**UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL**

**FACULDADE DE FARMACIA**

**DISCIPLINA DE TRABALHO DE CONCLUSAO DE CURSO**

# DEVELOPMENT OF A CELL-TARGETED LIPOSOME FORMULATION FOR SMALL CELL LUNG CANCER

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Orientador: Pedro Eduardo Froehlich

**"Nunca se afaste de seus sonhos, pois se eles se forem,**
**você continuará vivendo,**
**mas terá deixado de existir".**
***Charles Chaplin***

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## APRESENTAÇÃO

Os resultados deste estudo estão sob forma de artigo cientifico, o qual contem os seguintes tópicos: Introdução, materiais e métodos, resultados, discussão conclusão e referencias, e será submetido a revista European Journal of Pharmaceutics and Biopharmaceutics, após realização de mais alguns experimentos.

O trabalho esta de acordo com as normas da revista a ser publicada (em anexo).

Os experimentos foram realizados no Laboratório de biologia celular na University of East Anglia.

# Development of a cell-targeted liposome formulation for small cell lung cancer

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**A b s t r a c t**

Lung cancer is one of the most common cancers with small cell lung cancer (SCLC) representing 18% of the total lung cancer diagnoses. Conventional treatments include the administration of high dosages of cisplatin, but as most anti-cancer agents, this drug is not targeted which causes toxicity and undesirable severe side-effects which limit its clinical application. Liposome-based formulations have been recently exploited for improving the delivery and therapeutic effect of cisplatin (Lipoplatin®). In this study, we sought to generate a targeted liposomal formulation. The gastrin releasing peptide (GRP) receptor is over-expressed on many cancer cells including SCLC. A conventional liposomal cisplatin formulation was modified by adding a GRP receptor agonist to a pegylated lipid which was then inserted into pre-formed liposomes. However, the biological activity of peptide-modified liposomal cisplatin was comparable to the control liposomes when tested on GRP receptor positive SCLC cells (NCI-H345), and GRP receptor negative control cells (A549), using the MTS assay. ICP-AES analysis of cisplatin encapsulation efficiency showed encapsulation to be decreased in the targeted formulation. Fluorescence liposomes were also prepared to evaluate the uptake of the cells, using carboxyfluorescein as fluorescence agent. Results shows higher uptake by target- liposomes.

Keywords:

Small-lung cancer, Bombesin, Liposome, Cisplatin, Fluorescence

## 1. Introduction

According to the World Health Organization (WHO) is the major cause of death in developed countries in 2008 around 12.7 million new cancer cases were diagnosed worldwide resulting in 7.6 million deaths.[1, 2] Cancer is known medically as a malignant neoplasm, including a broad group of diseases, all involving unregulated cell growth. In cancer, cells divide and grow uncontrollably, forming malignant tumours, and invade nearby parts of the body. Cancer may also spread to more distant parts of the body through the lymphatic system or bloodstream.[1, 3] Determining the causes of cancer is complex. Many things are known to increase the risk of cancer, including tobacco use, certain infections, radiation, and lack of physical activity, obesity and environmental mental pollutants. [1] These can directly damage genes or combine with existing genetic faults within cells to cause the disease. Approximately five to ten *percent* of cancers are entirely hereditary.[3] It is believed that for a normal cell to be converted into a cancer cell mutations have to take place at different sites. [4] Two types of genes are of particular importance in the development of cancer: oncogenes, the genes that cause a cancerous phenotype, and the tumour suppressor genes which protect the cells from the development of cancer.[3] The oncogenes are often mutated or overexpressed in cancer cells and the tumour suppressor genes have often lost their function due to mutation.[3, 4] The most commonly diagnosed cancers worldwide are lung, breast and colorectal cancer. It is estimated that 18% of lung cancer diagnosed is small-cell lung (SCLC) also known as oat cell cancer.[5] In 16-32% of SCLC cases there is an overexpression of MYC proteins.[6] Through the encoding of nuclear phosphoproteins the MYC is a

transcription factor that regulates the transcription of many target genes that, cause increased cell growth, and also cause cell cycle progression.[5, 6] Gastrin-releasing peptide (GRP), the human homologue of the amphibian peptide bombesin, has a role in embryonic development and adult repair of bronchial epithelia.[6] GRP and neuromedin B are both produced by SCLC cell lines and small cancer lung cells also express receptors for these two peptides as well as bombesin receptor subtype 3.[7] An autocrine-stimulated growth loop is observed due to the secretion of the peptides and the subsequent binding to their receptors and receptor activation.[6, 7]

Chemotherapy is the treatment of cancer that uses anti-cancer drugs (ACD). It is often used with a curative intent or it may aim to prolong life to palliate symptoms.[8] Almost all the ACD are not target-specific, affecting all dividing cells therefore producing many side-effects.[9, 10] The ACD is classified according to their mechanism of action. In the case of SCLC the most commonly-used class of ACD is the alkylating agents, so-named because of their ability to alkylate many nucleophilic groups under conditions present in the cells.[10] Cisplatin and carboplatin are the most common alkylating chemotherapeutics used for the treatment of SCLC.[5] They impair cell function by forming covalent bonds with the amino, carboxyl and sulfhydryl groups in biologically important molecules. [10] Cisplatin forms stable adducts with DNA that are responsible for the drug's anticancer activity. [11] It is believed that cisplatin reacts with N-7 of guanine and intrastrand cross-links are formed, in this way cisplatin causes DNA bending and unwinding followed eventually by apoptosis. [12] After cisplatin has entered the cell the drug undergoes aquation to form $[Pt(NH3)2Cl(OH2)]^{+}$ and $[Pt(NH3)2(OH2)2]^{2+}$ due to the low level of

chloride ions inside the cell. [13]The principal limitations for their use are due to its severe toxicities, especially nephrotoxicity, neuropathy, ototoxicity and haematological toxicities.[10, 11]

For these reasons, an improved liposome formulation of cisplatin, Lipoplatin®, has been recently introduced in the clinical practice.[11] Lipoplatin (Liposomal cisplatin) is a liposome formulation containing cisplatin. [11, 12] The liposome is composed of a lipid shell made of dipalmitoyl-phosphatidyl glycerol (DPPG), soy, phosphatidyl-choline (SPC/*3*), cholesterol (CHOL), methoxy-polyethylene, and glycol-distearoyl phophatidylethanolamine lipid conjugate ($mMPEG_{2000}$-DSPE) in a ratio of cisplatin to lipids of aproximatelly 10% cisplatin and 90% lipids. [12, 14, 15] These liposomes contain cisplatin in the aqueous part. The PEG-coating increases the circulation of the lipoplatin formulation *in vivo as* preventing recognition by macrophages and immune cells favoring accumulation of the drug mainly in primary tumour sites and metasteses (EPR effect). [10, 11, 16] This accumulation of the drug in primary tumour is called passive targeting where the formulated drug makes use of the body's already existing mechanisms to reach the target site, can be achieved by encapsulating the drug in liposomes and making the liposomes resistant to opsonisation by plasma proteins; they will removed slowly from the circulation by the Mononuclear Phagocyte System (MPS) system and the drug can then circulate in the body for a longer time and can also make use of the Enhanced Permeability and Retention (EPR) effect within the tumour microenvironment.[17] Many anti-cancer drugs used in the clinic today have severe toxicity profiles because of their mechanisms of action are not tumour-specific and kill the healthy cells too.[18, 19] Liposomal formulations of anti-

cancer drugs are one way to try to avoid these toxicity drawbacks.[18, 20, 21] Preparation of a liposomal formulation as anti-cancer drug has a number of advantages including extension of the drug circulation time, increases the probability of drug accumulation in the tumour site through using the EPR effect and a consequential reduction in the toxicity drawbacks side-effects.[16, 18]

In order to make the drug delivery more specific different types of ligands can be coupled to the drug carrier. [21] Such ligands include antibodies, peptides, sugar moieties and folate.[22] Recent studies in nanomaterials have explored passive and active targeting strategies for enhancing intratumoral drug concentrations while limiting the unwanted toxicity to healthy tissues.[19]

One way to actively directed nanocarriers to tumour cells is by targeting the receptors that are over-expressed on their surface. However, the presence of receptor-targeting moiety on PEGylated liposomes limits the cellular uptake of liposomes due to receptor saturation. Considering that an ideal tumor targeted drug delivery system should not only selectively targeted delivery drugs to tumor, but also deliver the drugs into tumor cells with high efficacy.[19] For SCLC this can be achieved targeting the GRP receptors with specific ligands such as gastrin-releasing peptides (GRP), bombesin peptides. which have shown high affinity for these receptors.[19] There are two different subtypes of bombesin receptors, a GRP-preferring bombesin receptor and a neuromedin B-preferring bombesin receptor. [22] Bombesin and GRP share a common C-terminal heptapeptide sequence.[8, 19] Since bombesin receptors are overexpressed by many tumours including SCLC they can be used as targets for chemotherapeutics receptor-mediated tumor localization.[19] Studies

have shown specificity on the drug delivery and in use of therapeutic diagnostic using the bombesin like a target specific therapy.[19, 23]

In this study we modified a liposomal formulation of cisplatin by adding a small percentage of a Bombesin-$PEG_{2000}$-lipid able to specifically bind the GRP receptor and evaluated the response of targeted liposomes in receptor positive and receptor-negative cell line. The uptake of target and non-target liposomes was also evaluate using fluorescence target formulation in receptor-positive cell line.

## 2. Materials and Methods

*2.1 Materials*

1.2-dioleoyl-sn-glycerol-3 phosphoethanolamine-N-[Methoxy (Polyethylenoglycol-2000] (ammonium salt) (mPEG 2000 – DSPE)**,** 1,2 – dioleoyl–sn-glicero-3-phosphocoline (DOPC), 1,2-Diastearol-sn-glycero-3-Phosphoethanolamine-N-[Maleimide (Polyethyleneglycol-2000] (ammonium salt) (mPeg 2000-DSPE), Cholesterol (ovine wal)>98% by Avanti Polar Lipids, USA. Cis-Diammineplatinum (II) dichloride, G 25 Sephadex Phenazine ethosulfate, 5-(and-6)-Carboxyfluorescein (CF) from Sigma-Aldrich, UK. Chloroform from Fisher Scientific, UK. RPMI 1640, Dubelco modified eagle media (DMEM) by Gibco life technologies, UK. Hyclone fetal bovine serum, 96 microwell plate and T-75 flask by Thermo Scientific, UK. Penstrep (10.000/mL penicillin and 10mg streptomycin/0.9%Nacl) and Trypsin/EDTA 0.05%(0.25mg/100ml) by Gibco, Paisley, UK. CellTiter 96 AQueous MTS reagent powder. Promega, Southampton, UK. C-Bombesin (C-QWAVGHLM) peptide was generously donated by M. Giorgetti. Equipaments: UV-visible

spectrometer, Perkin Elmer, Lambda 35; BD accuri, Malvern Nano-ZS instrument, Malvern Instruments Ltd., Worcestshire UK; POLARstar Optima; BMG Labtech-Aylesbury, UK

*2.2 Methods*

*2.2.1 A549 – cells*

The A549 cell line, derived from a lung adenocarcinoma was from ATCC, USA. A549 cells grow adherently and do not express the GRP – receptor. They were used as negative control cells in this project.

A549 cells were maintained in DMEM medium with 10% FBS and 2% pen–strep in T-75 flasks. Cells were passaged washing with PBS followed by trypsinization. They were centrifuged (200 rcf, 5 min); the supernatant was removed and re-suspending in fresh media before passaging them onto either to 96 well plates or new flasks. Cultures were maintained in a water saturated incubator at 37 °C in an atmosphere of 95% air and 5% $CO^2$.

*2.2.2- NCI – H345 – cells*

The NCI – H345 cell line derives from a small cell lung carcinoma. The cells grow as mulitcell aggregates in suspension and express the GRP receptor.

Cells were maintained in RPMI 1640 medium with 10% FBS and 2% pen–strep in T-75 flasks they were passaged once a week and feed with fresh full media three days after passage. Cells were passaged by centrifugation (200 rcf, 5 min), removal of the supernatant and re-suspending in fresh media before passaging them onto either to 96 well plates or new flasks. One day after splitting the cells in the 96 well plates were treated with the different

formulations. Cultures were maintained in a water saturated incubator at 37 °C in an atmosphere of 95% air and 5 % $CO^2$.

*2.2.3 Synthesis of bomesin-PEG2000-DSPE lipid conjugate.*

35 mg of C-Bombesin (5 equivalents) was dissolved in 1mL DMSO and left at room temperature for one hour to complete dissolution. TCEP (bond breaker, Thermo Scientific) was added (1:100 v/v) and incubated for 1h to reduce any intramolecular disulfide bonds. 20 mg of DSPE-$PEG_{2000}$ – Maleimide (1 equivalent) was added to the peptide solution and reacted under stirring for 3 days at room temperature. The DMSO solvent was removed under high vacuum and the product re-suspended in 2 ml of water before dialysis for 3 days (MW 2,000) to remove unreacted peptide. Following dialysis the aqueous Bombesin-$Peg_{2000}$-DSPE suspension was lyophilised. The resultant powder was dissolved in chloroform and kept at -18 °C. The concentration of the DSPE-$PEG_{2000}$-Bombesin chloroform was determined by UV spectroscopy. 100 µL of the chloroform solution was diluted in 1 mL of chloroform and UV absorbance at 280 nm was measured on a UV-visible spectrometer (Perkin Elmer, Lambda 35). The total amount of Bombesin-$Peg_{2000}$-DSPE was determinate by using the Lambert-Beer equation using as extinction coefficient 5690.

*2.2.5 Bombesin -$PEG_{2000}$- DSPE insertion into pre-formed liposomes.*

Liposomes targeting ligands with 1% (57 DOPC:38 CHOL: 4 $PEG_{2000}$: 1 DSPE-$PEG_{2000}$-Bombesin, molar ratio) and 4% (57 DOPC:38 CHOL: 1 $PEG_{2000}$:

4 DSPE-$PEG_{2000}$-Bombesin, molar ratio) of the total lipid formulation were prepared.

An aliquot of the DSPE-$PEG_{2000}$-Bombesin chloroform solution, equivalent to 1% or 4% (molar ratio) was dried under rotary evaporation and high vacuum to form a film. The film was hydrated with the standard liposomal suspension loaded with cisplatin or carboxyfluorescein as described above in 2.3. The liposomal mixture was then heated at 60 °C for 1h to accelerate of the insertion of the micelles into the pre-formed liposomes.[24] Following heating, the liposomes were cooled on ice and the suspension dialyzed (MWCO 2.000) overnight against PBS, same osmolality as the liposomes, to remove any leaked cisplatin or carboxyfluorescein and unincorporated micellar bombesin-$PEG_{2000}$-DSPE from the external volume of the liposome suspension.

*2.2.4 Liposome Preparation*

Liposomes formulations were composed of: DOPC:CHOL:$Peg_{2000}$-DSPE (57:38:5 molar ratio).[25] For the Bombesin-Peg-DSPE, encapsulated with cisplatin, the molar ratio was 1 mol%, and encapsulated with carboxyfluorescein the molar ratio was 1 and 4 mol%.

Liposomes were prepared by the film hydration method followed by sequential extrusion.[25, 26] Vesicles were prepared by evaporation of the solvent from a $CHCl_3$ solution of the lipid; the dried lipid film was re-hydrated in a solution of cisplatin (1.5 mg/mL in RPMI culture medium) to obtain a final lipid concentration of 10 mg/ml. Control liposomes, without cisplatin, were prepared by re-hydrating film hydration in RPMI medium. The suspensions were vortexed until completely dispersion of the film and then freeze-thawed five times in liquid

nitrogen and 40 degree water bath.[27] The multi-lamellar polydisperse vesicles were then sized down by repeated extrusions (Liposofast, Avestin) at room temperature through polycarbonate membrane (Avestin).[25, 28] Three steps of twenty-one extrusions through filters of 0.2 mm, 0.1 mm and 0,05 mm pore sizes made the preparation of a narrow sized distribution of unilamelar vesicles.[25, 28]

Cisplatin that was not entrapped inside the liposome was removed by size exclusion chromatography (SEC) with Sephadex G-25 microcolumn (1 ml-syringe). The column was prepared by dispersing 1g of Sephadex G25 in 10 mL of RPMI. The mixture was left in the fridge overnight to swell the resin.[28] A cotton wool layer was inserted in the bottom of 1 mL syringes. The syringes were then filled with the Sephadex dispersion before they were placed in 15 mL Falcon tubes and centrifuged for 3-4 minutes at 2000 rcf.[28] After centrifugation more eluent was added and the falcon tubes containing the syringes were centrifuged again, 300 µL of liposome dispersions were eluted and centrifuged at 2000 g for three minutes without loss or dilution of material.[28] Cisplatin liposomes were passed 3 times through the column.

The final concentration of the cisplatin inside the liposome was measured by inductively coupled plasma–atomic emission spectrometry (ICP-AES). We don`t have this equipment so the sample were sent to the analytical department to be analysed.

Liposomes encapsulated with carboxyfluorescein were prepared by hydrating the dried film with 50 mM CF in 10 mM HEPES buffer at pH-7.4 and extruded as explained before. Non-encapsulated CF was separated from CF encapsulating liposomes using a Sephadex G-50 column using RPMI medium

as an eluent, which has the same osmolarity as the liposomes. They were passed 10 times through the column.

CF leakage experiments were recorded using a Varian Cary Eclipse. The excitation and emission wavelengths were set to 480 nm and 520 nm, respectively.

For CF we couldn`t precise the concentration, we just measure the final intensity inside the cells.

*2.2.6 Dynamic light scattering (DLS)*

DLS experiments were carried out on Malvern Nano-ZS instrument (Malvern Instruments Ltd., Worcestshire UK) and were analysed by Malvern Zetasizer 6.12 software. Measurements have been performed at 25 °C on 0.5 mL samples previously transferred into the plastic the plastic cuvette. Samples were diluted 10 times in buffer before the measurement.

*2.2.7 Zeta Potential*

Zeta potential measurements were carried out on Malvern Nano-ZS instrument (Malvern Instruments Ltd., Worcestshire UK) and analysed by Malvern Zetasizer 6.12 software. Folded capillary cells (Zetasizer) were used as measure cell. Runs were performed at 25 °C with a current rate of 215.3 kcps on 1 mL of the sample diluted 10 times in buffer.

*2.2.8 MTS – assay*

The MTS assay was used for the A549 and the NCI-H345 to evaluate the cell viability. 40 mg of MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-

carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) was dissolved in 20 mL of PBS 2:1 ratio (mg:mL; MTS:PBS) and 1.84 mg phenazine ethosulfate (PES) was dissolved in 2 mL of PBS in a 0.92:1 (mg:mL PES:PBS). The two solutions were then combined in a ratio of 20:1 (MTS:PES) and stored in dark containers at -20°C. To the culture wells containing cells in 0.2 mL of culture medium 20 µL of the MTS/PES solution were added. After two hours, the amount reduced formazan produced was assayed by measuring the optical density at 492 nm using a spectrophotometer (POLARstar Optima; BMG Labtech-Aylesbury, UK).

*2.2.9 Flow cytometer-assay*

Flow cytometer assay was performed using NCI-H345 cells to evaluate the uptake of the target and non-target CF liposomes, using BD accuri C6 equipment. 100.000 cells were incubating with different CF liposome formulation; non-target and target liposomes, 1 and 4mol% of Bombesin added. The cells were incubated with the formulations for two hours at 37 $^{0}$C 5% $CO_2$ and at 4 $^{0}$C. Liposomes that were not uptake by the cells were removed by washing them with cold isoosmolar PBS buffer.

The washes were performed by centrifuging the cells at 4 $^{o}$C for 8 minutes at 12000 rcf removing the supernatant and re-suspending the cells in 600 µL cold PBS buffer, this procedure was performed 3 times. The final cell suspension in PBS buffer was analysed by flow cytometer measuring fluorescence intensity, using the software BD accuri.

## 3. Results

*3.1 Characterization of control, cisplatin-loaded liposomes and CF liposomes*

Different liposomal formulation prepared were analysed by DLS and z-potential. Table 1 report the physical characterization of the liposomes and the efficiency of encapsulation of the cisplatin inside the liposomes. Control liposomes containing no cisplatin (Figure 1b) and cisplatin-loaded liposomes (Figure 1c) were monodispersed, as can be seen by the size distribution by intensity reported in Figure 3a and 3b. The graphs show a low range of distribution of liposome size indicating low Polydispersity (PDI) that is confirmed by the results demonstrated on table 1.

Results of physical characterization for CF liposomes are reported on table 1. Non-target and 1 mol% CF liposomes were monodispersed as can be seen by size distribution by intensity on Figure 3e and 3f, confirmed by the low PDI (table 1). However 4 mol% CF liposome had two populations as indicate by figure 3g , and confirmed by the elevate PDI (table 1).

The zeta potential almost zero indicates the stability of the liposomes in the suspension.

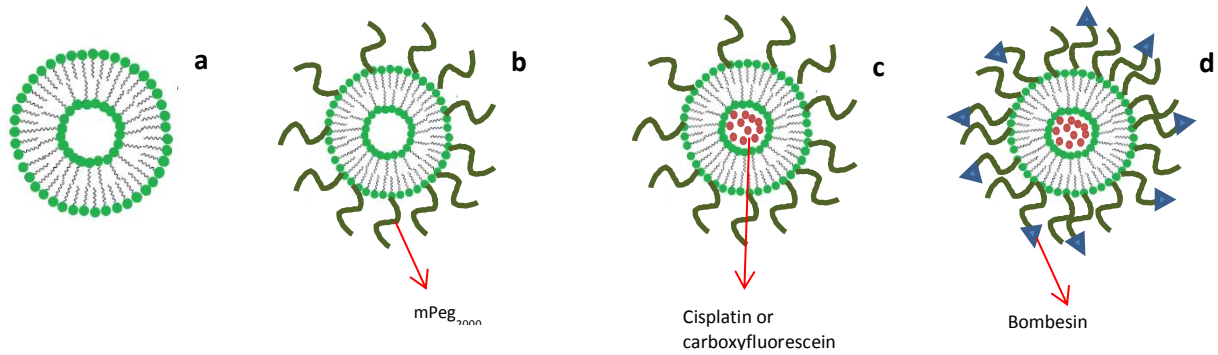


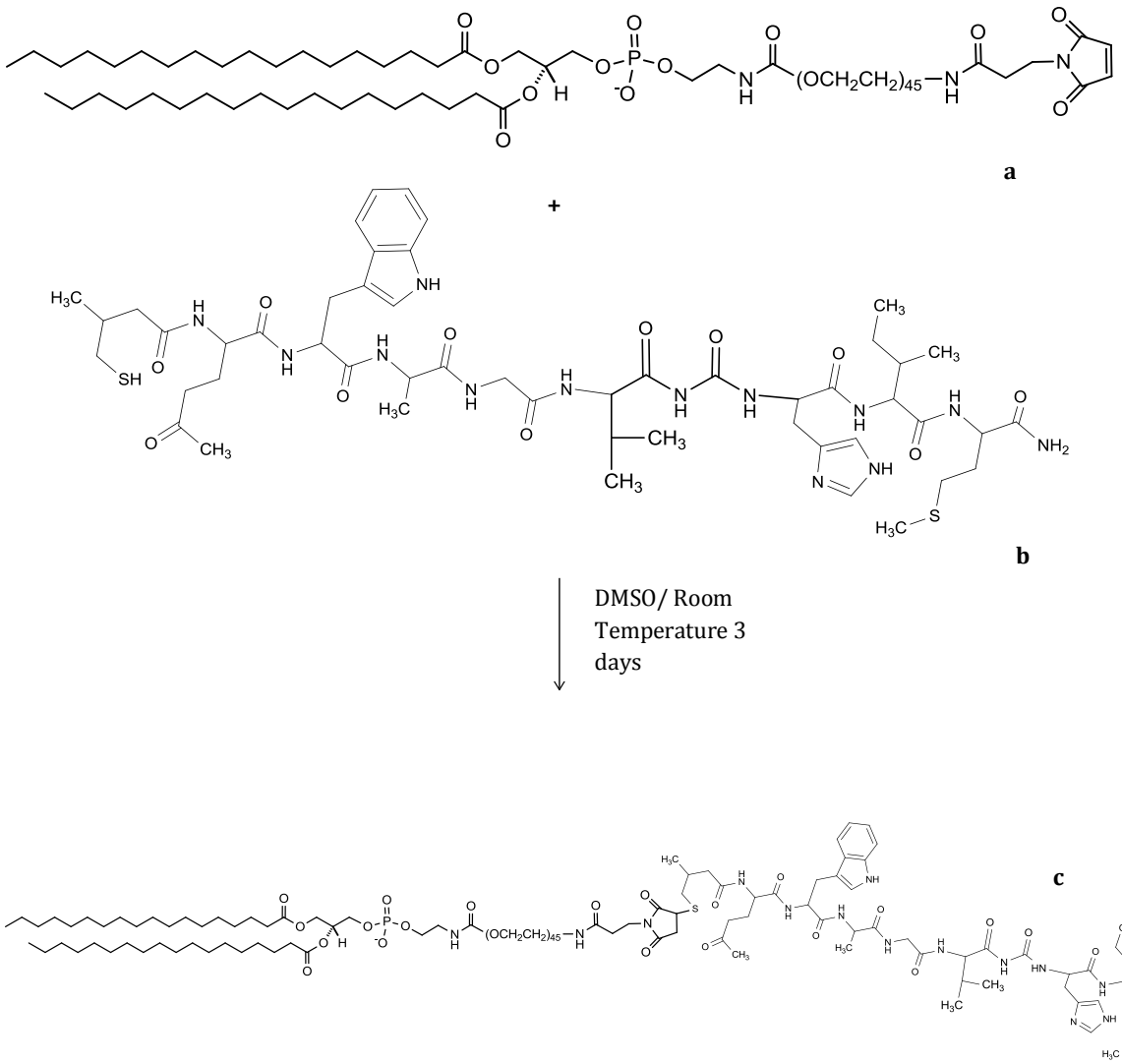

Figure 1 – Schemes of different liposomes formulation.
a – Normal structure of liposome – vesicle composed of a lipid bilayer; b – Liposome Pegylated;
c – Liposome load with cisplatin/carboxyfluorescein (cisplatin liposome; CF liposomes);
d – cisplatin/carboxyfluorescein liposome functionalized with Bombesin.

*3.2 Synthesis and characterisation of Bombesin -$PEG_{2000}$- DSPE*

Bombesin-$PEG_{2000}$- DSPE was prepared for later insertion into the pre-formed control pegylated liposomes loaded with cisplatin. The reaction is represented in Scheme 1. After the conjugation the product was purified by dialysis to remove unreacted peptide reactant. The Bombesin-$PEG_{2000}$-DSPE was characterized by Matrix-Assisted Laser Desorption/Ionisation (MALDI) Time of Flight (TOF) mass spectrometry (MS). Figure 2 represents the spectrum of the final product. The expected mass of the peptide is 3983.605 Da and the spectrum shows a peak at *m/z* 3922.233 and 3966.266, this difference can be attributed by the difference on final molecular weight of the $Peg_{2000}$-DSPE-maleimide that can be different of the one that we used to calculate because the polymerization reaction is hard to stop. On the spectrum (not shown this data) it was also detected the peptide and the $Peg_{2000}$-DSPE-maleimide but we assumed that almost everything was removed by dialysis and this amount represent a very small quantity.

Attempts to characterise the purity of Bombesin -$PEG_{2000}$- DSPE by reverse phase HPLC were unsuccessful due to suspected retention of the product on C18 and C8 columns (data not shown).

After dissolution in chloroform the final concentration and therefore yield of the product was evaluated by UV-spectrophotometer. Final concentration of the Bombesin -$PEG_{2000}$- DSPE solution was 1.849 mg/ml equating to an overall reaction yield of 90% which means that the reaction was almost completely having just 10% of lost in the process.




Scheme 1-Synthesis of the Bombesin -$PEG_{2000}$- DSPE

DSPE-$PEG_{2000}$-maleimide (a) – was reacted with Cysteine modified bombesin (b) to give bombesin -$PEG_{2000}$- DSPE (c)

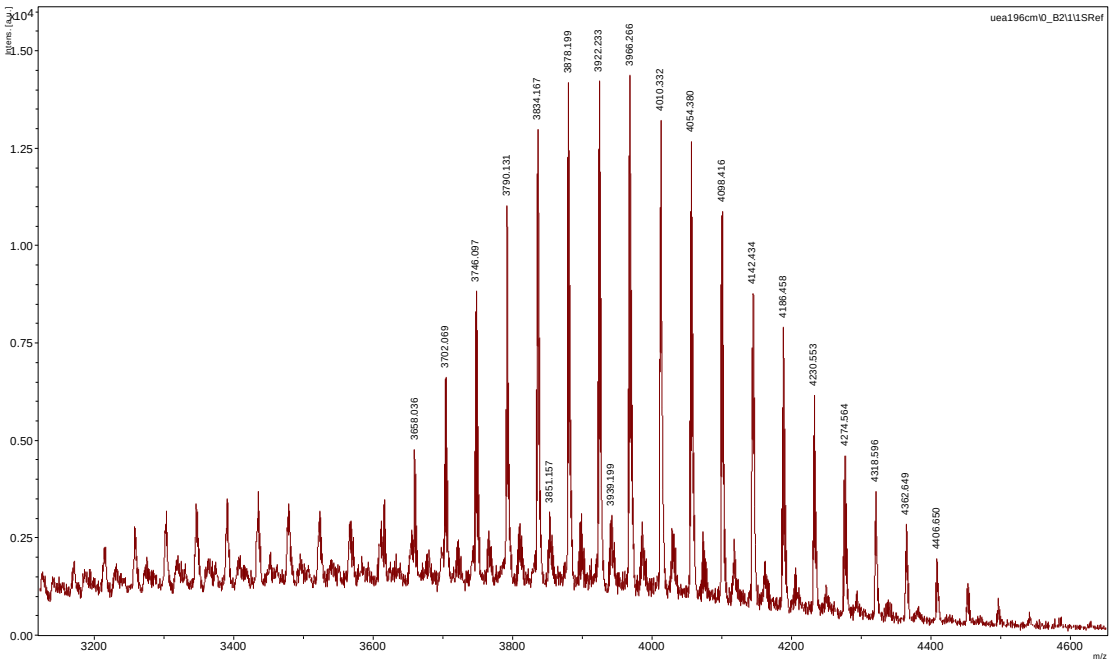



Figure 2 - MALDI mass spectrum of Bombesin -$PEG_{2000}$- DSPE

*3.3 Characterisation of Bombesin-modified liposomes.*

Bombesin-functionalized liposomes (Figure 1d) were prepared by adding the bombesin-PEG-lipid to preformed cisplatin-loaded liposomes or CF-load liposomes. As for control liposomes, the peptide modified liposomes were analysed by DLS. This preparative method, consisting of 3 different steps, has been optimized to obtain a monodispersed liposome suspension and the size has been checked throughout the process as shown in Figure 3d. As we can see on the figure the peptide did not fuse with the liposomes immediately, only after heat up as we can observe by the increase size of the liposomes, however there is still peptide that didn`t incorporate the liposomes and they are going to be removed by dialysis for 24h. Thus, bombesin-functionalized liposomes were characterized by more complex size distributions where bigger agglomerates are observed in Figure 3c. The main population were the target liposomes but as we can observe on the picture there is still a second pick but it represents

less than 1% so we assume it was pure. However when 4mol% bombesin-PEG-lipid was added we couldn`t manage to have an monodisperse liposome suspension as shown on figure 3f, even extruding again with 200 nm and 100 nm filter.

*4.4 Efficiency of cisplatin encapsulation*

Cisplatin encapsulation was lower than expected but it is reproducible for different replicates (n=3). However, the encapsulation of cisplatin in Bombesin-functionalised liposomes was half that of the control loaded liposomes.

**Table 1**
DLS analysis of Liposomes and efficiency of encapsulation.

| | **Size (nm)** | **PDI** | **Zeta Potential (mV)** | **Encapsulation Cisplatin (%) **** |
|---|---|---|---|---|
| **Liposome Control (n=3)** | 104.7±2.6 | 0.076±0.030 | -1.48±1.03 | n/a |
| **Cisplatin Liposome (n=3)** | 101.3±1.9 | 0.093±0.018 | -3.23±1.26 | 11.1±0.6 |
| **Cisplatin Liposome + 1% Bombesin-DSPE-PEG (n=1)*** | 121.6±3.9 | 0.421±0.037 | -1.74±1.01 | 5.02 |
| **CF Liposome (n=3)** | 110.4±2.7 | 0.139±0.014 | -1.15±0.012 | n/a |
| **CF Liposome 1% Bombesin-DSPE-PEG (n=2)** | 126.1±1.5 | 0.213±0.001 | -2.72±1.9 | n/a |
| **CF Liposome 4% Bombesin-DSPE-PEG (n=2)*** | 329.6±5.8 | 0.461±0.035 | -2.91±1.5 | n/a |

n/a = not applicable; PDI = Polydispersity
*Sample had multi-populations as can be seen on figure 3c and the 121nm represents the continuous peak that is most abundant population.
**Encapsulation was calculated using the real concentration of the cisplatin solution used to prepare the liposomes compare with the real concentration of cisplatin find in the liposomes. Real concentration of cisplatin was given by ICP-AES analysis of the samples.

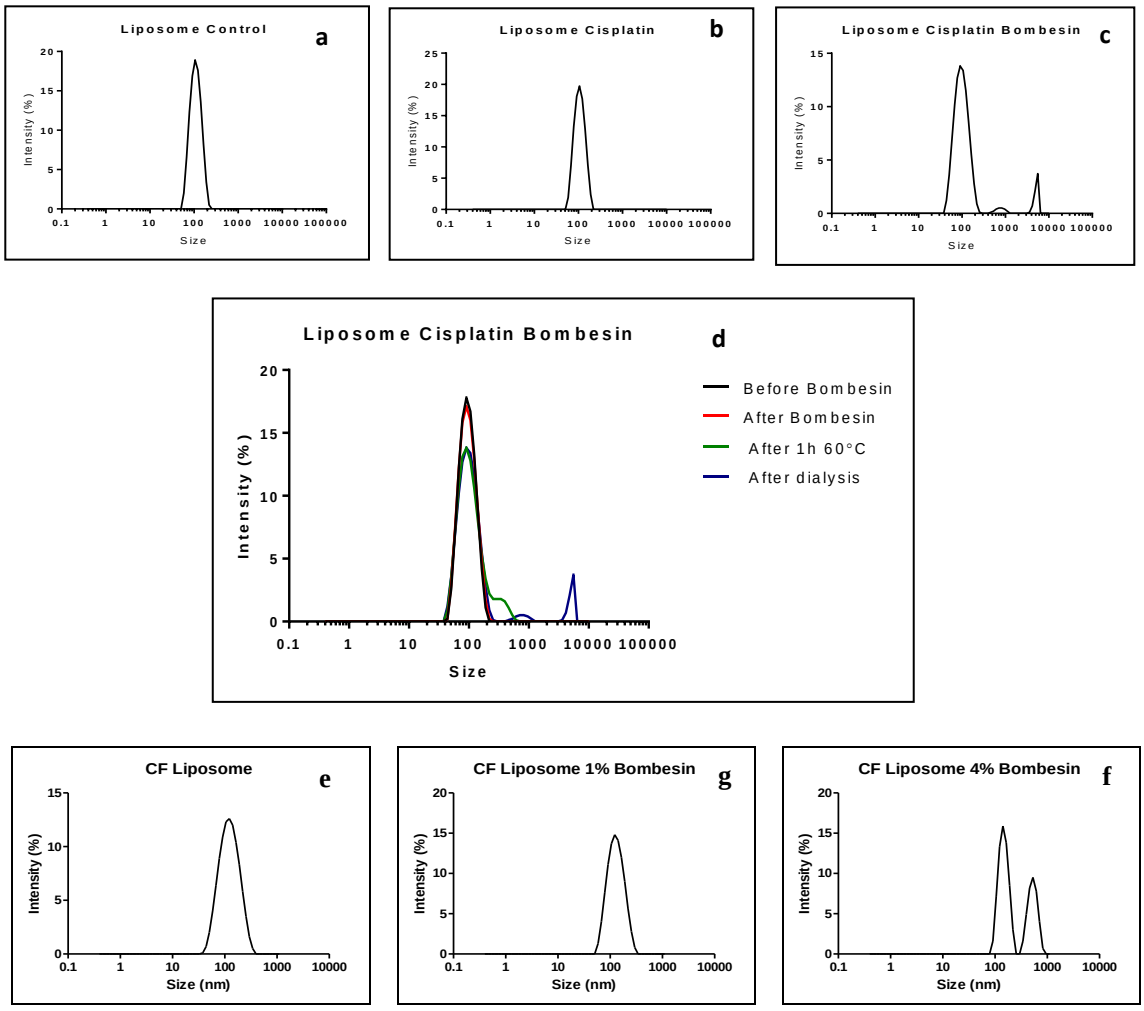



Figure 3 – DLS of different liposomal formulation prepared. The graphs represent the size distribution of the liposomes.

- Cisplatin liposome without Bombesin-$Peg_{2000}$-DSPE
- Cisplatin liposome just after add the Bombesin-$Peg_{2000}$-DSPE .
- Cisplatin liposome-Bombesin after 1h at 60°C.
- Cisplatin liposome-Bombesin after purification – Dialysis 24hrs.

*4.3. Cellular effect following incubation with conventional and Bombesin liposomes*

In order to evaluate the effectiveness of the different formulations prepared MTS cell assay was performed, as described on section 2.3, on the two different cells lines used on this study NCI – H345 and A549.

Different concentrations of each formulation were added to the cells to evaluate the dose-response. Figure 4 represents how much cells were alive, cell viability, after exposed to different concentrations of the different formulations. Results with the liposome control, figure 4a and 4b, shows that this formulation didn`t have any effect on the cells and no IC50, half maximal inhibitory concentration of a substance, was calculated for them. For the others formulation IC50 was calculated and it is demonstrated on table 2.

Evaluating the response of the cells to the others formulations the A549 cells showed a dose-dependent response for cisplatin solution (figure 4d); H345 shows a dose-response for the formulation too, (figure 4c) however the cell viability for the A549 was 1. For the cisplatin liposome the A549 show a lower dose-response effect having about 11% of cell viability; H345 shows a dose-response for the formulation too, the response was almost the same as it was for the cisplatin solution, table 2 shows the results of IC50 calculated, that reflects the response of the cells showed in the graphs.

Figure 5a and 5b represents the cell viability of the NCI-H345 and A549, respectively, when exposed to the cisplatin liposome-Bombesin formulation. As we can see the cell viability is 100% or more, which means that there was no effect of the formulation over the cells. Although an IC50 value was not able to be calculated for the bombesin-liposome formulations it is clear from the trend in the cell viability graphs that the cisplatin did not significantly affect the viability of either cell line. For example, the mean viability of A549 cells at the highest cisplatin concentration was 112% and for H345 199%.

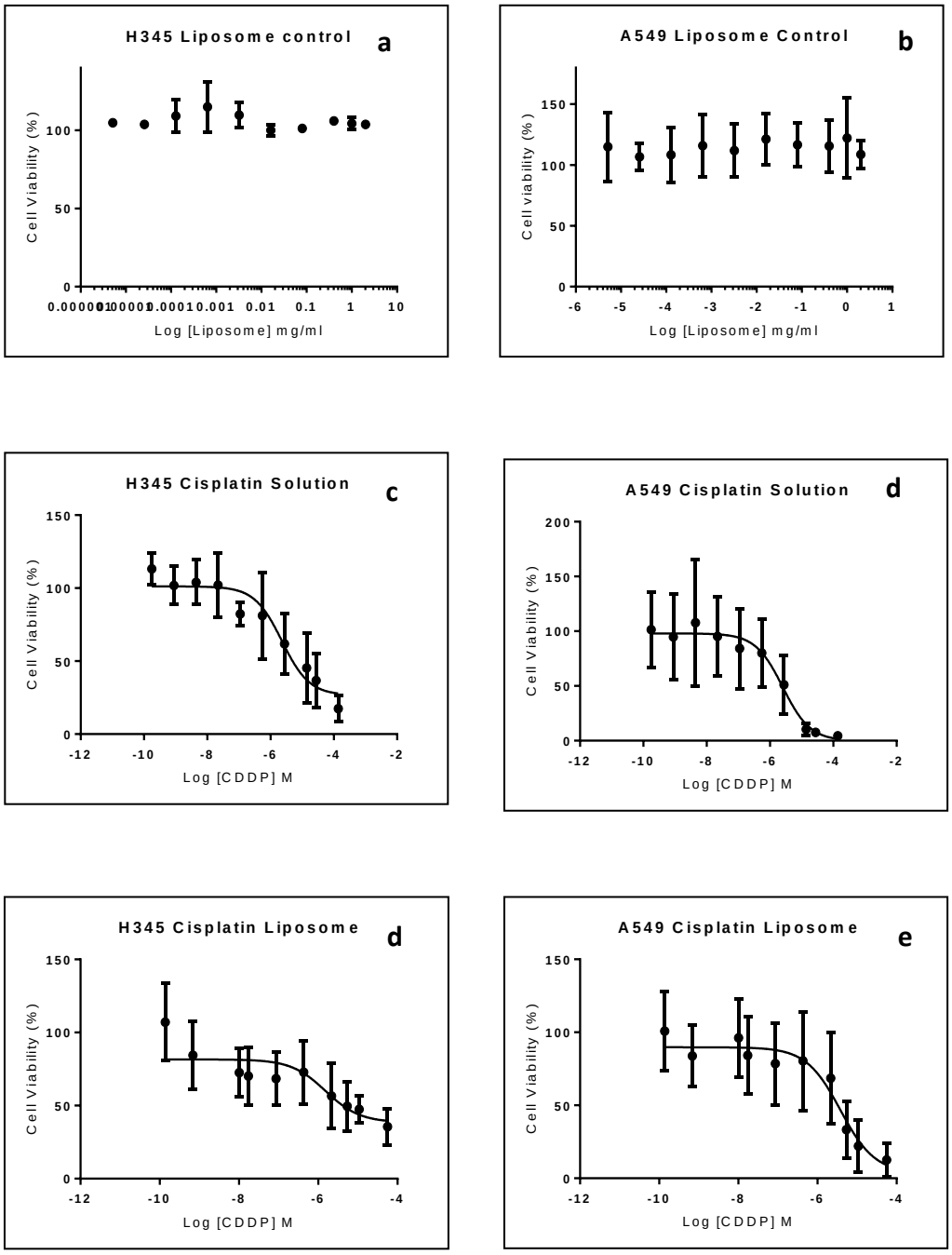



Figure 4– Dose-response of the cells with different treatments. Results for three representatives experiments. GraphPad Prism 5 was used to acquire the data.

**Table 2**
Results of IC50 from three representatives experiments.

| | IC50 Cisplatin Liposome (μM) | IC50 Cisplatin Solution (μM) | IC50 Cisplatin Liposome-Bombesin (μM) |
|---|---|---|---|
| **H345** | 0.00716±0.00055 | 2.71±2.31 | * |
| **A549** | 3.58±2.27 | 2.39±1.34 | * |

*not possible to calculate

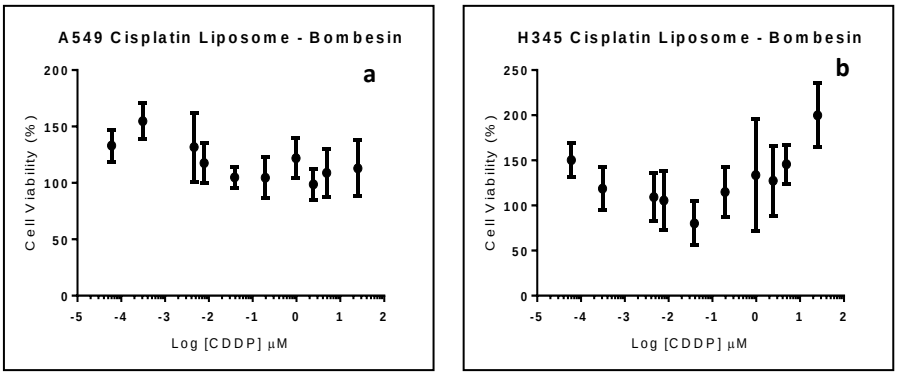



Figure 5–Dose-response of the cells for target liposomes. Result of one representative experiment.

*4.4 Cellular effect following incubation with conventional and Bombesin CF liposomes*

The evaluation of the effectiveness of target and non-target CF liposomes was performed by flow cytometry assay in order to evaluate the uptake of the CF liposomes by NCI-H345 cell, as described on section 2.2.9.

Figure 6 represents the fluorescence intensity measure in the cells; more fluorescence means more liposome uptake. Target liposomes had two different results, with 1 mol% Bombesin we have the higher uptake even at 4 °C

(figure 6b). With 4mol% Bombesin we had the lower uptake (figure 6c). Cells incubated at 4 °C had the lower uptake as it was expected.

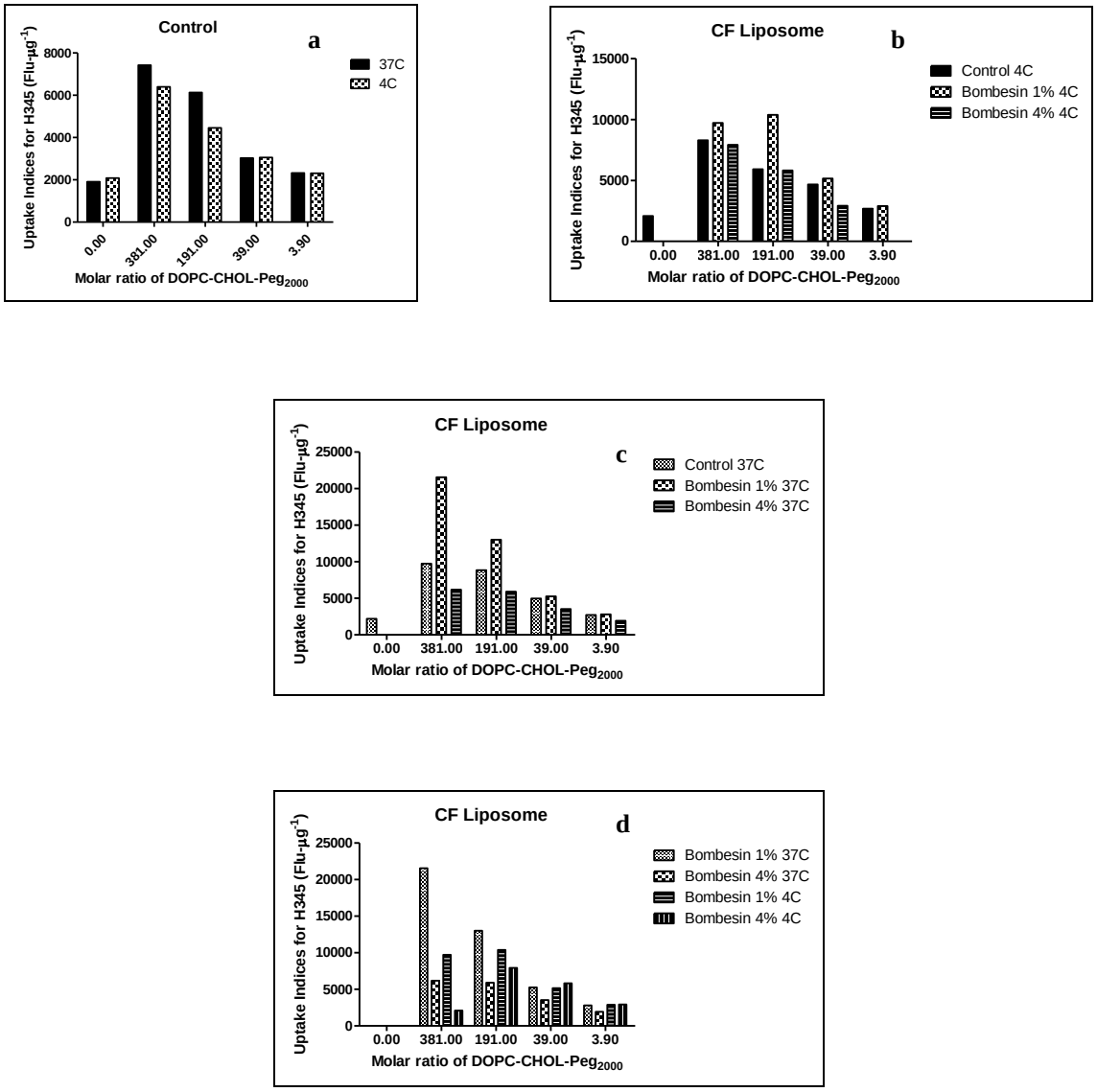



Figure 6– Quantitative observation of effect of different amounts of Bombesin-PEG$_{2000}$-DSPE on the uptake of CF liposomes by NCI-H345.

## 4. Discussion

The characterization of the liposomes showed a monodisperse liposome suspension with a slight negative zeta potential for all the three different formulations. The zeta-potential is almost zero indicating the stability of the suspension. On biological application nanoparticles should be sized between 5

and 150 nm, smaller than 5 nm it will be removed very quickly by renal clearance and bigger than 150 nm can accumulate in the spleen, liver and bone marrow.[29] The mean size of the liposomes, control and cisplatin, that were prepared for the experiment were about 100nm and CF liposomes were about 110 nm, except 4 mol% Bombesin that size was around 329 nm. The detection of a liposomal diameter greater than the pore diameter is common and happens because pegylated liposomes are more rigid structure making more difficult to reduce the size, but they are in the size range used in biomedical application. The Pegylation is a strategy used to reduce the formation of protein corona forming a `brush` that does not permit the proteins coat the liposomes. Other advantage of using Pegylated liposomes is because it has the ability to passively accumulate in tumour tissues through Enhanced Permeability and Retention (EPR) effects, however can lead to another problem that is reduce the extent of interaction with the target cells. [30] To overcome this problem ligands like peptides are added at the terminal ends of PEG moieties to produce active targeting PEG-liposomes.[30] The peptide attached to the liposomes on this study was a Bombesin analogue where an N-terminal cysteine was added to bombesin to enable conjugation to a maleimide functionalised pegylated DSPE lipid. This peptide was chosen because the SCLC expresses the receptor for Bombesin, the GRP. [6] The peptide has previously been shown to target specific-prostate-tumour that overexpresses the GRP receptor.[18] The study shows that if the peptide is conjugated with gold-nanoparticle it can be used as theranostics therapy because the target nanoparticle will accumulate on the specific tissue (prostate). [18]

Synthesis of the bombesin-$Peg_{2000}$-DSPE conjugate was successful as demonstrated by analysing the molecular weight of the final product, that should be around 3983.605 g/mol (bombesin = 1043 PEG2000-DSPEmaleimide = 2940.605) . The MALDI-TOF spectrum shows a range of different molecular weights close to average molecular mass (44 g/mol). The broad range of spectral peaks is due to the presence of a range of polymeric moieties, each separated by ABC Daltons, which represents one ethylene glycol unit. This happens because the $PEG_{2000}$ component of the DSPE-$PEG_{2000}$-maleimide is polymeric and the polymerization reaction is very hard to stop leading to some of the polymers have more molecules of ethylene glycol than others.

The post-insertion method used to fuse the peptide into the liposomes is described in section 3.4. It involves the application of pre-formed liposomes to a thin film of peptide-PEG-DSPE. To achieve the final formulation, some steps have to be performed. When the liposome suspension is added to the Bombesin-$Peg_{2000}$-DSPE film it is expected that the peptide-PEG-lipid will form micelles in the aqueous suspension and that fusion of micelles with the liposomes will produce a colloidal mixture where the targeting peptide resides only on the outer leaflet of the liposome. Immediately after hydration of the film we observed no difference in DLS analysis of the liposomes, which can mean that the micelles didn't incorporate the liposomes immediately. To induce the fusion of the micelles with the liposomes a heating step was performed.[24] After heating to 60 degrees it was possible to see that the liposomal suspension was no longer monodispersed, because not all the micelles had incorporated into the liposomes. To remove any excess of lipid-peptide not encapsulated in the liposomes we dialysed the sample against an isoosmolar buffer for 24 hrs.

DLS results showed that the most abundant size population was similar to the cisplatin-loaded liposomes before hydration of the thin film and so micelle incorporation was presumed to have occurred. For CF liposomes we have similar results when added 1 mol% Bombesin-$Peg_{2000}$-DSPE, but when added 4 mol% Bombesin-$Peg_{2000}$-DSPE there were two populations, even after dialysis and extruding again the liposomes we were not able to reduce the size and have a monodisperse suspension. This could be attributed to the fact that in higher concentration is harder to the peptide attach the liposomes because is more difficult to find space on the surface of the liposomes and is very common have an accumulation of peptide in one side of the liposomes surface. [19] Figure 3f shows the size distribution of the liposomes with big size and having two populations making harder the entrance in the cells because the liposomes are two times bigger than the recommended for biological use and will probably be eliminated by renal clearance or will be recognized by macrophages two things that we should avoid in a formulation. [31]

Dose response cytotoxicity studies were performed on NCI-H345 and A549 cells. The viability of the cells was evaluated by MTS assay after 72 hrs, which is a standard time scale of exposure for anti-cancer drugs.[32] Liposome control, with no encapsulated cisplatin, did not show any toxic effect on the cells while cisplatin solution and cisplatin-loaded liposomes have a dose-dependent response. A549 showed almost the same response to the both formulations, while for the H345 the liposome formulation is more effective, as shown by the lower IC50 on Table 2. However these results are not striking because of the low efficiency of encapsulation of the cisplatin (Table 1) and an optimization on the process needs to be performed. As we can observe in figure 4 the NCI

H345 is more resistance cell line than A549 using the highest concentration of cisplatin there is less than 10% of cell viability and for the NCI H345 using the same concentration we have more than the double of cell viability. It was already been reported that NCI H345 (SCLC) have resistance for the chemotherapy, one of the options to increase the response to the treatment is use multi-drug treatment, but still is not enough to cure the disease. [6, 33, 34] The resistance of the NCI H345 on the chemotherapy treatment is one of the main reasons to develop a target formulation and increase the delivery of the drug inside the cells increasing the cells death.

Bombesin-functionalized liposomes loaded with cisplatin were tested and didn`t show to effect for both cell lines used. It was expected not to have an enhanced effect on A549 cells because the cells do not expresses the GRP receptor which would allow NCI-H345 cells to internalise greater numbers of liposomes through GRP receptor-mediated endocytosis. Effect on the NCI H345 was expected since these cells should express the GRP receptor and hence to be targeted. We speculate that the low encapsulation efficiency of cisplatin in the targeted liposomes explain the poor outcome from the MTS assay. As mentioned above, cisplatin encapsulation efficiency was very low also in the control formulations and needs optimization. For the cisplatin-liposome-Bombesin the encapsulation efficiency decreased by 50% with respect to the control compromising the outcomes from the biological results. However, we cannot rule out that the bombesin-functionalized liposomes are not specifically interacting with the GRP receptor expressed on the cells. If the Bombesin is not being recognized by the GRP receptor the liposomes are not entering inside the cells, and no dose-response effect will be observed. A third hypothesis is that

the NCI H345 cells do not express the GRP receptor in our hands. This is less likely as the over-expression of the GRP-receptor has been reported in the literature. [21]

Flow cytometer simultaneously measure and then analyse multiple physical characteristic of singles particles including size, relative granularity or internal complexity and fluorescence intensity. [31, 35] In this study we use this method to evaluate the uptake of the liposomes by cells measuring the fluorescence intensity.

Since cisplatin liposomes didn`t show goods results we use a fluorescence agent, CF to load the liposomes and investigate enhanced uptake into NCI-H345. As we can see on section 4.4 the results using fluorescence target liposomes were the ones that we expected since the beginning of the work, target liposomes had bigger uptake by the cells than the non-target, control liposomes, as it can be seen on figure 6c. The results showed better uptake when 1 mol% Bombesin was added. It was already been described on literature that the concentration of the peptide on the formulation is a critical point. [19] Two reasons can explain that, one was already been discussed, about the size of liposomes and the other one is that the receptor could saturated blocking the entrance of the liposomes inside the cells. Concentrations lower than 1 mol% and between 1 and 4 mol% should be tested to determinate the ideal concentration of the peptide to be attached on liposomes, use different concentration of peptide will also show if the bad results using 4 mol% Bombesin was the saturation of the receptor or it was the formulation that should be optimized.

Comparing percentage of liposomes added and temperature we could see that 1 mol% Bombesin at 37 $^{o}$C have the higher uptake different from the results when liposomes were load with cisplatin. Target cisplatin-liposomes didn`t showed good results when it was tested on the cells and we suggest that the cells were not expressing the GRP receptor, but after analyse results obtained using CF liposomes we could visualized that the target liposomes worked. This results gives more support to the hypothesis that the target cisplatin liposomes didn`t worked because of the lower encapsulation of the drug. Next steps of the work should be optimize the encapsulation of cisplatin, or use another drug, and optimize the concentration of $Peg_{2000}$-DSPE-Bombesin added to the liposome. In the present work others concentrations were not tested because the amount of peptide that we had was not enough. Experiments using scrambled peptide should also be done as well to evaluate the specificity of the peptide that we are using.

## 5. Conclusion

Liposomes were prepared and used on the experiments performed on the cells. The encapsulation of the cisplatin inside the liposomes presents to be a problem, having a lower percentage of encapsulation especially with cisplatin liposome-Bombesin. The lower encapsulation of the cisplatin reflect on the results of the cell viability assays. Fluorescence liposomes were prepared to investigate the uptake by the receptor-positive cells and evaluate if target liposomes have more uptake than non-target. Experiments were successfully executed with 1 mol% Bombesin liposome having the best uptake. The next step is try add different concentrations of Bombesin on the formulation to find the ideal concentration and optimize cisplatin load inside liposomes.

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Numbering and lettering of figures should be carefully accepted in order to ensure readability after photographic reduction of the figure. Use only standard symbols to mark datapoints. Explanation of curves and symbols should be in the legend rather than part of the drawing, unless this is detrimental to clarity. Indicate SI units of measure in parentheses.

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***References***

References must be arranged as follows:

[1] A.– L. Cornaz, P. Buri, Nasal mucosa as an absorption barrier, Eur. J. Pharm. Biopharm. 40 (1994) 261– 270.

[5] C. Lanczos, Applied Analysis, Prentice-Hall, Englewood Cliffs, NJ, 1967, pp. 272– 280.

[10] D.M. Barends, Stability of active ingredients, in: H. Mü ller, W.H. Oeser (Eds.), Drug Master Files, Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, Germany, 1992, pp. 121– 128.

[14] E.A. Balazs, Ultrapure hyaluronic acid and the use thereof, U.S. Patent 4,141,973 (1979).

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