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Development of targeted nanocapsules against breast cancer cells.

by

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Abstract

Breast cancer is now the most diagnosed cancer worldwide, having surpassed lung cancer diagnosis as stated by American Society of Clinical Oncology. Chemotherapy comes with many adverse effects that can include hair loss, depression, endometriosis or heart problems or memory loss. Functionalized nanocarriers with different ligands such as antibodies for cancer treatment has been proposed and studied to improve drug delivery efficiency, especially with liposome vesicles, which present many advantages such as great biocompatibility and biodegradability.

This project has successfully synthesized 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) nanocapsules functionalized with Immunoglobulin G and encapsulated cytotoxic drugs in order to assess their effectiveness in future cell viability studies.

Liposomes were prepared following the lipid film hydration protocol. Three drugs were encapsulated in these steps: 1,4-Phenylenebis(methylene)selenocyanate (80 µM), Gemcitabine (5 µM) and Doxorubicin (2.5 µM) for five different treatments and a final lipid concentration of 15 mM. The vesicles were downsized with an ultrasonic probe sonicator.

In order to functionalize the liposomes, 3 strategies were tested: a direct addition of IgG to de liposomes, using EDAC and NHS and finally the PEGylation of liposomes' suspensions were centrifuged in order to eliminate free drugs and IgG.

Dynamic Light Scattering showed an homogeneous size distribution of 29.07 nm in average and a polydispersity index of 0.206 and cryo-TEM imaging allowed to visualize that most of the liposomes were small unilamellar vesicles.

By analyzing the thermodynamic properties of empty liposomes and the three functionalization strategies by Differential Scanning Calorimetry technique, it has been proved that the three methods for functionalization were effective by observing an increase in the liposomes Tm and a decrease in enthalpy.

Finally, the presence of IgG in the liposomes' membrane was confirmed by means of ELISA tests.

Acknowledgements

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Finally, I would like to thank my family for their constant support every long day and every sleepless night, and for being by my side in each of the decisions that have brought me this far.

Summary

Abbreviation Index

ADC	Antibody Drug Conjugate
α-IgG	Anti Immunoglobulin G
***cryo*-TEM**	*Cryo*-Transmission Electron Microscopy
DLS	Dynamic Light Scattering
DOX	Doxorubicin
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DSC	Differential Scanning Calorimetry
EDAC	1-etil-3-(3-dimetilaminopropil) carbodiimide
ELISA	Enzyme-Linked Immunosorbent Assay
EPR	Enhanced Permeability and Retention
GEM	Gemcitabine
HPLC	High-Performance Liquid Chromatography
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IgG	Immunoglobulin G
MWCO	Molecular Weight Cut-Off
NHS	N-Hydroxysuccinimide
NPs	Nanoparticles
PBS	Phosphate-buffered saline
PEG	Polyethileneglycol
***p*-XSC**	1,4-phenylenebis(methylene)selenocyanate
Tm	Main Transition Temperature
Tp	Pre-Transition Temperature

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1. Introduction

1.1. Breast Cancer

Breast cancer is not only the most occurring cancer in women but is now the most diagnosed cancer worldwide, having surpassed lung cancer diagnosis as stated by American Society of Clinical Oncology (ASCO).[1] More than 2.26 million new cases of breast cancer in women were diagnosed in 2020.[2] In Europe, it represents the 13% of all the cancer types, making it the most occurring cancer type. That same year, breast cancer was responsible for 91,826 deaths and the European Commission declared it the first cause of cancer death among women in Europe.[3]

There are several ways to classify breast cancer types based on the type of cell that turned into a tumor, its size, how much it has spread or the type of receptors that it possesses.[4] It can be non-invasive, meaning that the tumor has not spread beyond the breast tissue where it originated; invasive, when it spreads across surrounding breast cells; metastatic, a kind of invasive cancer that spreads to other parts of the body like lungs, bones, liver or brain. Breast cancer can also be recurrent, appearing sometime after treatment in the breast or in another part of the body.[4,5]

Regarding the type of tissue that holds the tumor it can be distinguished between[4–7]:

- Ductal carcinoma: This is the most common type of breast cancer (80%), and it starts at the ducts that transport milk towards the nipple.
- Lobular carcinoma: It is the second most common type (10%), and it is more often found in both breasts than other types of breast cancer. It starts at the glands that produce milk.
- Inflammatory breast cancer: Although rare, it can occur when cancer cells block lymph vessels in the skin.
- Much less common are Paget's disease, which starts at the ducts and spreads towards de areola; Angiosarcoma, in blood or lymph vessels or Phyllodes tumor which develop in the connective tissue of the breast.

Breast cancer can also be classified depending on the type of tumor found in the tissue. The 3 most common classes are:[8]

- Hormone receptor positive: 33% of tumors express estrogen (ER positive) and/or progesterone (PR positive). This type of cancer depends on these hormones to grow and are more frequent after menopause.
- HER2 positive: These types of cells depend on the human epidermal growth factor receptor 2 (HER2*)* gene to grow and comprise 15-20% of breast cancers. HER2 positive tumors grow more quickly, and they can also be hormone receptor positive at the same time.
- Triple negative: A triple negative cancer does not express ER, PR or HER2. 10% to 20% of breast cancers are triple negative and it is often related to a mutation in the *BRCA1* gene. It is more common in young adults and particularly Black and Hispanic women.

1.1.1. Challenges of Breast Cancer

Depending on the kind of breast cancer and the extent to which it has spread, it is treated one way or another. The common treatments for all breast cancers are surgery, chemotherapy, hormonal therapy, and radiation. Patients often get more than one treatment, the combination of which is personalized to adjust to the person's specific situation.[9] For instance, it is common practice for nonmetastatic cancers to be treated with surgical methods in combination with radiation therapy to prevent recurrence.[10] Metastatic breast cancer is incurable as for today and the current objectives are life prolonging and symptom palliation.[10,11]

Surgery is a type of local therapy where cancer tissue is cut out by operating the tumor out of the breast (lumpectomy), removing axillary lymph nodes, or removing the breast altogether (mastectomy) followed by a reconstruction of the breast. These procedures have the risk of hematoma, infections, skin necrosis, injury to blood vessels or nerve injury, which can lead to sensory and motor defects.[12]

As mentioned above, radiation therapy is used in tandem with surgery, and it has been performed to treat breast cancer since 1895. In some cases, this combination of therapies decreases the recurrence and mortality caused by breast cancer.[13] Sometimes surgical methods are not the right approach as a treatment, depending on the cancer, and patients require chemotherapy, which uses cytotoxic agents to kill or prevent the growth of cancer cells in the form of pills, intravenous doses or both, as well as hormonal therapy using modulating agents to inhibit the synthesis of progesterone, estrogen or other

hormones.[10,13] These strategies may help decrease the tumors before surgery, but they come with several adverse effects (Figure 1) that go from change of body weight or fatigue to more serious problems such as endometrial cancer or heart problems.[14]

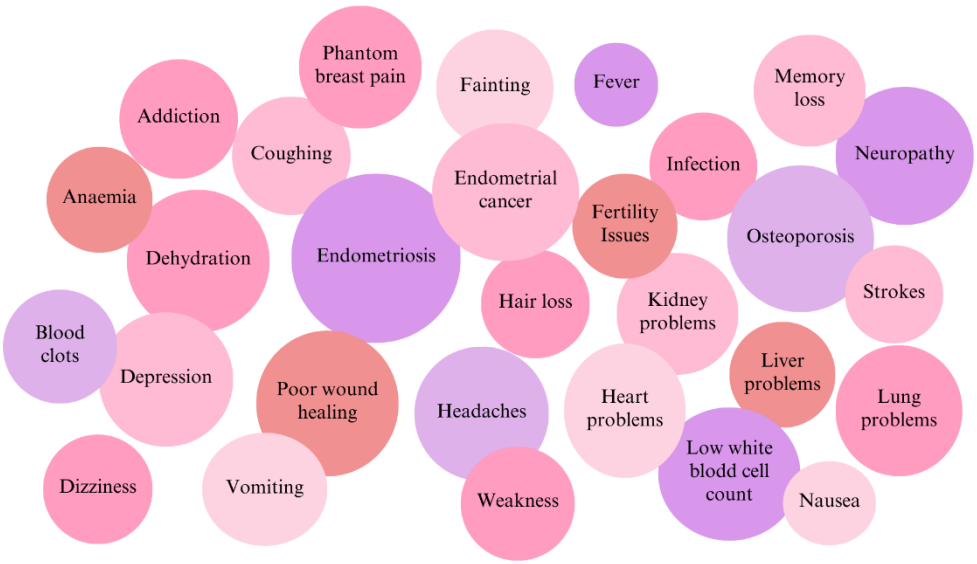

Figure 1. Collection of the most severe effects of traditional breast cancer therapies.

1.2. Targeted Therapies: The "Magic Bullet".

Chemotherapy agents need to penetrate different barriers (e.g., skin, gastrointestinal tract, or endothelial system) in order to reach the tumor and do so at a concentration able to kill it.[15] Because of the non-selective activity on non-tumoral cells, chemotherapy agents often cause multiple of the secondary effects mentioned above.[16] In order to minimize the damage to healthy cells, improve the therapy's effectiveness and decrease the patient's pain in the process, targeted therapies started to become a promising line of study.

The concept was introduced by Nobel prize Paul Ehrlich back in 1907, who assured that it was "possible to kill a specific microbe in the body without harming the body itself", with a "magic bullet".[17] Breast cancer targeted therapies use drugs specifically designed to target receptors that tumors overexpress and disrupt the signaling responsible for cancer growth, division, proliferation and other cell cycle pathways (Figure 2).[18]

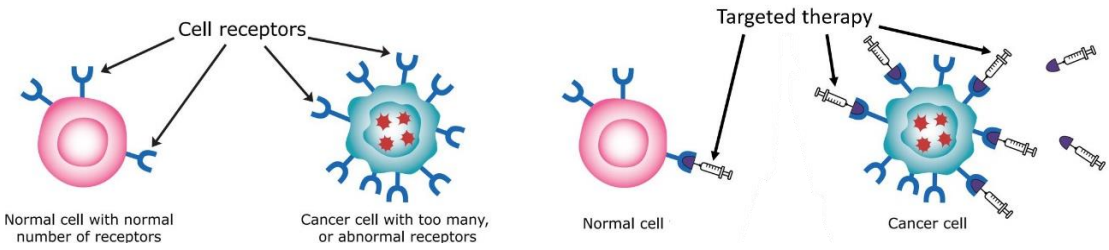

Figure 2. Schematic representation of targeted therapies' mechanism of action. Credit: Facing Our Risk of Cancer Empowered, 2023[19]

The two most common types of targeted therapies are[20]:

Small-molecule drugs. They are typically used when the target is inside the cell, seizing its small size to easily penetrate the cell. Small molecule inhibitors interfere with the intracellular signaling of tyrosine kinases, like EGFR, HER2/neu, and VEGF receptors, but because of their short half-lives (in the order of hours), daily dosing is required to see results.[21] Although more specific than chemotherapy agents, molecular inhibitors are not as efficient and become ineffective after a use time period, presenting the challenge of drug resistance.[22] Strategies to overcome this problem involve the synthesis of multitarget anti-cancer drugs[23], new generation drugs against resistance mutations[24] or combination therapies.[25]

Monoclonal antibodies. Antibodies can work in many ways: as drugs, blocking a specific antigen outside the cancer cell stopping its proliferation or inducing apoptosis; carrying small drugs (ADC, antibody-drug conjugate), radiation or natural killer cells. They can also be used to help the immune system signaling white cells to fight against the cancer cells. This practice is called immunotherapy.[26–28]

Although these types of targeted therapies have shown an improvement in patient's recovery and overall survival[29], they may cause severe side effects, like in the case of monoclonal antibodies which can cause the apparition of autoimmune problems[30], and their effectiveness is still hindered by cells' drug resistance and its rapid degradation even before reaching the target.[31]

1.2.1. Drug Carriers or Nano vectors

Nanocarriers for cancer treatment has been proposed to improve drug delivery efficiency, minimize dose quantity and decrease side effects.[32] They have shown many advantages in comparison with conventional treatments, such as the ability to penetrate biological barriers, the protection they offer to therapeutic drugs against degradation, the ability to

transport a certain concentration of multiple drugs towards a target and their specific accumulation on tumors by enhanced permeability and retention (EPR) effect.[33–36]

These nanoparticles (NPs) have different surface characteristics, shapes and sizes that will influence the efficacy of the drug delivery system. Usually, the size range goes from 10 to 100 nm so they can achieve EPR more easily. Lower than 10 nm can leak outside and damage normal cells and higher than 100 nm are more likely to be phagocyted.[15,37]

There are different types of nanocarrier systems, typically classified as organic or inorganic. Singh *et.al*[38] categorized them in three classes (Figure 3): Lipid-based carriers, including nano-emulsions, solid lipid nanoparticles, phospholipid micelles and liposomes; inorganic nanoparticles like iron oxide and gold NPs or nanotubes and single-layer graphene; and polymeric nanoparticles, such as Poly (lactic-co-glycolic acid (PLGA)[39], methoxy-polyethylene glycol–poly (glutamic acid) (mPEG–PGA)[40], PEG-poly(ω-pentadecalactone-co-N-methyldiethyleneaminesebacate-co-2,2' thiodiethylene sebacate (PEG-PMT)[41] and poly (D,L-lactide-co-glycolide)-polyethyleneimine (PEI-PLGA)[42] each with different properties, advantages and disadvantages.

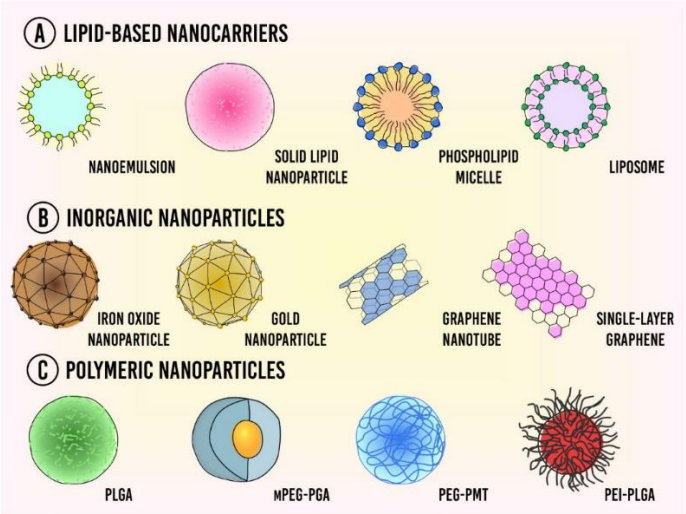

Figure 3. Different types of nanocarriers used to encapsulate drugs. Credit Lôvo et.al. 2021[43]

1.3. Liposomes

Liposomes are nanoparticles that present many advantages with respect to other drug delivery systems: their main characteristic is their biocompatibility and biodegradability, being widely used as cell membrane models to study biological systems.[44–46] Furthermore, they present less side effects, longer drug circulation time and they enhance

the drug permeability and retention in tumor tissues.[47,48] Apart from drugs, lipid vesicles are also good carriers for enzimes[49] and nucleic acids like DNA[50,51] or mRNA.[51]

1.3.1. Classification

Liposomes are formed by phospholipids (usually phosphatidylcholines) with varying degrees of stability and rigidity determined by their phase transition temperature (Tm) which depends on chain length and degree of unsaturation. They are composed of a polar head group oriented towards the aqueous interior of the vesicle and the external medium, and a hydrocarbon chain that forms the lipidic bilayer[52,53] (Figure 4-A).

They are classified (Figure 4-B) by both their size and lamellarity. If they are made of one lipidic bilayer they can be: small unilamellar vesicles (SUVs) of less than 50 nm, large unilamellar vesicles (LUVs) of higher than 50 nm and giant unilamellar vesicles (GUVs) of sizes between 10 and 100 µm. When they are composed of more than one bilayer, they can be classified as large multilamellar vesicles (MLV), formed by concentric bilayers of phospholipid of sizes between 2 and 4 µm and multi-vesicular vesicles (MVV) of sizes from 0.5 to 5 µm.[53–55]

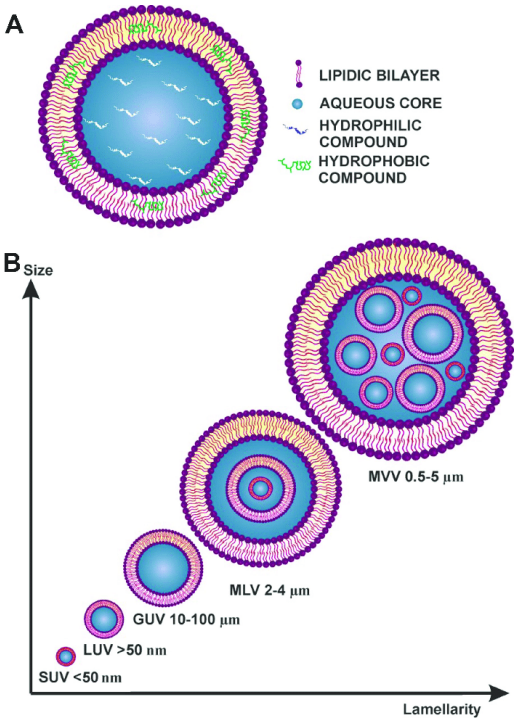

Figure 4. Graphic representation of liposome composition (A); Classification of liposomes by increasing size and lamellarity (B). Credit[55]

1.3.2. Liposomes Preparation Methods

Liposomes preparation methods typically consist of two steps. First, the formation of the vesicles for which there are 3 main strategies: thin film hydration,[56] detergent removal,[57] and reverse phase evaporation method.[58]

Thin Film Hydration: Phospholipids are dissolved in an organic solvent, evaporated over the Tm to form a film layer that will be hydrated with a buffer swelling the liposomes into MLVs.

Detergent Removal: Lipids are solubilized using a detergent solution to form micelles. The successive removal of the detergent forms unilamellar vesicles.

Reverse Phase Evaporation: Lipids are dissolved in an organic solvent to form inverted micelles and an aqueous solution is added to create a microemulsion. The organic solvent is evaporated to promote the formation of LUVs.

Secondly, the formed vesicles are downsized and processed to form smaller unilamellar vesicles from bigger and multilamellar with one of 3 different methods: sonication, with bath and probe techniques; extrusion, with pore containing membranes of the desired size, and high-pressure homogenization method, where the liposomes suspension is injected with a high-pressure flow to make the particles to collide with a metal wall.[59–61]

1.4. Functionalization of Liposomes

Treatments with encapsulated drugs in liposomes are not always effective because of different factors like low drug release or poor cellular uptake.[62] In order to actively target tumor cells with liposomes, their surface is functionalized with different ligands to recognize specific antigens that tumors overexpress. [63]

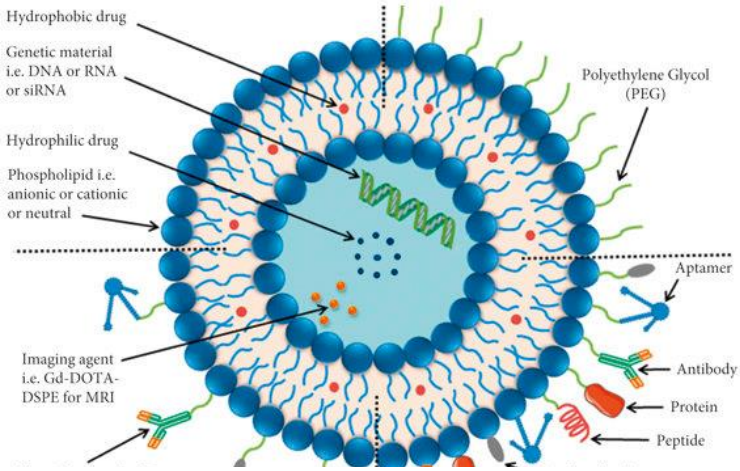

Figure 5. Schematic representation of the different ligands that can be coupled to an antibody. Credit[64]

In Figure 5 it can be seen that liposomes can be functionalized with peptides, which can have different functions like cell-penetrating peptides (CPP) and cell-targeting peptides (CTP),[64] with aptamers (small single stranded DNA or RNA sequences) that have been tested to have good anti-proliferating activity in breast cancer cells,[65] small molecules like folate[66] or with antibodies or its fragments to form immunoliposomes. Because of their high specific recognition to a wide variety of antigens in cancer cells, immunoliposomes are promising formulations that are being studied for cancer treatment. However, they present several challenges that difficult clinical applications such as highly variable and unpredictable EPR effect and internal stimuli effects on the vesicles which are difficult to monitor and control.[67–71]

1.5. Characterization Techniques

1.5.1. Microscopy

There are three microscopy techniques widely used for liposome characterization: Fluorescence microscopy, to visualize vesicles alone and their interaction with cellular systems and atomic force microscopy, which is able to measure size distribution, morphology and stability and *cryo*-Transmission Electron Microscopy (*cryo*-TEM). *Cryo*-TEM is a variation of TEM in which a solution is vitrified into a thin aqueous film using a cooling medium such as liquid nitrogen. This is one of the best and most utilized ways to visualize liposomes' lamellarity, size and shape. This technique avoids the need of chemical fixation, dehydration, or other sample preparation methods of conventional TEM that can modify the morphology of vesicles.[72,73]

1.5.2. Dynamic Light Scattering

Dynamic Light Scattering (DLS) technique is used to determine the size distribution profile of particles in suspension or solution typically in the sub-micron range. The sample is illuminated with a laser beam and because of the Brownian motion of the particles, the fluctuation of scattered light is detected. It gives information about the hydrodynamic radius of the liposomes as well as the polydispersity.[74]

1.5.3. Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) is a thermal analysis technique which measures the heat flow of a sample as a function of temperature or time in relation to a reference cell when heated at the same time. When the sample suffers a physical alteration, like a phase transition, the heat needs to flow to maintain the same temperature, represented in a thermogram as endothermic or exothermic.[75] It gives information about glass transition and melting temperatures, crystallization, specific heat capacity among others.[76]

For liposomes, each peak corresponds to a phase transition temperature typically the main transition (Tm) from the gel phase to the fluid phase, which corresponds to the highest heat flow. In most cases a pre-transition temperature (Tp) can be seen at lower temperatures, corresponding to the change from the gel phase to an intermediate rippled

state. It is not always observable since it is very sensitive to sample preparation and impurities[77] (Figure 6). These transitions are typically called Lβ' →Pβ' → Lα.[78]

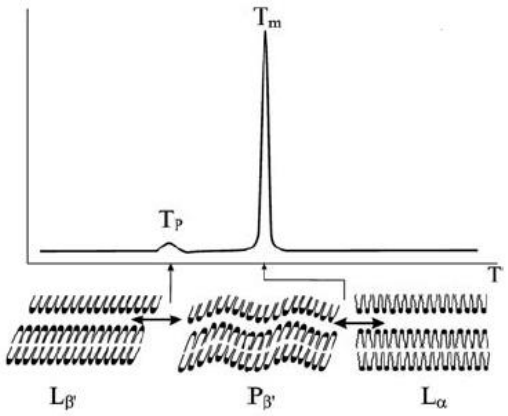

Figure 6. Thermogram representation of lipids' pre-transition temperature from gel to ripple state and the main transition from gel to liquid. Credit [78]

DSC is a useful technique to determine the effects of the interactions between liposomes and peptides. Different kinds of information can be extracted from a thermogram when analyzing lipid-protein systems. Firstly, changes in Tm indicate that a peptide has changed the physicochemical properties of the liposomes' membrane. A decrease in Tm corresponds to a favored transition and a fluidizing effect because of the presence of the peptide. On the other hand, changes in the peak area correspond to changes in the enthalpy (ΔH) of system's transition. When ΔH decreases, is an indicator of a change in the liposome caused by the peptide.

Lastly, differences between peaks' shapes are related to homogeneity: normally broader transition peaks are related to peptide-rich domains.[79]

1.5.4. Enzyme-Linked Immunosorbent Assay

Enzyme-Linked Immunosorbent Assay (ELISA) is a plate-base technique in which a plate is coated with the antigen of interest, which in the case of immunoliposomes it can be the antibody on the membrane, or the molecules encapsulated. Then a secondary antibody (α-Ab) that recognizes that antigen is incubated with the proteins so there is a binding between each other. The α-Ab is tagged with an enzyme that upon recognition will provoke a color change indicating the positive presence of antigen. Figure 7 represents the four types of ELISA tests: direct, indirect, sandwich and competitive assays.[80]

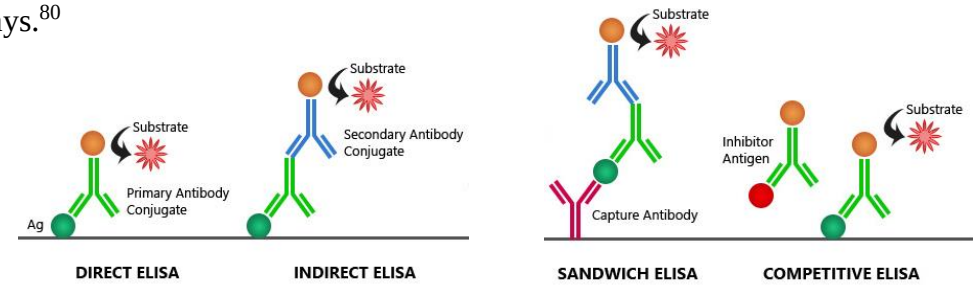

Figure 7. Simple representation of the four types of ELISA tests: direct, indirect, sandwich and competitive. Credit [80]

2. Objectives

The two main objectives of this work are:

1. Synthesize and functionalize 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) nanocapsules with Immunoglobulin G (IgG) in order to be targeted against breast cancer cells.
2. Encapsulate cytotoxic drugs in the vesicles and study their encapsulation efficiency to assess their effectiveness in future cell viability studies.

This will be achieved by pursuing the following specific objectives:

1. Develop DPPC–based vesicles and load them with five different treatments via dry-film hydration protocol.
2. Optimize the sonication conditions for the successful formation of SUVs.
3. Characterize the nanoparticles defining their size and morphology via Dynamic Light Scattering and *cryo*-Transmission electron microscopy.
4. Evaluate the encapsulation efficiency with time of *p*-XSC drug via inductively coupled plasma mass spectrometry during 4 weeks.
5. Test three different functionalization strategies with IgG found in bibliography.
6. Confirm the presence of IgG in the liposomes' membrane by means of Enzyme-Linked Immunosorbent Assay tests.

3. Experimental Procedure

3.1. Materials and Equipment

The phospholipid used was 1,2-dipalmitoyl-sn-glycero-3-phosphocholine 16:0 (**DPPC**) (Avanti Polar Lipids, Inc., USA, CAS 63-89-8) and the solvents were Chloroform (**$CHCl_3$**) (Merck Millipore, KGaA Germany, HPLC grade), Methanol (**MeOH**) (Scharlab, Spain, HPLC grade) and Phosphate-buffered saline (**PBS**) buffer prepared with $Na_2HPO_4 \cdot 2H_2O$ (10 mM), KH_2PO_4 (1.8 mM), NaCl (137 mM), and KCl (2.7 mM).

The reagents for the synthesis of immunoliposomes consisted of 1-etil-3-(3-dimetilaminopropil) carbodiimide (**EDAC**) (Merk Millipore, KGaA Germany, CAS 25952-53-8), N-Hydroxysuccinimide (**NHS**) (Merk Millipore, KGaA Germany, CAS 6066-82-6), O-(2-Aminoethyl)-O′-(2-carboxyethyl) polyethylene glycol (**PEG-3000**) (Merk Millipore, KGaA Germany, CAS 187848-66-4) and Immunoglobulin G Monoclonal Rabbit (**IgG)** (Origene, RA032S).

The drugs encapsulated were 1,4-phenylenebis(methylene)selenocyanate (***p*-XSC**) (CAS 85539-83-9), Gemcitabine **(GEM)** (CAS 95058-81-4) and Doxorubicine **(DOX)** (CAS 23214-92-8)

The equipment needed was a **rotatory evaporator** (Heidolph Instruments GmbH & CO., Laborota 4001)**, centrifuge** (REMI Elektrotechnik LTD, India, NEYA 16R), **centrifugal concentrator tubes** (Vivaspin 20, MWCO 300 kDa, Merk Millipore, KGaA Germany), **ultrasonic bath** and **ultrasonic probe sonicator** (Branson Digital Sonifier 250).

For the performance of ELISA tests, the reactants used were **rabbit monoclonal IgG** (H+L chain), **goat polyclonal antibody** (secondary antibody), **powder milk** (Central Lechera Asturiana), Horseradish Peroxidase (**HRP**) (Origene, R1364HRP), 3,3', 5,5-tetramethylbenzidine chromogen solution (**TMB**) (ThermoFisher Scientific Inc. Life Technologies, 002023) and HCl, 0.1 M as **TMB stopper solution**.

The necessary equipment consisted of **immuno-plates** (Thermo Fisher Scientific Inc., Maxisorp, 442404), **microplate shaker** (VWR International, LLC., 12620-928), **thermo-shaker** (Grant Instruments, PHMP-4) and a **microplate absorbance reader** (Bio-Rad Laboratories, Inc., iMark, 1681130) with the software Microplate Manager 6 BioRad.

3.2. Synthesis of Nanocapsules

Liposomes were prepared following the lipid film hydration protocol: a stock solution of DPPC 100 mM (Figure 7-A) was prepared with cooled chloroform and added to a round bottom flask. A cold mixture of $CHCl_3$:MeOH (2:1) was added (7 mL) before evaporating the solvent in a rotatory evaporator for 45 minutes at 45ºC, which is above the phase transition temperature of DPPC (Tm= 41ºC). The lipid film was slowly formed at the bottom of the flask in multiple layers of lipid as the solvent evaporated.

The film was hydrated using PBS buffer (pH= 7.4) to reach a final concentration of liposomes of 15mM by sonication in an ultrasonic bath for 15 minutes at 45ºC to form the lipid vesicles (Figure 7-B) and do so in their fluid phase (Figure 7-C).

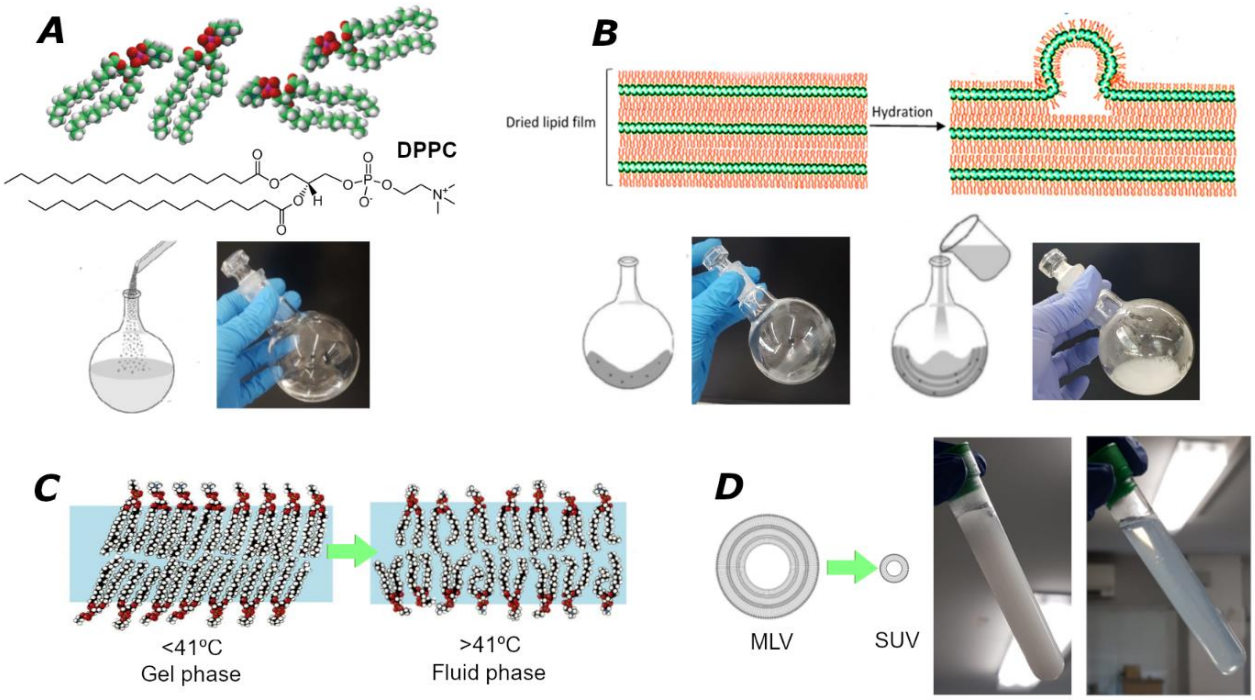

Figure 8. Schematic representation of the structure of DPPC (A), the vesicle formation process by film hydration (B), the state transition temperature of DPPC (C) and the process of downsizing multilamellar vesicles to small unilamellar vesicles (D).

After sonication, the vesicles obtained were downsized from large multilamellar vesicles to small unilamellar vesicles (Figure 7-D) using an ultrasonic probe sonicator for a total sonication time of 8 minutes with an amplitude of 40%. To minimize overheating, on/off sonication was performed in intervals of 20 seconds and submerging the liposomes solution in an ice bath for the first 5 minutes.

3.3. Drugs encapsulation

The process of encapsulation of drugs inside the liposomes, can occur in the film layer formation step of the nanocapsules or in the film hydration step depending on the hydrophobicity of the drug. Five different treatments (Table 1) were obtained using 3 different drugs (Figure 8): 1,4-phenylenebis(methylene) selenocyanate (*p*-XSC)[81], a selenium-based drug which has proven to be an effective chemo-preventive agent, was introduced with DPPC in the film layer formation step and Doxorubicin (DOX) and Gemcitabine (GEM), typically used as chemotherapy drugs against breast cancer[82,83], were and added in the hydration step. Concentrations have been selected from previous studies of the research group.

Table 1. Summary of the five different treatments encapsulated and their final concentration.

	Treatment	Concentration
1	*p*-XSC	80 µM
2	GEM	5 µM
3	DOX	2.5 µM
4	*p*-XSC + GEM	80 µM, 5 µM
5	*p*-XSC + DOX	80 µM, 2.5 µM

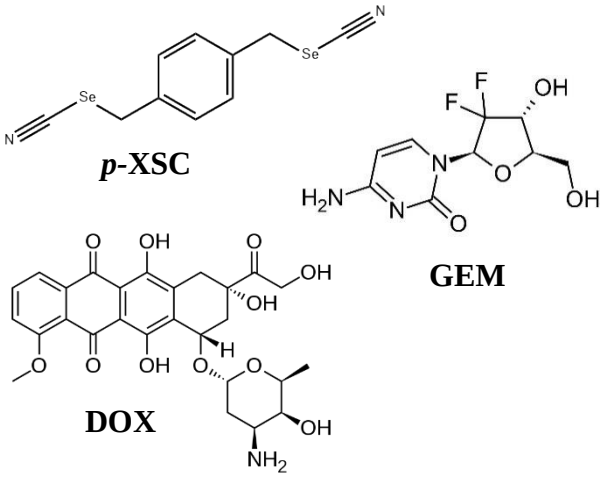

Figure 9. Structure of the three different drugs: p-XSC, GEM and DOX

3.4. Synthesis of Immunoliposomes

The immunoglobulin of interest to target liposomes against breast cancer cells is anti-EpCAM, an antibody used for the recognition of the epithelial cell adhesion molecule. In this work, rabbit monoclonal IgG was used as a more economical alternative for the research study to test the different functionalization reactions.

In order to obtain the immunoliposomes, 3 strategies were tested: direct addition of IgG to the liposomes; using EDAC and NHS, a condensing reagent and an additive used in the conjugation of peptides[84] and finally the PEGylation of liposomes, which is a spacer link procedure typically used in liposome targeting.

The molar ratios of the reactants (Table 2) were taken from bibliography[84–86] and kept constant in all the experiments. The functionalization reactions were incubated for 2 hours at 25 ºC with magnetic stirring.

Table 2. Molar ratios (mol) of the reactants for the functionalization of liposomes with IgG

	DPPC	EDAC	NHS	IgG	PEG
Strategy 1	602	0	0	1	0
Strategy 2	602	700	700	1	0
Strategy 3	602	700	700	1	1

In order to eliminate not encapsulated drugs and free IgG, the liposome suspensions were centrifuged for 2 hours at 9000 rpm using centrifugal concentrators of a molecular weight cut-off (MWCO) of 300 kDa, in order to recollect the free reagents in the filtrate and recuperate the liposomes from the membrane. The vesicles were washed twice with PBS and resuspended to the desired concentration.

3.5. Characterization

The size distribution profiles, and polydispersity of the vesicles were determined after sonicating for 15 minutes above 41ºC by DLS and their morphology was characterized by *cryo*-TEM.

Changes in phase transition temperatures were monitored before and after functionalization using DSC.

3.6. ELISA Tests

To confirm the presence of antibodies in the liposomes an indirect ELISA was performed. ELISA is a type of colorimetric immunoassay that is preferred when analyzing liposomes, as they can interfere with other types of protein assays. The principle of this study is to detect IgG bound to liposomes by recognition of this protein with an anti-IgG secondary antibody. Liposome suspensions were sonicated in an ultrasonic bath for 15 minutes at 45 ºC before starting the ELISA protocol which was as follows:

Step 1. Coating: Plate's wells (Figure 10) were coated with 200 µL of non-targeted liposomes for negative control (represented as DPPC (-) in Figure 10), liposomes suspensions of the three different functionalization strategies (represented as Strategy 1 to 3 in Figure 10) and IgG for the positive control (represented as IgG (+) in Figure 10). Consecutive dilutions of 1/5 (Figure 10, blue arrows) were done to represent ELISA's sigmoidal curve. The plates were incubated for 2 hours or 12 hours at 25 ºC at 300 rpm.

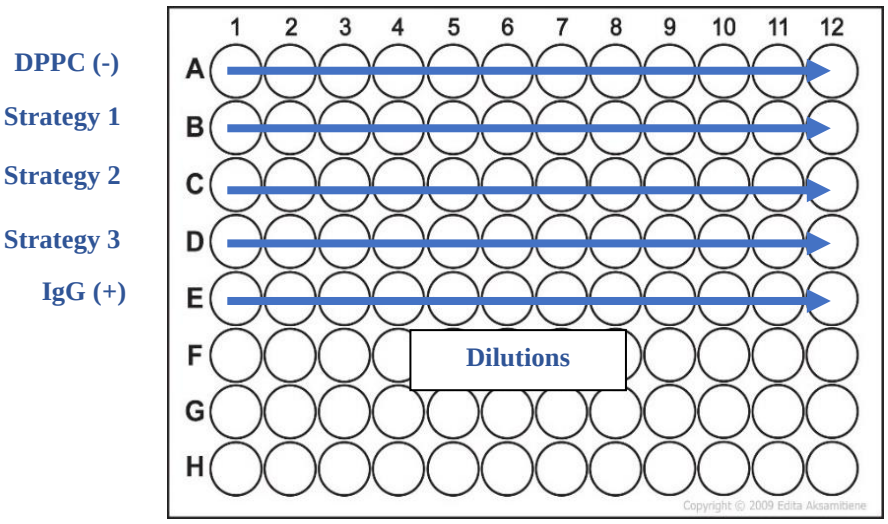

Figure 10. Schematic representation of the ELISA microplates used in the protocol.

Step 2. Washing: After those times, the plates were washed 3 times. with 200 µL of PBS with agitation for 30 seconds at 300 rpm.

Step 3. Blocking: After the coating step it is important to wash the wells and block those gaps in the plate to prevent the adhesion of secondary antibody and result in false positives in the readout. The blocking step was performed with 200 µL of 2% milk solution in PBS and left incubated for 1 hour at 25 ºC at 300 rpm.

Step 4. Binding: The wells then were coated with 100 µL the secondary antibody (1mg/mL) containing horseradish peroxidase (HRP) enzyme at two different dilutions: 1:10,000 and 1:5000 in PBS and left incubating for 1 hour at 25ºC at 300 rpm.

Step 5. Washing: The wells were thoroughly washed as described in Step 2.

Step 6. Oxidation reaction: 100 µL of TMB chromogen solution was added and kept in the dark for 30 minutes at room temperature without agitation. In the case of antibody-antigen recognition, HRP oxidizes by enzymatic action the TMB substrate (colorless) to a blue product (Figure 11).

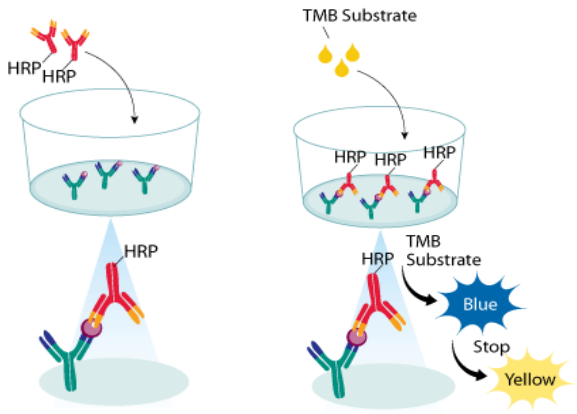

Figure 11. Schematic representation of HRP enzymatic action to TMB substrate. Credit R&D Systems

Step 7. Reaction stopping: TMB reaction is stopped with 100 µL of HCl 0.1 M.

Step 8. Measuring: The absorbance of the wells was measured at 450 nm with microplate absorbance reader and the software Microplate Manager 6 BioRad.

3.7. Encapsulation Study

The evolution of *p*-XSC release was monitored centrifuging the liposomes dispersion every week for 1 month and analyzing the content of drug in the filtrate using ICP-MS for the detection of ^{78}Se isotope.

The encapsulation efficiency is defined as the concentration of drug inside the liposomes over the initial concentration used to make the formulation. It was calculated using equation (1), being *Ci* the initial concentration of *p*-XSC and *W1* and *W2* the concentrations of the drug in the two filtrates after the centrifugation through the 300 kDa filters as described in section 3.4.

$$\%\ Encapsulation = \frac{Ci - (W1 + W2)}{Ci} \qquad (1)$$

4. Results and Discussion

4.1. Dynamic Light Scattering

In order to optimize the ultrasonic probe sonicator parameters, a first sonication test was performed with the following parameters: sonication time: 2 minutes, pulse on/off: 1s/2s, Amplitude: 10% without submerging the solutions in an ice bath. This methodology was

not enough to obtain an homogeneous liposomes suspension as seen in DLS size distribution (Figure 10, Red).

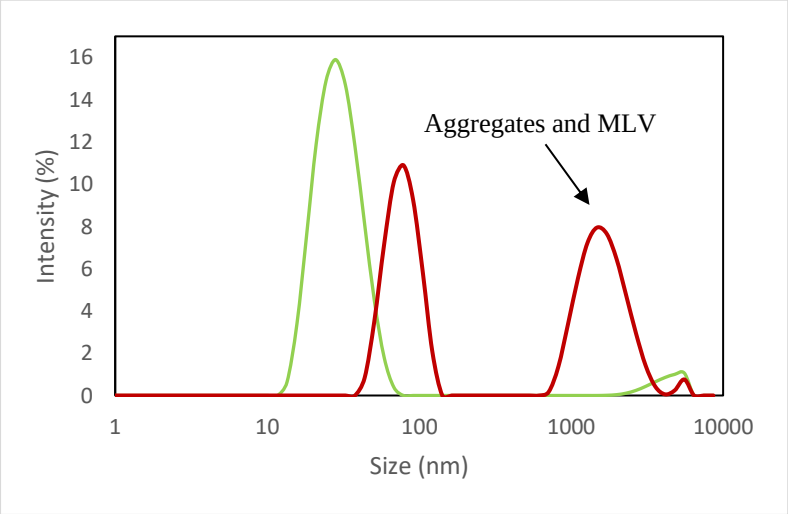

Figure 12. Size distribution profile of heterogeneous liposomes (red) and homogeneous liposomes (green).

The average diameter was 157.1 nm (± 18) which is above of the liposomes size to be considered SUVs, and a polydispersity index of 0.763, higher than the acceptable value of 0.3,[87] with large aggregates of vesicles remaining (Table 3). After testing different parameters, it was observed that to obtain an homogeneous solution of liposomes it was necessary a sonication time of at least 8 minutes in total, 5 of which in an ice bath, with pulses on/off of 20 seconds and an amplitude of 40 %. The average size was reduced to 29 nm (±10,) and a polydispersity index of 0.206, showing that homogeneous SUVs were successfully synthesized.

Table 3. Average size, Polydispersity index and Standard deviation (SD, n=3) of heterogeneous and homogeneous liposome dispersions.

Conditions	Average Size (nm)	SD (nm) n=3	Polydispersity Index
Time: 2 minutes Pulse:1s/2s Amplitude: 10%	157	18	0.763
Time: 8 minutes Pulse: 20s/20s Amplitude: 40%	29	10	0.206

4.2. *Cryo*-Transmission Electron Microscopy

It was seen that the vesicles adjust to the size determined by DLS (Figure 11) and although multilamellar vesicles can be observed (yellow arrow) the majority of liposomes are unilamellar.

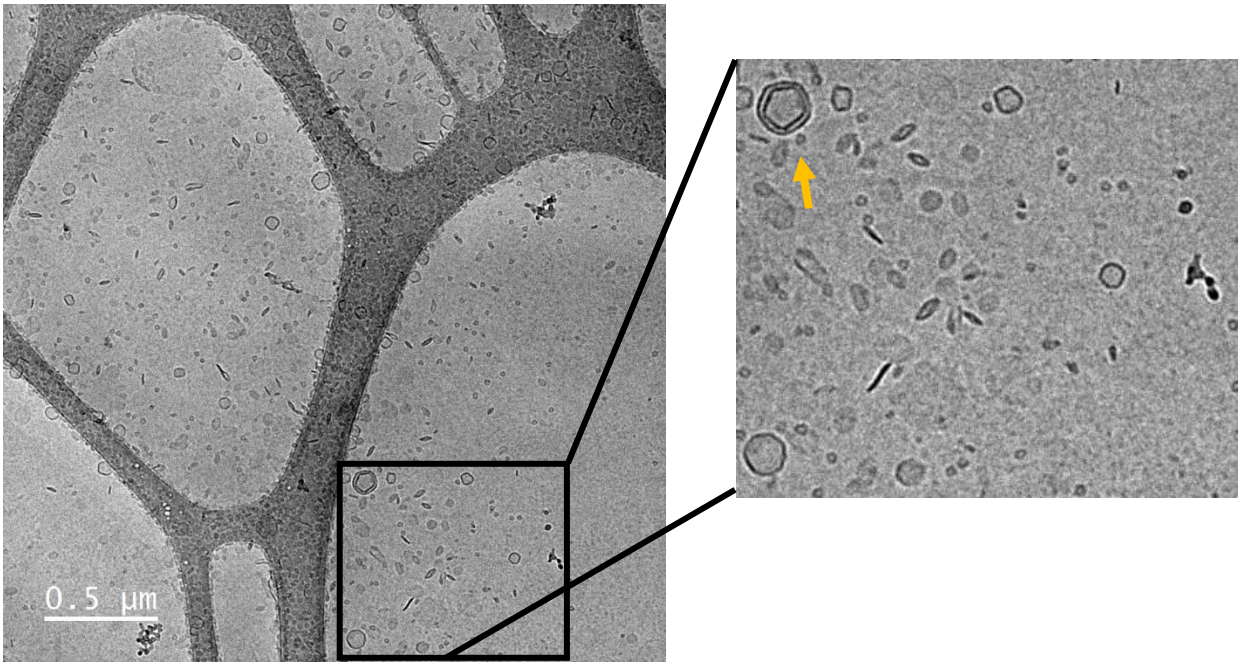

Figure 13. cryo-TEM images (Magnification x10k) of liposomes (left), and a close-up of uni and multilamellar vesicles (right)

Coinciding with bibliography, liposomes did not stay entirely spherical due to the collapsing of the vesicles, sometimes creating hexagonal or even square-like shapes (Figure 12, blue arrow). It can be observed (Figure 12) the presence of disc-like shapes (white arrows) formed by either the breaking of the vesicles or the formation of micelles during the preparation steps that require the temperature to be above phase transition temperature (dry film formation, hydration, or sample preparation).[88–90]

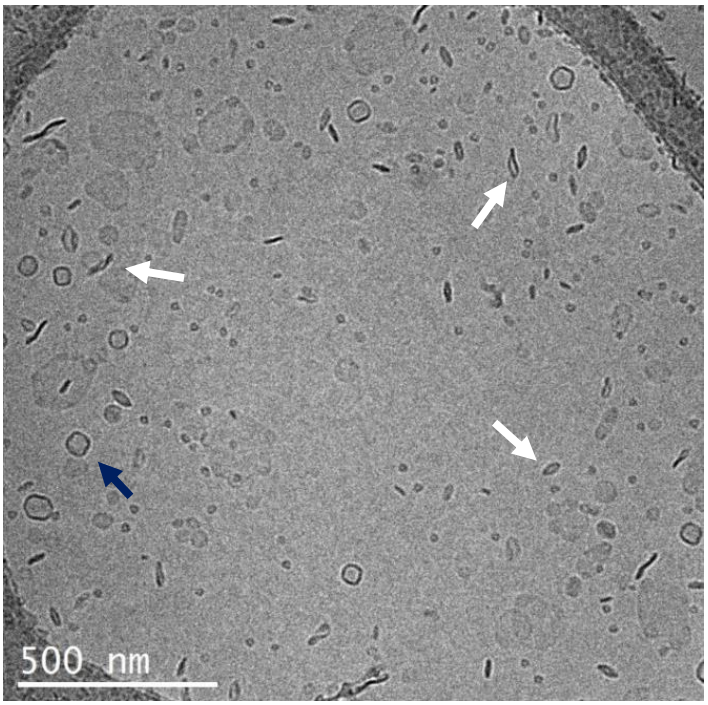

Figure 14. cryo-TEM image (Magnification x12k) of liposomes showing disc-like shapes.

4.3. Differential Scanning Calorimetry

In this experiment, the thermogram (Figure 14) shows different endothermic peaks corresponding to Tm and also Tp. Firstly, we can observe that the main transition temperature for DPPC is slightly higher than typical (41ºC) but still falling in the acceptable range for this phospholipid (41ºC-43ºC).[91] On the other hand, changes in the peaks corresponding to the targeted liposomes can be seen for the three functionalization strategies.

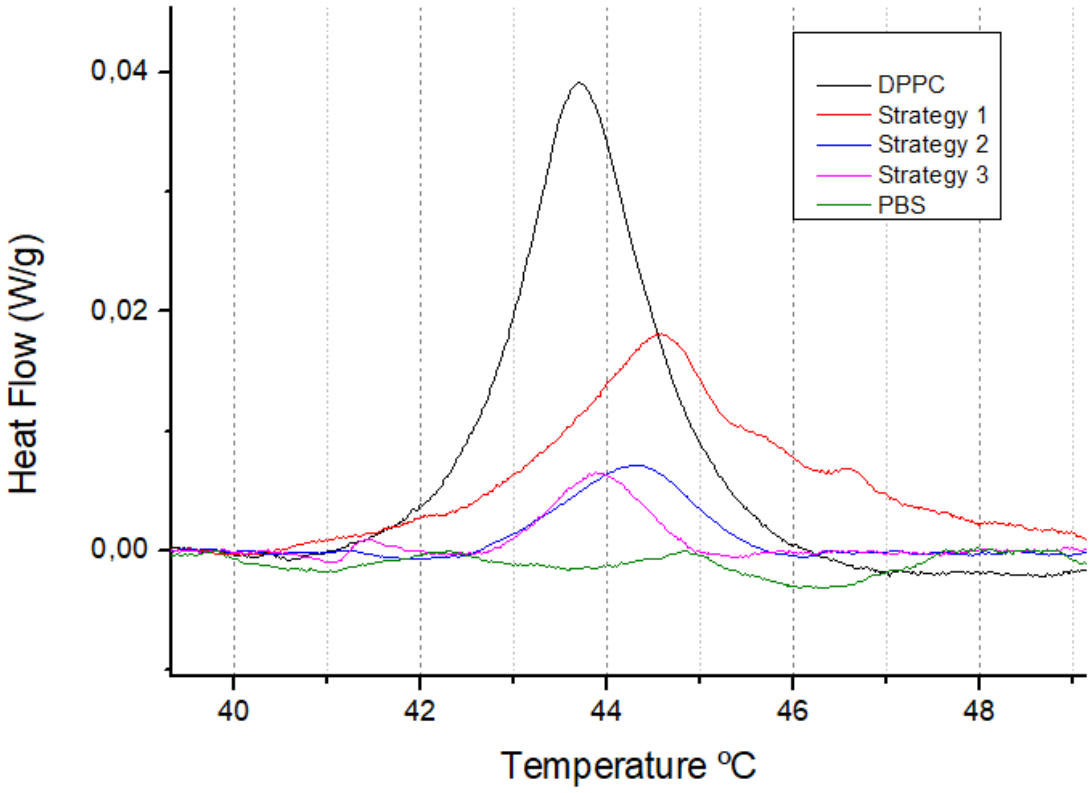

Figure 15. DSC thermogram of Heat Flow (W/g) of liposomes with the different targeting strategies

The change in Tm (Table 4) corresponds to that stated in bibliography[79] for liposome-protein systems which is typically in the order of 1ºC or less and the decrease in enthalpy is also an indicator of disturbances in the liposomes caused by the addition of immunoglobulins.

Table 4. Different main transition temperatures and enthalpies for DPPC and functionalized DPPC liposomes.

	Tm (ºC)	ΔH x 10^{-3} (J/g)
DPPC	43.6	443
Strategy 1	44.6	301
Strategy 2	44.3	72
Strategy 3	43.9	43

4.4. Enzyme-Linked Immunosorbent Assay

As explained in Section 3.6, a positive signal was observed in the wells of the three types of DPPC functionalized liposomes that should have antibodies indicating that there was a binding between the IgG in the liposomes' membranes and the secondary antibody. Figure 16 shows the change in color from blue (Figure 16-A) to yellow (Figure 16-B) due to the TMB substrate and HRP reaction before and after it stops. It was found that 2 hours of incubation of liposomes is enough for the initial coating of the immunoassay by obtaining the same absorbance profile as for 12 hours.

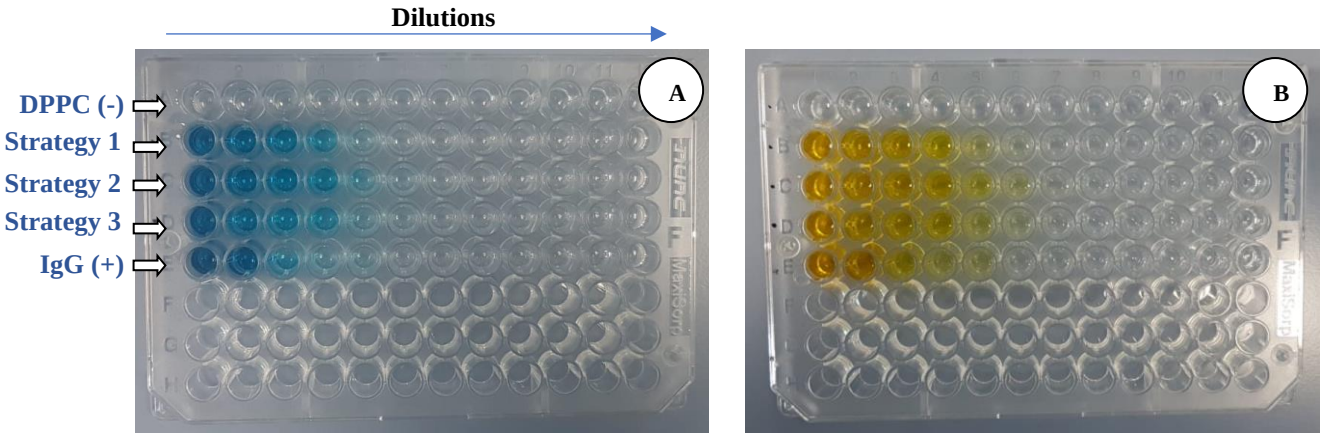

Figure 16. ELISA plates of liposomes coated with IgG with the 3 different strategies when adding TMB substrate (A) and after stopping the reaction (B) while doing consecutive 1/5 dilutions. Incubation time: 2 hours.

ELISA data is usually represented with absorbance vs log of concentration or dilution of the sample to obtain a sigmoidal curve (in the present case log sample dilution was used). Figure 17 shows the curves of the negative control (DPPC (-), blue curve in Figure 17), and liposomes targeted with the three strategies, confirming that the three functionalization strategies were successful.

It was seen that applying the α-IgG at a 1:10,000 dilution produces severe fluctuations in the absorbance curve, proving to be too diluted for this assay. The curves shown were obtained using a 1:5000 dilution of the secondary antibody (Figure 17).

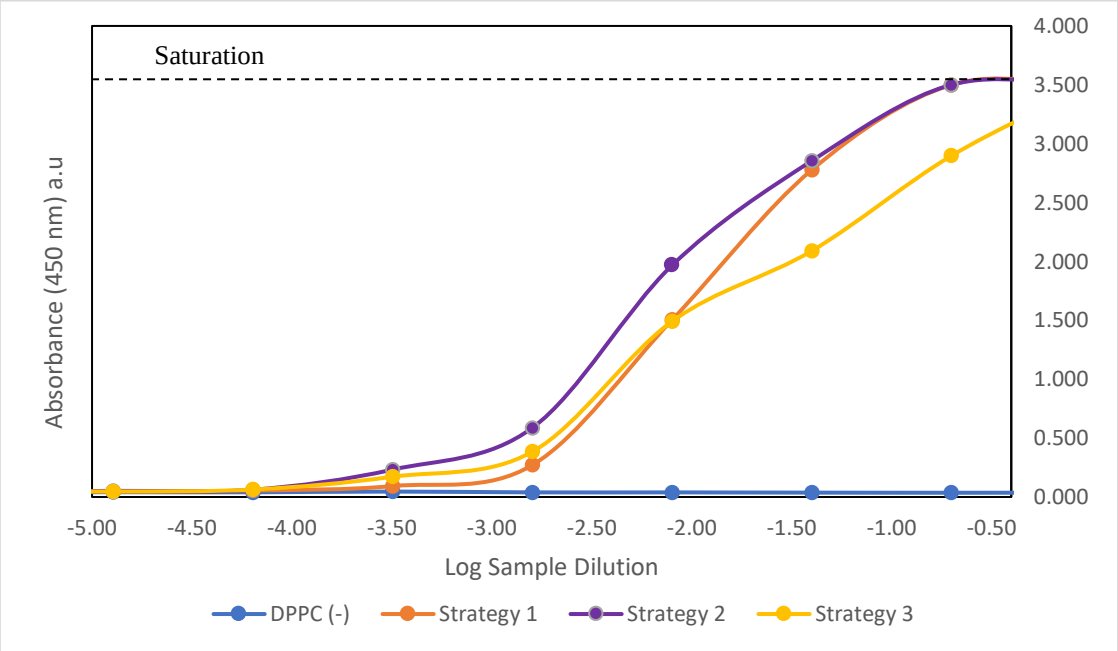

Figure 17. ELISA curves of un-targeted DPPC (-) and liposomes coated with 3 methodologies. Coating incubation 2 hours. α-IgG dilution 1:5000. Measured at 450 nm.

At first glance it appears that the number of antibodies in liposomes decreased following Strategy 2 > Strategy 1 > Strategy 3 however there are still fluctuations in the sigmoidal curve that difficult the proper comparison between methodologies and quantification of IgG.

IgG would be quantified by the determination of the half maximal effective concentration (EC_{50}), which is a measure of the concentration needed to obtain 50% of total binding.[92] In order to optimize the assay and be able to determine this value, more dilutions of the liposomes are needed to improve the sigmoidal curve.

On the other hand, detection of IgG in the centrifugation washes described in section 3.4 was also tested. It was observed the decrease in IgG after two centrifugations as in the example on Figure 18, however free IgG was not completely washed from the liposomes' treatments meaning that more centrifugations through filters are needed.

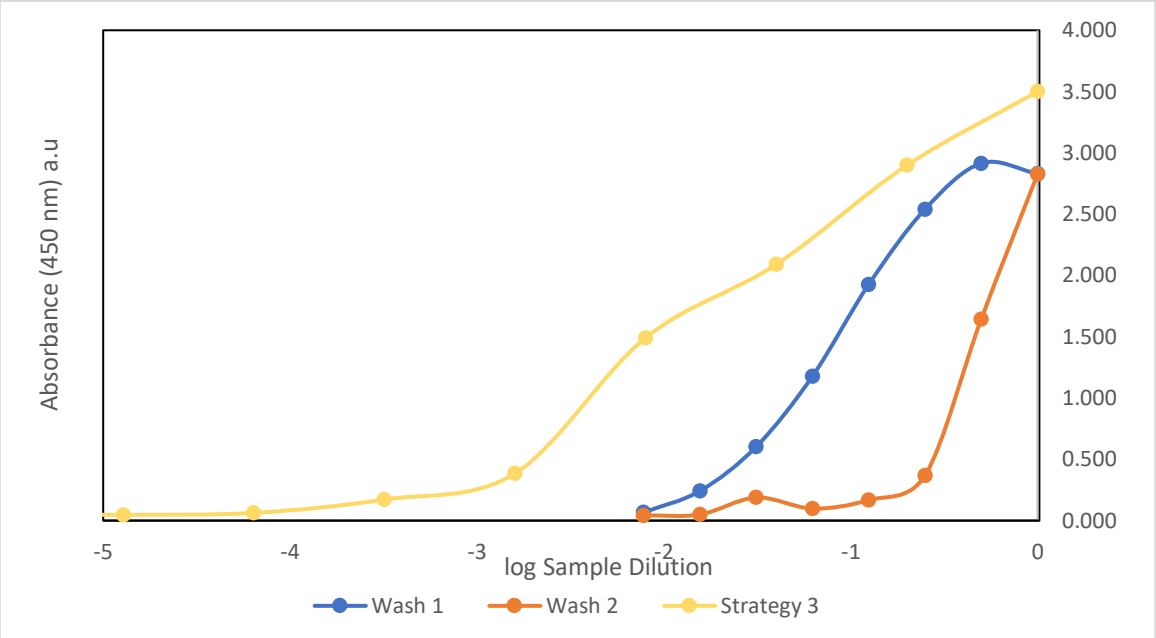

Figure 18. ELISA curves Liposomes targeted with Strategy 3, and the 2 washes. Coating incubation 2 hours. α-IgG dilution 1:5000. Measured at 450 nm.

4.5. Encapsulation Study

4.5.1. Inductively Coupled Plasma Mass Spectrometry

The encapsulation efficiency of *p*-XSC, calculated by means of equation 2, was maintained above 86 % throughout 4 weeks for the 3 types of treatments tested (Figure 19), which coincides with similar values found in bibliography, meaning that liposomes systems for this drug are stable up to a month. The efficiency varies depending on the properties of the liposomes, like membrane rigidity and composition, the type of agent to encapsulate and its properties and the encapsulation methodology, but encapsulation efficiencies above 90% are achievable.[93–95]

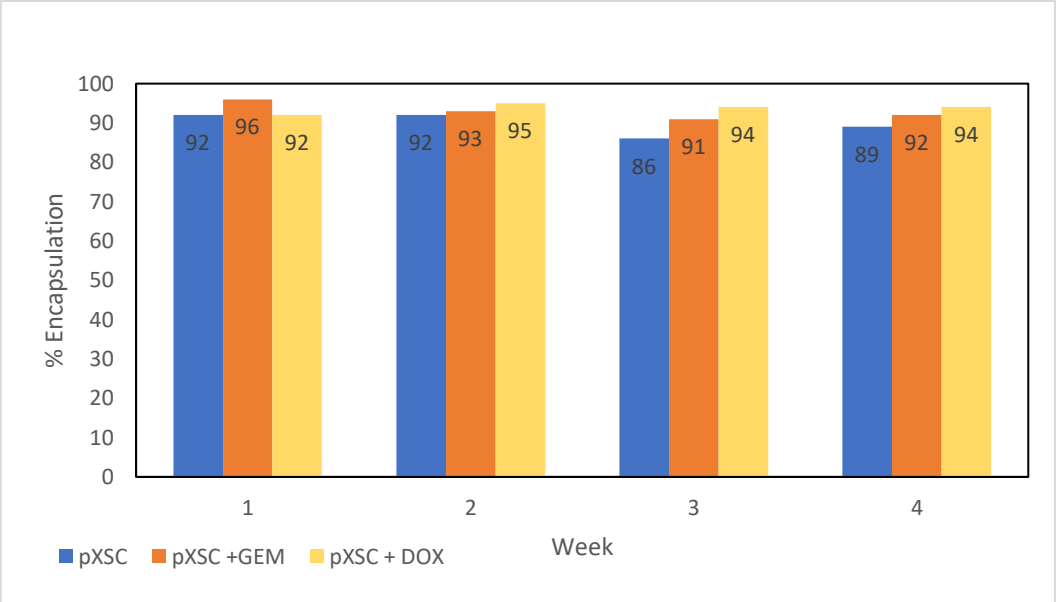

Figure 19. Encapsulation efficiency of p-XSC inside the liposomes in each week for a month.

On the other hand, it was seen that DPPC liposomes centrifugation with centrifugal concentrators is effective in the separation of the non-encapsulated *p*-XSC. A test of 4 washes were performed and it was seen that at the third the concentration of drug was below the limit of detection, so in the following liposomes' synthesis, only 2 washes were performed each week. Figure 20 is an example of the decrease in free *p*-XSC concentration after successive centrifugations starting from an initial concentration of 25,128 ppb to 1,778.59 ppb and finally 137.81 ppb.

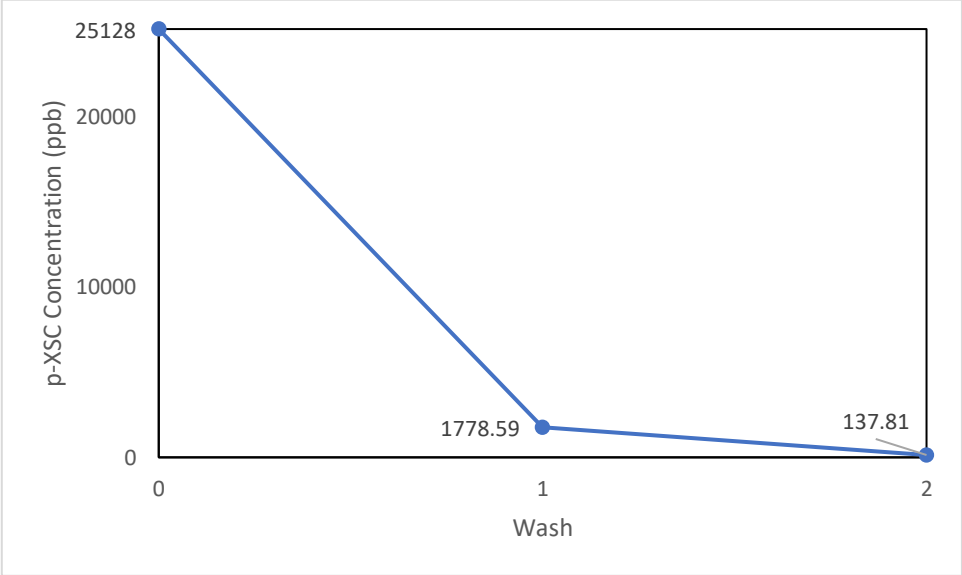

Figure 20. Graphic representation of the decrease of p-XSC concentration with 2 centrifugations.

5. Conclusions

In this work, immunoliposomes of DPPC and IgG for testing in cell viability assays against breast cancer cells have been successfully synthesized and characterized.

Specific conclusions are as follows:

1. The dry film hydration method followed by downsizing with an ultrasonic probe sonicator has been successful in the preparation of DPPC liposomes.
2. The sonication conditions (Sonication time: 8 minutes, Pulse: 20s/20s, Amplitude 40%) found for this type of liposomes allows the formation of liposomes of less than 50 nm.
3. DLS profiles have shown that the liposomes synthesized have an average hydrodynamic diameter of 29 nm (± 10) with a polydispersity index of 0.206.
4. *cryo*-TEM images have shown that the DPPC liposomes formed are small unilamellar vesicles in their majority.
5. DPPC liposomes with encapsulated *p*-XSC are stable with an encapsulation efficiency above 86% during 4 weeks for the three different treatments tested.
6. Differential Scanning Calorimetry has shown that the three different strategies of DPPC liposome functionalization are effective, since there were changes both in Tm and ΔH, and their presence was confirmed by ELISA tests.

6. Future Works

Regarding DPPC, studies should be carried out testing different DPPC/Drug concentrations and also study different compositions of phospholipids for the liposomes' formation.

In terms of drug encapsulation, the development of an HPLC method for the study of GEM and DOX release out of the liposomes should be developed to fully define the encapsulation efficiency of DPPC liposomes.

As for the ELISA procedure, it is recommended to optimize the methodology to be able to properly quantify the amount of IgG by lowering DPPC and IgG concentrations, diluting the secondary antibody and/or coat wells with more dilutions which will allow the improvement of the curve.

Finally, the performance of a cell viability test to compare the cytotoxicity of the drugs on cancer cells and the drugs delivery by the immunoliposomes synthesized should be carried out using anti-EpCAM as the targeting antibody.

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