fluorescence at excitation and emission wavelengths of 550 and 590 nm, respectively, as described in ''Materials and Methods'' section. The data are expressed as RFU per 1×10^6 cells (mean±S.D. obtained from triplicates), and are representative of three independent experiments.

Chapter 3 – Enhanced delivery via enzyme-triggered release: Phospholipase A_2 sensitivity studies.

3.1 - INTRUDUCTION

The ultimate goal of chemotherapy is to increase the survival time and the quality of life of the patient but, for this purpose, the systemic toxicity of the chemotherapeutic agents needs to be reduced (Danhier et al., 2010). One approach to this issue is to design systems that can entrap or complex the active agent for ultimate delivery to the desired site. Many long-circulating delivery systems have been designed to stably encapsulate drugs, increase blood circulation half-life and tumor biodistribution (Immordino et al., 2006), in order to improve antitumor activity and reduce toxicity. Despite increased accumulation of long circulating liposomes into tumors due to the EPR effect, they still accomplished modest increased therapeutic efficacy (Andresen et al., 2005). Specific drug delivery to tumors can further avoid toxic effects at non-target sites and increase the antitumoral potential. Concerning this, a major focus of research has been the development of methods to target liposomes to the desired sites of action. There are strategies to perform site-specific delivery: active targeting and triggering(Andresen et al., 2005). Both solutions take advantage from the acquired knowledge of the tumor microenvironment and its numerous differences compared with normal tissue (Danhier et al., 2010). A higher degree of liposome accumulation by active targeting can optimize the problems with the drug distribution. However, for many liposomal drugs, the rate of drug release is not optimal and clinical utility is limited. On the other hand, stimuli-responsive nanocarriers release their cargo upon arrival at the tumor site, since the nanocarriers are transformed by a unique stimulus. This is especially important when the stimuli are specific to the pathology, allowing the nanocarrier to respond to the unique tumoral extracellular microenvironment.

It is well known that there are significant differences between normal and malignant tissues in the expression of enzymes (Cho et al., 2008; Zhu et al., 2011). Enzymes play a central role in cell regulation, and so they are important targets for drug development and for therapeutic strategies. When a determinate enzyme is found at higher concentrations at the target site the drug delivery systems can be programmed to

deliver drugs via enzymatic conversion of the carrier. In this work, differences in secretory phospholipase A_2 ($sPLA_2$) expression were chosen to exploit the drug release capacity of our formulations. Recent studies demonstrated that $sPLA_2$ expression and activity is increased in prostate, breast, and pancreatic cancers. By instance, the Group IIa $sPLA_2$ was found to be up-regulated in breast cancer and was associated to their malignant potency (Mannello et al., 2008; Yamashita et al., 1994). Furthermore, there is limited $sPLA_2$ in the systemic circulation, except in the case of septic shock or inflammation.

Membrane phospholipids consist of a glycerol backbone, to which 2 long-chain fatty acids are bound at the sn-1 and sn-2 positions and a phosphate-containing head group at the sn-3 position (Figure III.6) (Laye & Gill, 2003). The PLA_2 family consists of a wide variety of enzymes that hydrolyze the sn-2 ester bond of phospholipids, releasing a lysophospholipid and a free fatty acid (Jørgensen et al., 2002). The mammalian PLA_2 enzymes have been broadly classified into three groups: (1) low-molecular-weight Ca^{2+}-dependent secretory phospholipase A_2 ($sPLA_2$); (2) high-molecular- weight Ca^{2+}-dependent cytosolic phospholipase A_2 ($cPLA_2$); and (3) Ca^{2+}-independent phospholipase A_2 ($iPLA_2$) (Laye & Gill, 2003).

$sPLA_2$ represents a group of esterases between 14–19 kDa, which require millimolar concentrations of Ca^{2+} for enzymatic activation and are secreted from cells being consequently found extracellularly (Laye & Gill, 2003; Zhu et al., 2011). Despite these shared features, there were ten subtypes of $sPLA_2$s named IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XII identified in humans (Andresen et al., 2005). The most studied $sPLA_2$ subtype in humans is type IIA that is up-regulated in infectious and inflammatory diseases. In fact, sPLA2-IIA and other family members play a key role in inflammation mediation and have been suggested to exhibit a central role in both tumor development and progression (Arouri & Mouritsen, 2011b; Zhu et al., 2011). Within the $sPLA_2$ subfamily there are differences between the types of phospholipids cleaved, for example, it has been shown that sPLA2-IIA mainly acts upon anionic rather than neutral phospholipids (Laye & Gill, 2003).

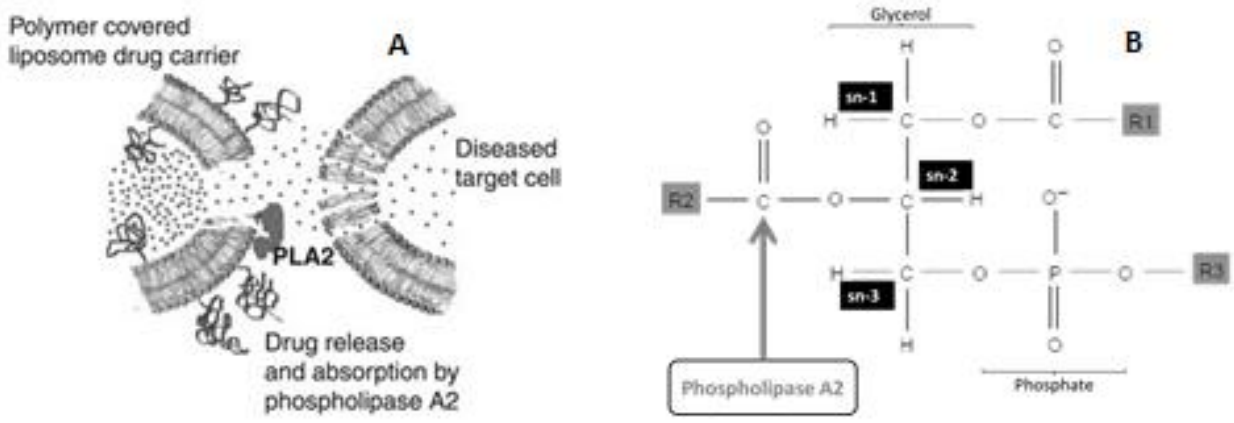

Figure III.6 - Triggered liposomal drug release by PLA_2 in diseased tissue. A) Schematic representation of the principle: Polymer-covered liposomes accumulate in the tumor microenvironment; subsequently, the overexpressed PLA2-catalyzes degradation of the liposomes and leads to a site-specific release and absorption of the encapsulated drugs at the target site. (Jørgensen et al., 2002) B) Representation of the phospholipid structure with phospholipase A2 cleavage site.(Laye & Gill, 2003)

The goal of this part of the work was to determine the effect of $sPLA_2$ on the rate and extent of drug release from liposomes. This was accomplished by examining the functional activity of $sPLA_2$ on liposomal degradation. For this purpose the release of the encapsulated epirubicin, induced by sPLA2-mediated lipid degradation, was assessed by measuring its intrinsic fluorescence during time course. A substantial structural similarity exists between different sources of $sPLA_2$ (Andresen et al., 2005) and, in this studies snake $sPLA_2$ (from *Naja mossambica mossambica*) was used as a convenient model to prove the concept of $sPLA_2$ as a site-specific trigger (Kini, 2003).

3.2 - RESULTS AND DISCUSSION

A liposomal drug carrier for clinical use must be able to efficiently balance stability in circulation (drug retention) with the ability to make the drug bioavailable at the disease site (drug release) (Maurer et al., 2001). In liposome systems, the drug is not bioavailable while associated with the liposome, and failure to release the drug from the carrier in a timely manner may result in a reduced therapeutic effect relatively to the free drug (Allen & Cullis, 2004). Drug may be released from liposomes inside the tumor microenvironment in response to a secretory phospholipase A_2 ($sPLA_2$) stimulus. The major advantage of this mechanism of delivery is that it does not require the binding of liposomes to the tumor cells. $sPLA_2$ is up-regulated in the tumor microenvironment, and is, in this way, able to mediate lipid degradation and release

epirubicin from liposomes. The effect of $sPLA_2$ on the rate and extent of drug release from liposomes was determined using $sPLA_2$ from *Naja mossambica mossambica* as described in "Matherials and Methods". $sPLA_2$ induced the release of epirubicin and, for 180 minutes, the increase in fluorescence resulting from this leakage and dilution of the drug in aqueous solutions was monitored. The resulting drug release curves are represented in Figure III.7.

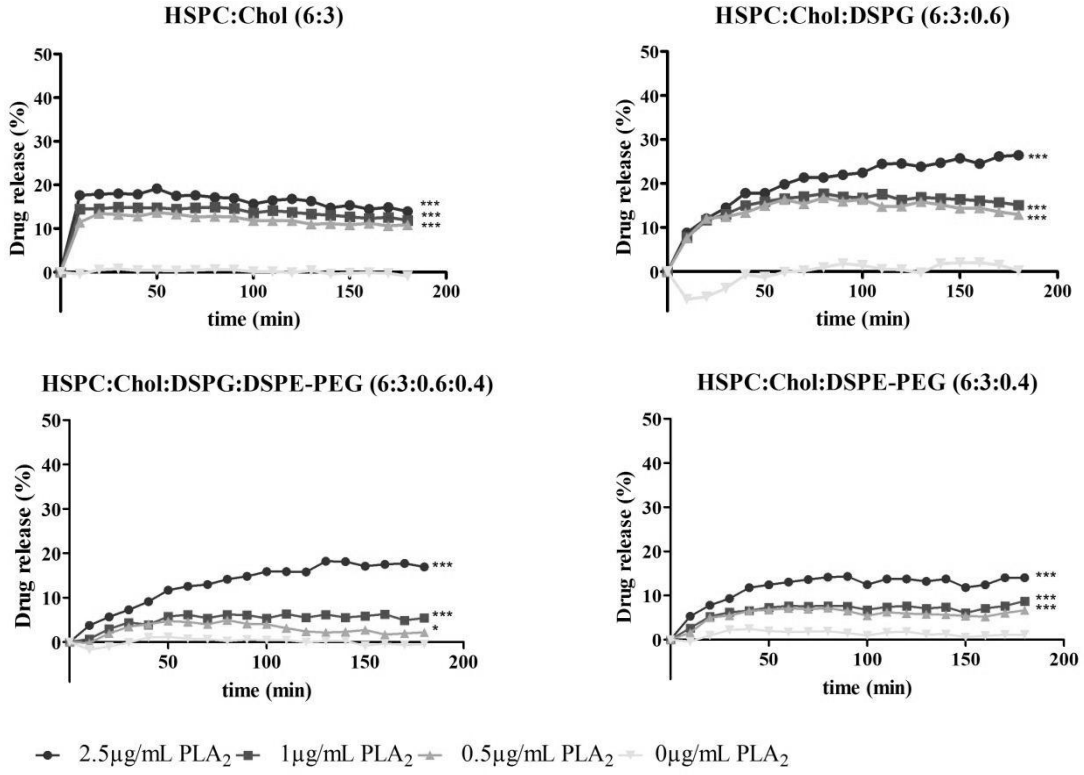

Figure III.7 – Comparison of sPLA2-mediated epirubicin release from different liposome formulations. sPLA2 from Naja Mossambica mossambica (snake venom) was used as a model for the human behaviour of sPLA2 . The effect of sPLA2 (0-2,5 µg/mL) on the release of epirubicin from different loaded liposomes (final epirubicin concentration= 5µM) was determined by fluorescent intensity changes in the media for 0–180 min at 37◦C. Fluorescence intensity was obtained by measuring the fluorescence at excitation and emission wavelengths of 470 and 585 nm, respectively. The results correspond to the mean±SD obtained from triplicates and are representative of at least three independent experiments. $P<0.05$ (*); $P<0.01$ (**); $P<0.001$ (***).

Drug release from control samples (liposomes not treated with the enzyme) remains stable over time, with values close to zero. As shown in Figure III.7, treatment with $sPLA_2$ induced a rapid and significant increase in the drug release, when compared to that obtained with non-treated controls. The concentrations of $sPLA_2$ used in these studies ranged from 0.5 to 2.5 µg/mL. The lower concentrations correspond to the amount of $sPLA_2$ in tissues under normal physiological conditions (0.025 to 0.5

µg/mL). Only in cancer and inflamed tissues these sPLA2 levels are increased 2- to 1000-fold (Zhu et al., 2011).

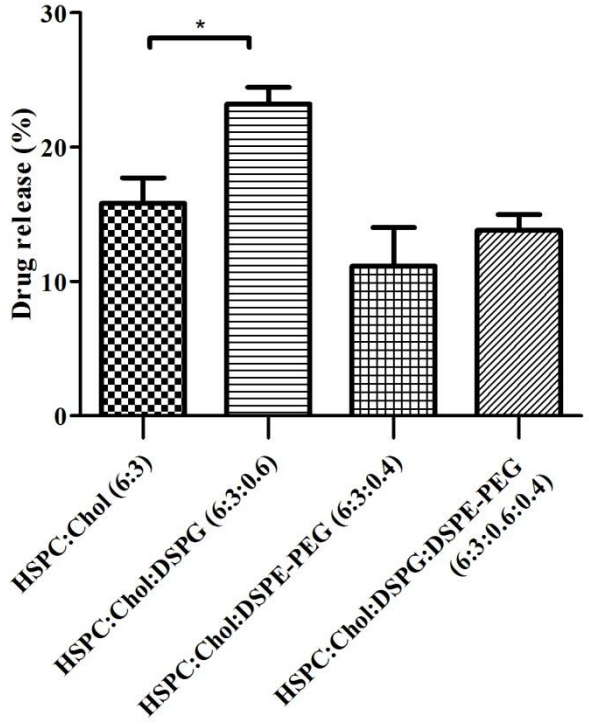

Figure III.8 - Effect of DSPG and PEG on sPLA2-mediated epirubicin release from different liposome formulations. sPLA2 from *Naja Mossambica mossambica* (snake venom) was used as a model for the human behaviour of sPLA2 . The effect of DSPG and DSPE–PEG on sPLA2-mediated (2,5 µg/mL) release of epirubicin from different liposomal formulations (final epirubicin concentration = 5µM) was determined by fluorescent intensity changes in the media after 180 min at 37◦C. Fluorescence intensity was obtained by measuring the fluorescence at excitation and emission wavelengths of 470 and 585 nm, respectively. The results correspond to the mean±S.D. obtained from at least two independent experiments, each done in triplicate. $P<0.05$ (*).

Different kinetics and degrees of release were observed for the tested formulations. In fact, the enzyme activity is controlled by the physical properties of the substrate, for example, it has been shown that $sPLA_2$-IIA acts much more on anionic phospholipids rather than neutral ones. The choice of PLA_2 from *Naja mossambica mossambica* venom was based on its large similarity, in terms of structure and catalytic function, with mammalian enzymes (Kini, 2003). Moreover, the interaction of phospholipids with $sPLA_2$ from snake venoms is much well-characterized than their interactions with human $sPLA_2$. Although the kinetics of $sPLA_2$ from snake venoms and from humans may vary, the overall behavior is similar (Arouri & Mouritsen, 2011b).

The addition of DSPG (approximately 6% of the total lipid) to the formulations significantly increased the $sPLA_2$-mediated epirubicin release when compared to that

obtained with formulations composed of HSPC and cholesterol (Figure III.8). This observation is most probably due to the preferential activity of this enzyme on anionic phospholipids.

On the other hand, the incorporation of PEG into the liposomes resulted in a lower activity of the enzyme, as shown by the smaller drug release (Figure III.8). PEG might prevent the hydrolysis of lipids because this polymer has the ability to shield the liposomes surface, consequently avoiding the action of $sPLA_2$. However, several studies have demonstrated that there is a higher $sPLA_2$ activity on PEGylated liposomes and that the degree of hydrolysis increases with increased amounts of PEG (Zhu et al., 2011). Several justifications were used to explain this effect: $sPLA_2$ can indeed reach the surface of pegylated liposomes; DSPE-PEG presents an anionic nature and this nature may enhance the enzyme activity; and PEG alters the structure of the membrane surface promoting an enhanced binding or activity of $sPLA_2$ (Andresen et al., 2005). Regarding this, further studies must be done to clarify this issue.

Concerning the presence of cholesterol, previous studies associated the incorporation of large amounts (more than 20 mol%) of this lipid into the liposomes to the reduced activity of $sPLA_2$. In fact, secretory PLA_2 is unable to hydrolyze liposomal formulations such as Doxil® and DaunoXome® (Andresen et al., 2005). However, other studies showed that liposomes containing 33% of cholesterol (which is approximately the amount on ours formulations) presented high sensitivity to the enzyme activity (Zhu et al., 2011). This information could be important to improve the degree of liposome degradation and consequently the clinical relevance of this strategy.

Our results demonstrated that sPLA2 has the ability to induce the degradation of the liposomes promoting the epirubicin release. We also show that the presence of anionic phospholipids in the formulation can increase the sensitivity of liposomes to the enzyme activity. This strategy could be of great importance since the distribution of epirubicin throughout the tumor could further enhance the therapeutic effect, due to the action of the free drug in tumor cells that do not directly interacted with the liposomes.

IV- FINAL CONCLUSIONS AND PERSPECTIVES

Nanotechnology constitutes a promising strategy for medical applications. Between these applications, oncology has been a notable area of interest. Currently available chemotherapy agents are time-tested, and confer good disease-free survival for a limited period of time. Nevertheless, non-target tissue toxicity and drug resistance curtails the utility of these agents. These problems have awakened the need for drug delivery system capable of a more effective chemotherapeutic approach. Therefore, the focus on nanotechnology in cancer treatment has intensified. From the different drug delivery systems, liposomes have received a great attention.

The main goal of this work was to develop a drug delivery system that had the ability to specifically transport and deliver epirubicin to breast cancer cells, enhancing the amount of drug within the tumor, improving the pharmacokinetics and biodistribution of the drug and avoiding toxicity to normal cells. For this purpose, liposomes composed of HSPC, cholesterol, DSPG and DSPE-PEG were prepared and the formulations were optimized for a posterior application as drug delivery systems. The efficiency of liposomes as a drug delivery system for anticancer therapeutic applications has been correlated to their physico-chemical properties. Regarding this, we evaluated parameters like the encapsulation efficiency, the mean size and drug release. The reported data showed the following conclusions:

- The lipid composition of liposomes determined the encapsulation efficiency of the drug. Liposomes with lower amounts of cholesterol presented an increased ability to encapsulate epirubicin. Addition of a negatively charged phospholipid (DSPG) resulted in an increased encapsulation efficiency of epirubicin. Coating the liposomes with a hydrophilic polymer (PEG) promoted a slight decrease in the drug encapsulation efficiency.
- Liposomes formulations presented sizes near the 150 nm, making these liposomes suitable for intravenous administration.
- Membrane permeability is regulated by the lipid composition. HSPC, cholesterol and DSPG formed liposomes with a reduced leakage of epirubicin. Although the incorporation of DSPE-PEG into liposomes resulted in a lower drug retention capacity, this effect was abolished in the presence of 10% of FBS, constituting a good indication for *in vivo* applications.

In the second part of this work we made use of the MDA-MB-231 cell line to evaluate the *in vitro* cytotoxic activity and drug intracellular uptake of liposomal epirubicin. MDA-MB-231 cells were used as model for an aggressive type of breast cancer. The *in vitro* studies demonstrated that both the conventional and the stealth liposomes presented a reduced antitumoral efficacy when compared to the free drug, since epirubicin is able to directly pass through cell membrane while liposomal-epirubicin has an additional barrier to the drug diffusion. Moreover, the tested formulations presented a limited cellular association, which most probably contributes to their reduced antitumoral activity. However, these observations do not take in account the *in vivo* biodistribution and pharmacokinetics, which are much more unfavorable for a free drug than for a liposomal-drug**.**

In order to reverse this limitation, two strategies were proposed in this work. Failure to release the drug from the carrier may result in a reduced therapeutic effect relative to the free drug. Regarding this, the development of systems that have the ability to specifically release the drug in response to a stimulus, may constitute a good strategy to overcome this limitation. In this context, we evaluated $sPLA_2$ as a stimulus to release epirubicin from the liposomes. $sPLA_2$ catalyzes the hydrolysis of phospholipids and is up-regulated in some type of tumors, like breast cancer. These drug release studies were a proof of concept on enzymatic triggering release strategies and allowed the following conclusions:

- $sPLA_2$ stimulus significantly increased the leakage of epirubicin from the liposomes.
- Lipid composition is an important factor that determines the liposomal sensitivity to the enzyme. Addition of DSPG to the formulation resulted in an increased sPLA2 activity, showing that this induced drug release strategy is dependent on the lipid composition.

The second one consisted in a targeted-delivery strategy that could probably improve the internalization of the liposomal epirubicin by the tumor cells, via a receptor-mediated endocytosis process. High levels of the epidermal growth factor (EGF) receptor are found in breast tumors with high proliferation rates and poor outcomes. Regarding this, we proposed an approach involving the specific targeting of liposomes to this receptor, using an anti-EGFR Affibody® (antibody mimetic with

several advantages for intravenous applications) as a targeting moiety. Unfortunately, it was not possibly to complete this goal.

Overall, these findings suggests that HSPC:Chol:DSPG:DSPE-PEG (6:3:0.6:0.4 molar ratio) formulation can be developed to combine a great encapsulation efficiency with the benefits of long circulation half-lives. This formulation may be used as a $sPLA_2$-sensitive system, to control drug release, and as targeting approach, to improve the specific cell-liposome interaction. The resulting multifunctional nanocarrier would increase the liposomal-epirubicin uptake, by tumoral cells with EGFR expression, and help the release of epirubicin, from liposomes which do not interact with these cells, in response to the $sPLA_2$ activity. However, it is essential to carry out further studies to prove this concept. It is necessary to determine if anti-EGFR Affibody® is a suitable targeting strategy for breast cancer and a more profound knowledge of the $sPLA_2$ activity on stealth liposomes is recommended. Finally, the *in vivo* antitumoral activity must be evaluated to fully understand the potential of this therapeutic strategy.

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