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Carrier Systems for Vitamin D

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“Work gives you meaning and purpose and life is empty without it.”

Stephen Hawking

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Ao meu Pai (*in memoriam*).

Carrier systems for Vitamin D

Abstract

There has been an increased interest aiming to the conception of delivery systems to encapsulate, protect and release lipophilic active compounds such as vitamins. In this particular case vitamin D, which numerous studies both *in vitro* and *in vivo* had already shown the ability to inhibit cell proliferation in several cell types, including carcinomas of the breast, prostate, colon, skin, brain and myeloid leukemia cells. Furthermore, has also been demonstrated that vitamin D may potentially induce apoptosis and inhibit angiogenesis and also tumor invasion and metastases.

In this study nano-scale drug delivery systems using polymeric nanoparticles and liposomes were developed to incorporate vitamin D increasing its bioavailability for cancer therapy anticancer drug. The polymeric matrix was prepared with poly(lactic-co-glycolic acid) (PLGA) which is FDA approved and is often employed in drug deliver due to its biocompatible and biodegradation properties. The produced PLGA nanoparticles were stable within the size range of 170-180 nm, low polydispersity index, negative zeta potential and spherical shape observed by TEM. The PLGA/vitamin D nanoparticles had shown an encapsulation efficiency of approximately 75%. For this carrier system, the *in vitro* cytotoxic studies were performed on human pancreatic duct cell line (HPNE). The PLGA/vitamin D nanoparticles showed to have reduced cytotoxic effect on these cells comparing to vitamin D free.

The liposomal system composed of 1-phosphatidyl-2-oleoyl-sn-glycero-phosphocholine (POPC) presented a mean size of 77 nm and zeta potential between -6 and 0 mV. The encapsulation efficiency for vitamin D resulted in approximately 67%. Also physical stability tests were performed for over 3 weeks which proved to be a reliable system to protect the drug content. In conclusion, both systems presented promising results that lead to the continued research of the vitamin D encapsulation on these selected nanocarriers.

Keywords: nanoparticles, PLGA, liposomes, cholecalciferol, vitamin D, encapsulation efficiency

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List of abbreviations

1,25 (OH) D – 1,25-dihydroxycholecalciferol
25 (OH) D – 25 hydroxycholecalciferol
DLS – Dynamic Light Scattering
DMEM – Dulbecco's Modified Eagle Medium
 d_p – Hydrodynamic Diameter
DSC – Differential Scanning Calorimetry
EE – Encapsulation Efficiency
FDA – Food and Drug Administration
FTIR - Fourier Transform Infrared Spectroscopy
 $f(k_A)$ – Henry's Function
 IC_{50} – Half Maximal Inhibitory Concentration
 η – Viscosity
HPNE - Human Pancreatic Duct Cell Line
 K_B – Boltzmann Constant
MLV - Multilamellar Vesicles
MPS - Mononuclear Phagocytic System
MW – Molecular Weight
NMR - Nuclear Magnetic Resonance Spectroscopy
O/W – Oil in Water
PB – PrestoBlue
PBS – Phosphate Buffered Saline
PC - Phosphatidylcholine
PDI – Polydispersity Index
PEG - Polyethylene Glycol
PLA - Polylactide
PLGA - Polylactic-co-glycolic Acid
SEM – Scanning Electron Microscopy
SD – Standard Deviation
SRB – Sulforhodamine B
SUV – Small Unilamellar Vesicles
TEM – Transmission Electron Microscopy
 U_E – Electrophoretic Mobility
VDR – Vitamin D Receptor
 ξ – Zeta Potential

1 Introduction

1.1 Motivation

Liposoluble compounds like vitamins are commonly found in food or used as excipients in the well-known industries such as pharmaceutical, cosmetics or even in the food industrial field. Vitamins are very sensitive compounds to manage, so they need to be protected of the environmental conditions that may affect their chemical integrity and their physiological effects.

The existing solution is their encapsulation that allows preserving their native properties for longer. These encapsulation systems are developed at nano-scale in order to improve shelf life time, to an active control of the delivery compound and prevent the existence of side effects. Regarding the different administration pathway, liposoluble vitamins may imply different requirements on their use.

Vitamin D is a good example as it is liposoluble and its major functions are the increase of intestinal absorption of calcium and promotion of normal bone formation and mineralization. It is already evidenced that vitamin D is of great importance and is associated with a wide range of diseases, such as cardiovascular diseases, diabetes, rickets, osteoporosis, osteomalacia and secondary hyperparathyroidism and cancer. Several studies demonstrated that vitamin D may potently induce apoptosis and inhibit angiogenesis and also tumor invasion and metastases (Field & Newton-Bishop, 2011). Vitamin D is present in two main chemical forms, namely, ergocholecalciferol or activated ergosterol or vitamin D₂ and cholecalciferol or activated 7-dehydrocholesterol or vitamin D₃.

1.2 Main Objectives

Nowadays the attempts to engineer efficient local and targeted drug release agents are mainly focused on nanoparticle systems. In order to overcome the water solubility limitation of the vitamin D is necessary to develop a suitable delivery system. Encapsulation techniques through controlled release and small doses administration are

able to reduce the hypervitaminosis syndrome and the possible appearance of side effects. The main strategies available to formulate liposoluble vitamin carriers are lipid based formulations, where vitamins are solubilized in a matrix entrapment by polymer.

The objective of this work is to design and develop nanocarriers capable of encapsulating vitamin D₃ to be tested as anticancer drug. The selected nanosystems were PLGA nanoparticles and liposomes. These systems were characterized and evaluated as nanocarriers of vitamin D for its delivery into pancreatic normal cells.

Few studies about these systems have been published for vitamin D.

To our knowledge, any study was developed on vitamin D-loaded PLGA nanoparticles for its controlled release into cancer cells.

1.3 Thesis Organization

This thesis is divided in 5 chapters each associated to the following content.

Chapter 1, which is the present chapter, explains the main motivations to formulate and develop nanocarriers for Vitamin D. Additionally, the main objectives of this work are defined.

Chapter 2 presents the state of the Art. It induces the properties of vitamin D and its biological functions, and its relation with cancer, the studies that have been developed so far on the vitamin D encapsulation, in particular for nanoparticles and liposomes as drug delivery vehicles.

Chapter 3 presents the materials, and methods used throughout the work. Specifically preparation of PLGA nanoparticles and liposomes as well as the principles of the physicochemical characterization of the nanoparticles. The description of the cytotoxicity studies was also included.

In Chapter 4 includes the main obtained results and their further discussions for the PLGA and liposomal systems developed for the encapsulation of vitamin D.

The overview of this study, its main conclusions and additional future perspectives are presented on Chapter 5.

2 State of the Art

2.1 Vitamin D

Vitamin D is a liposoluble molecule and its major functions are the increase of intestinal absorption of calcium and promotion of normal bone formation and mineralization (Gueli et al., 2012). Vitamin D has two main chemical forms, vitamin D₃ (cholecalciferol) or vitamin D₂ (ergocalciferol) (Henry, 2011) (Wiseman, 1993). The chemical structures are shown in figure 1.

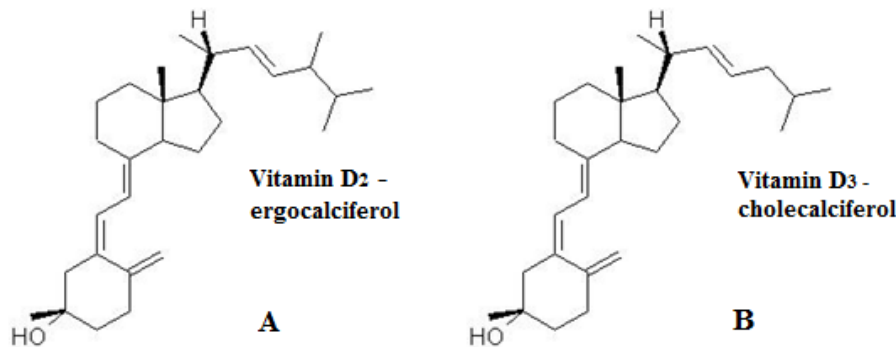


Figure 1 – Chemical structure of vitamin D₂ (A) and vitamin D₃ (B) (University of Bristol, 2007).

Vitamin D needs to bind to a receptor, known as the vitamin D receptor or VDR, which allows the connection to its genetic effects, and also have been identified in several tissues and cells (Field & Newton-Bishop, 2011). This vitamin is considered a steroid hormone and is synthesized in human skin from 7-dehydrocholesterol in the presence of UV. Firstly the vitamin D that enters in the human body through skin or by ingestion in the diet is still biologically inactive. It requires two obligate hydroxylations, first in the liver to 25-hydroxyvitamin D, and then in the kidney to 1,25-dihydroxyvitamin D (M. F. Holick, 2006). Vitamin D travels in the blood bounded to an α -globulin and is present in two main chemical forms, namely ergocalciferol or activated ergosterol or vitamin D₂ and cholecalciferol or activated 7-dehydrocholesterol or vitamin D₃ (Gueli et al., 2012). Vitamin D₃ is synthesized in the skin after light

exposure and displays several chemical structures, such as calcitriol that is the non-hydroxylated and inactive form. Another variation is calcidiol or 25-hydroxyvitamin D, which is the monohydroxylated and the blood circulating form that is responsible for several different actions related with the control of calcium and phosphorus homeostasis, internal transport, bone metabolism and renal calcium re-absorption, as well as the task to control the blood pressure and insulin secretion (Gonnet et. al. 2010). The metabolized structures of the vitamin D inside the body are presented in the figure 2.

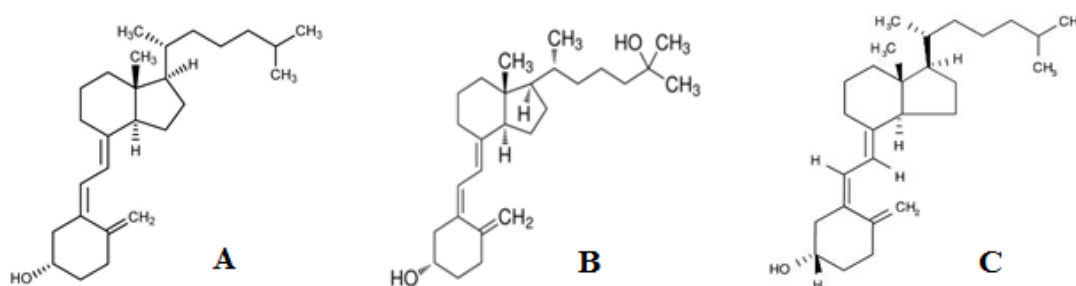


Figure 2 – Chemical structure of; A) cholecalciferol; B) calcifediol; C) calcitriol (University of Bristol, 2007).

Besides that, in enterocytes, calcidiol regulates calcium and phosphorous absorption by its attachment with its cellular receptor. The other main chemical form known is the vitamin D₂ which is formed by the action of the UV irradiation with ergosterol (Gonnet et al., 2010).

More precisely the action of cholecalciferol is important to regulate the concentrations of calcium and phosphate ions in the blood serum. Also has a relevant function in skeletal homeostasis, either by controlling bone metabolism by regulation of the osteoblast differentiation, proliferation and apoptosis. Still, plays an important role in the expression of specific bone proteins and growth factors in the upcoming forming bone tissue (Ignjatovic et. al. 2013). On the other, ergocalciferol is synthesized in plants. Although its susceptible to oxidation and is also isomerized to isotachysterol in the presence of light and under acidic conditions (Patel & San Martin-Gonzalez, 2012).

Cholecalciferol and ergocalciferol are equally effective as inhibitors of lipid peroxidation, since they have the same side chains as cholesterol and ergosterol (Wiseman, 1993). Additionally, important information is asserted by the work of Wiseman (1993), where cholecalciferol, 1.25-dihydrocholecalciferol, ergocalciferol and

7-dehydrocholesterol may act as membrane oxidants through the membrane stabilization against lipid peroxidation with the interaction between their hydrophobic rings (Wiseman, 1993).

The supply of vitamin D is affected by several factors, such as the geographic, seasonal, genetic factors and some diseases that will disturb the metabolism and others that cause bad levels of absorption (Kimlin et al., 2007). It has been estimated that 1 billion people worldwide have vitamin D deficiency or insufficiency (Michael F. Holick, 2009). Vitamin D deficiency or insufficiency happens mainly in developing countries and can cause rickets (Rajakumar, 2003).

The main source of vitamin D for most of the population comes from the sun exposure with a small amount acquired from food and supplements (Heaney et. al. 2003).

Vitamin D₃ is the preferable form of vitamin D (Armas et. al. 2004). Additionally, the usual indicator of the vitamin D status in the body is the vitamin D₃ or 25 (OH) D. Moreover, this metabolite has the advantage of longer half-life time, approximately 3 weeks, when compared to other vitamin D metabolites. The vitamin D₃ status is determined through measurements in the plasma or serum (Deeb et. al. 2007).

Epidemiological studies suggest an important role in autoimmune diseases, infections, cardiovascular disease and cancer (Woloszynska-Read et. al. 2011).

A relationship between vitamin D and cancer does exist and is strongly supported by epidemiological, clinical and laboratorial studies (Mocellin, 2011). Several cancers may be vitamin D sensitive such as brain, breast, bladder, colon, rectal, endometrial lining, esophagus, gallbladder, kidney, lungs, ovaries, pancreas, prostate and stomach since they express the 1- α -hydroxylase that is indispensable to convert 25 (OH) D into 1,25 (OH) D (M. F. Holick, 2006). One possible explanation is that 1,25-dihydroxyvitamin D₃ appears as a regulator of gene expression and signal transduction in every tissue. Besides that, the presence of functional VDR in human tumors and cancer cell lines may be indicated as target for drug delivery therapy since those cell lines may be capable of transform the compound into his active form (Welsh, 2012). This information allows assuming that vitamin D might be connected with growth mechanism of these different types of cells.

For the next chapters of this work, cholecalciferol may be referred also as Vitamin D.

2.2 Nanocarriers for Vitamin D

Vitamin D is a highly lipophilic molecule that cannot be directly dispersed into an aqueous solution. Instead, it needs to be incorporated into an appropriate colloidal system prior to dispersion (Gonnet et al., 2010). In order to explore for targeting purpose and also to improve his bioavailability is necessary to review the carrier systems to perform a sustainable release (Li et. al. 2011). In the literature review, there is some lack of information regarding the nano or micro encapsulation of vitamin D₃. Nevertheless, there are some studies referring the use of cholecalciferol and calcitriol in drug delivery. Furthermore, several case studies report the attempts that have been done in the matter of carrier systems referring as vitamin D₃ and its metabolites as main drugs for carrier design systems. A few number of papers portraits the microencapsulation of cholecalciferol as a drug model or as a nutrient supplementation. Still, in another research study, it was described the encapsulation of cholecalciferol into hydrophobic alginate nanoparticles for sustained release form (Li et al., 2011). In the work of Luca *et. al.* to enhance the viability and function of the pancreatic islet cell, cholecalciferol was encapsulated into cellulose acetate microspheres and tested as an anti-oxidizing agent (Li et al., 2011). Other formulation was presented by Nguyen *et. al.* where were used calcitriol loaded micro-particles using poly(vinyl-neodecanoate-crosslinked-ethyleneglycol dimethacrylate) as polymer with the purpose to ensure the extension behaviour of the anti-cancer concentration after local hepatic injection (Nguyen et al., 2007).

Another carrier system proposed was vitamin D₃ as a nutraceutical model for sustained release purpose and it is based on oleoyl alginate ester. The results obtained reveal that when the vitamin D concentration was increased it enabled the increase of loading capacity, but in the meantime the loading efficiency was descendent. Although, these nanoparticles were able to release vitamin D when located in the target, so it is

possible to pronounce positively that the oleoyl alginate ester nanoparticle is a sustained carrier system for vitamin D (Li et al., 2011).

Recently a study developed by Almouazen *et. al.* displays the link between vitamin D₃ and anticancer application. In this case, was carried out a formulation of vitamin D₃, with metabolites cholecalciferol and calcitriol, using polymeric nanoparticles. These nanoparticles were used as carrier system and also studied to evaluate the *in vitro* treatment behaviour. In more detail, PLA was used as biodegradable polymer and it was concluded that these nanoparticles had shown an antiproliferative activity for over 10 days against breast cancer (Almouazen et. al. 2013).

2.3 Encapsulation of Hydrophobic Drugs

Encapsulation technology is the process to capture active agents into a carrier material, as a way to improve and protect bioactive molecules for further delivery (Nedovic et. al. 2011).

The clinical utility of most conventional chemotherapeutics is limited either by the inability to deliver therapeutic drug concentrations to the target tissues or by severe and harmful toxic effects on normal organs and tissues (Drummond et. al. 1999).

The advantages of encapsulating drugs in nanocarriers comprise an increase in the dissolution rate of hydrophobic or drugs with poor solubility, the surface properties allows target delivery and immune system evasion, controlled and sustained drug release. When these factors act together provide less drug dosing frequency and improve the therapeutic effect extending *in vivo* therapeutic half-life (Cheow & Hadinoto, 2011) (Cho et. al. 2008). The ideal drug delivery system approach is the one that act on the selected target. Although the nanoscale drug delivery systems may act on the target by two strategies. The active strategy is where the carrier system possesses the specific ligand for the existing receptor in the target tissue. The other strategy relays on the passive targeting, where the carrier system moves to the inflamed tissues and release the drug in providing higher concentration on site without induce toxicity (Malam et. al.

2009). Even though, some authors suggest the importance of active targeting strategy due to the specific molecules conjugated at the surface of the carrier. These vector molecules recognize the overexpression of biomarkers in cancer cells enhancing the affinity for the delivery of the drug carrier at the specific site (Karra & Benita, 2012).

Another important feature is the ability to avoid the recognition of the P-glycoprotein, which is one of the main drug resistance mechanisms by the attachment to the specific receptors to pass in into the cell (Larsen et. al. 2000). Therefore, nanotechnology may cause a significant impact on drug delivery (Farokhzad & Langer, 2009). The drug loading allows the optimization parameters in controlled drug delivery (Speiser, 1991). By encapsulation of potential drugs inside nanocarriers it is possible intensify their therapeutic index by enhancing their release time and also their pharmacokinetic profile. Besides the solubility and the stability of the drug is enhanced (Langer, 1998). Vitamin D is lipo-soluble thus it is essential to increase the solubility in water (Li et al., 2011).

In this work, to encapsulate vitamin D was firstly necessary to select an appropriate delivery system. Depending on the hydrophobic nature of vitamin D, a hydrophobic polymer matrix is crucial to the drug loading succeed (Makadia & Siegel, 2011). In that order PLGA is identified as primary choice. Polymers like PLGA have the benefit of biodegradability and biocompatibility (Danhier et al., 2012). The application of PLGA as outcome successfully as biodegradable polymer resultant of the monomers lactic acid and glycolic acid derived from its hydrolysis (Kumari et. al. 2010). Besides, in other studies PLGA show competence to encapsulate drugs of poor solubility (Liu et. al. 2010). PLGA-based nanoparticles have the advantage of controlling the drug release by the extra time that they remain in the blood circulation resulting in the increase of the anti-tumor activity (Averineni et al., 2012). Furthermore, the polymer matrix offer rigidity to the overall structure making them more stable than liposomes (Pinto-Alphandary et. al. 2000). PLGA-based nanoparticles are colloidal systems and the separation of the encapsulated from the non-encapsulated nanoparticles is executed by centrifugation. Through this process is not so easy to fully control the accurate determination of the drug content. Another disadvantage that is related to the PLGA-based nanoparticles is the high initial burst release of the drug which might be due to the existence of drug adsorbed to the nanoparticle surface. Several studies had shown that when the nanoparticles are inside the body, there is a change in their

structure. This is the result of the complex interaction verified between the nanoparticles and the biological systems and the behavior change (Danhier et al., 2012) (Kumari et al., 2010). To minimize these pitfalls, another drug delivery system was studied and characterized for vitamin D, to accomplish a better understanding of the advantages and drawbacks of both systems.

Liposomes or phospholipid vesicles are introduced as drug carrier system where it seems that are widely investigated in such applications. They are nontoxic and non-immunogenic lipid molecules and are able to encapsulate hydrophobic molecules in lipid bilayers given them the capacity to deliver oil-soluble compounds (Storm & Crommelin, 1998). In liposomal formulations, physicochemical properties as surface charge, functionality or size are well controlled by adding or mixing commercially available lipid molecules. This is advantageous over other carriers which need additional chemical modifications to change their structure. Nonetheless, physical and chemical limitations had been noted especially in cancer therapy due to oxidation and hydrolysis of the phospholipids molecules causing drug loss, short residence time in blood and even alterations in size caused by aggregation and fusion (Lasic, 1998) (Stark et al. 2010). Nevertheless, the biocompatibility at cellular level is higher in liposomes when compared to some of the nanoparticles formed by the synthetic polymers (Y. Liu et al., 2010).

As drug delivery systems used currently liposomes and nanoparticles are the primary choice for the rational delivery at nano-scale (Malam et al., 2009) (Y. Liu et al., 2010).

For this work, there have been proposed two systems to comprehend and to test the advantages reported in the literature and to understand their interaction with vitamin D.

2.3.1 Polymeric-based Nanoparticles as Carriers

Nanoparticles are usually defined as solid colloidal particles that can be prepared by both polymerization methods and combined with preformed polymers (Vauthier & Bouchemal, 2009). These particular systems may be relevant as active vectors for its

ability to release drugs. Its subcellular size allows a higher intracellular uptake and at the same time can be biocompatible with tissues and cells when combined with materials which embody biodegradable and biocompatible characteristics (Mora-Huertas et. al. 2010).

Due to the particular environment of the tumor site the use of nanoparticles can influence the cancer therapy by the accumulation of a higher drug concentration at the site. In addition, the possible side effects of the chemotherapeutic agents are reduced (van Vlerken & Amiji, 2006). Moreover, it can be added imaging contrast agents to these nanoparticles that facilitate the overview to their address to the specific site (Parveen et. al. 2012).

Other benefits that may be mentioned are the high drug encapsulation efficiency, protection against factors as pH, light and the reduction of tissue irritation due to the action of the protective polymer coating. Also the low polymer content compared to other systems nano-systems such as nano-spheres (Pinto Reis et. al. 2006).

Polymeric nanoparticles already support an extensive background as drug carriers especially in the pharmaceutical field, where the main aspects that need to be analyzed are mean size, zeta potential, encapsulation efficiency, active release, nano-dispersion stability and *in vivo* and *in vitro* pharmacological performance behaviors (Mora-Huertas et al., 2010).

Currently the synthetic polymers in use are polylactide (PLA), poly D, L-lactide-co-glycolide (PLGA) and polyethylene glycol (PEG) and the biological polysaccharides are chitosan, cyclodextrin and dextrans (Chan et al., 2009). Besides, synthetic polymers already have their formulations and methods of production well described and adapted to several types of drugs such as hydrophobic or hydrophilic and also to small molecules or macromolecules. Still, they provide protection of drug from degradation, the possibility to modify the surface properties, and also the possibility of better interaction with biological materials and the ability to reach the specific organs or cells (Danhier et al., 2012). Some examples of compounds constituted by polymeric nanocarriers as chemotherapeutics agents are given by Cho *et. al.* (2008) such as Abraxane, Xyotax, CT-2106, PK1 and PK2 (Cho et al., 2008).

2.3.2 Poly Lactic-co-Glycolic Acid (PLGA) as Biodegradable Polymer

Biodegradable polymers may be derived from natural or synthetic origins and are synthesized *in vivo*, either enzymatically or non-enzymatically or both, in order to produce biocompatible, toxicologically safe by-products which are further eliminated by the already existing metabolic pathways (Makadia & Siegel, 2011). In the category of synthetic biodegradable polymers used in drug delivery is included the poly lactic-co-glycolic acid (PLGA), which is the one to be used in this particular work and is already widely used as drug delivery vehicle. For this specific polymer some requirements are applied, where biocompatibility is one of the clearly relevant properties. This property is related to the biological environment and also to the tolerability that exists in the interaction of the specific drug-polymer tissue (Shive & Anderson, 1997). The parameters that are going to be directly involved with the successful fate of the polymeric nanoparticles are mainly the size, size distribution, surface charge, drug encapsulation efficiency among others (Feng, 2004). Controlling the size of the PLGA nanoparticles is very important because is necessary to adjust drug-polymer complex and also imaging agents can be incorporated in order to achieve imaging probes of the cancer drug targeting (Kumari et al., 2010). The zeta potential is the indicator of the surface charge which will play an important role on the interaction with the charge of membrane cells (Hans & Lowman, 2002). Moreover, small volume and high viscosity are the targets to accomplish if higher encapsulation efficiency is desired (Maa & Hsu, 1997) (Yeo & Park, 2004). It is currently observed for polymeric nanoparticles that an increase of the polymer concentration will cause an increase in the encapsulation efficiency value. This can be explained by the higher viscosity and fast solidification rate achieved by the increase of polymer concentration which leads to lower porosity preventing the drug diffusion from polymer channels and thus a low initial burst release (Yeo & Park, 2004).

PLGA is a FDA approved polymer and non-toxic, which is a copolymer of poly lactic acid (PLA) and poly glycolic acid (PGA). It is already well defined, in respect to design and performance, and PLGA is an acronym for poly D, L-lactic-co-glycolic acid where D- and L-lactic acid forms are in equal ratio (Danhier et al., 2012). The

hydrolysis of these monomers in water originating glycolic acid and lactic acid that will forward be eliminated through the Kreb's cycle (Lu et al., 2009).

PLGA is soluble with several common solvents as chlorinated solvents, tetrahydrofuran, acetone or ethyl acetate. For example in water, PLGA is degraded by hydrolysis of its ester linkages (Makadia & Siegel, 2011).

Additionally, PLGA is one of the most employed carriers for sustained drug release, where it is possible to control relevant parameters such as polymer molecule weight, ratio of lactide to glycolide and drug concentration to achieve the desire dosage upon the drug type (Mohamed & van der Walle, 2008). The release of the incorporated drug molecule and surface erosion are due to the change of the physical properties of PLGA. Those physical properties previously studied, are linked with several factors such as initial molecular weight, the ratio of lactide to glycolide, the size of the carrier, exposure to water, surface change and storage temperature (Houchin & Topp, 2009). As basic standard, higher content of PLGA leads to faster rates of degradation, yet with the exception of 50:50 ratio of PLA/PGA which exhibits the higher degradation interval. This is due to the presence of methyl side groups in PLA that enables more hydrophobicity properties than PGA, where in the other hand with higher lactide content PLGA copolymers are less hydrophilic, which leads to absorb less water resulting in a slowest degradation rate. Another important consideration is the molecular weight and polydispersity index that are directly associated with the mechanical strength of the PLGA (Makadia & Siegel, 2011). Yet, the PLGA composition and molecular weight were related with nanoparticle size, where nanoparticles with 50:50 PLGA achieve a lower mean size when compared to 75:25 PLGA (Konan et. al. 2002). PLGA carriers allow the release either by diffusion through the polymer barrier, or by erosion of the polymer, or by a combination of both mentioned mechanisms (Shive & Anderson, 1997). However has been studied that the release is assumed to be biphasic, where initially by diffusion and later by degradation or erosion of the polymer matrix itself (Panyam et al., 2003).

Furthermore, PLGA carriers tolerate the modification of their surface to direct the delivery to tumour or even other tissues (L. Liu et al., 2004).

For the preparation of PLGA nanoparticles, the most common method is the emulsification-solvent evaporation technique, which has been successfully applied for hydrophobic drugs (Hans & Lowman, 2002). The hydrophobic nature of PLGA allows

hydrophobic molecules as cholecalciferol to be adsorbed by hydrophobic interactions (Kerleta, 2010).

This technique allows the encapsulation of hydrophobic drugs by the dissolution of the polymer and the active compound in an organic solvent, in this particular case ethyl acetate as usually is the first option (Mora-Huertas et al., 2010). The existing oil (O) in water (W) emulsion or O/W is prepared by adding water and surfactant, here is Pluronic® F127, to the polymer solution. Pluronic® F127 is constituted by copolymers arranged in A-B-A structure where is comprehended as ethylene oxide-propylene oxide-ethylene oxide. Additionally Pluronic® F127 enhances solubility, helps the metabolic stability and also enables remaining more time in the blood stream (Batrakova & Kabanov, 2008). The successfully incorporation of Pluronic® F127 is due to the hydrophilic-lipophilic balance (HLB) values of the ethylene oxide-propylene oxide-ethylene oxide derivatives and the availability of the carrier matrix (Santander-Ortega et. al. 2006).

Regarding the external phase, the solvent used is water and PLGA is chosen as the stabilizing agent. In order to obtain better dispersion stability, stabilizer agents may be used in the diluted solutions, thus the dilution phase is a mixture of water with Pluronic® F127. Then the nano-sized droplets are accomplished by sonication or homogenization. Still for the preparation of nano-particle, the organic phase is emulsified under vigorous agitation in the aqueous phase which results in the evaporation of the organic solvent. After the waiting time of that step, the solvent is evaporated and the nanoparticles are collected after centrifugation. Furthermore, it has been studied that the nano-particle size is correlated to the shear rate used previous in the emulsification process, the chemical composition of the organic phase, the polymer concentration, the drop size and also the oil-to-polymer ratio (Moinard-Chécot et. al. 2008).

Other example that focuses the advantages of polymeric nanoparticles as drug delivery systems is the work by Musumeci *et. al.* where the aim of the work was to improve the drug solubility in a delivery system for the intravenous administration of docetaxel, which shows poor water solubility. Polymeric nanoparticles were prepared using as matrix PLA and PLGA, at different molecular weights, where in the final results they conclude that system was able to entrap the docetaxel and also to provide a sustained drug release (Musumeci et al., 2006). Is found in literature that PLGA

nanoparticles are considered as valued carrier system for the encapsulation of several anti-cancer agents as well as paclitaxel, 9-nitrocamptothecin, cisplatin, triptorelin, dexamethasone, xanthone, etc. (Danhier et al., 2012) (Hans & Lowman, 2002).

2.3.3 Liposomes as Carriers

Liposomes are micro-scale spherical vesicles constituted by phospholipids, which enclose liquid compartments within their structure of lipid bilayers. Besides, they are able to encapsulate both hydrophilic and lipophilic materials (Hsieh et. al. 2002).

Liposomes are widely employed as nano-carriers, and are very attractive vehicles because of its interaction with the host cell due to the use of phospholipids that are the main structural component of the membranes (Khan et. al. 2008) (Cheow & Hadinoto, 2011). The incorporation of the drug into liposomes is achieved by several mechanisms as formation with an aqueous solution saturated with the soluble drug, through the use of organic solvents and solvent exchange mechanisms, by the use of lipophilic drugs, and with pH gradient methods (Y. Liu et al., 2010). Furthermore, the incorporation of the water soluble drugs is in the aqueous core on the other hand the oil soluble compounds are entrapped in the liquid bilayer (Cheow & Hadinoto, 2011). The liposomal lipid bilayer along with the mentioned properties have the ability to minimize toxic side-effects that are usually associated with the use of chemotherapeutic agents (Khan et al., 2008).

As advantages, the liposomes can be produced on a large scale and are composed of well tolerated components (Müller et. al. 2002). Also they are widely used due to high biocompatibility, friendly pharmacokinetic profile and the flexibility available of surface modification by variation of the preparation methods (Torchilin, 2005) (Y. Liu et al., 2010). Moreover, they provide the attractive biological property to deliver pharmaceutical purpose inside individual cellular compartments (Torchilin, 2005).

Liposomes, mainly constituted by phospholipids, may be used in either active or passive targeting strategies. They get into the interstitial space from the bloodstream and are very useful because of his competence to receive additional molecules in the

external surface of the lipid bilayer becoming easier to manipulate them (Malam et al., 2009). Additionally, liposomes provide the possibility to use ligands as targeted nanotechnology-based drug delivery systems. This system excludes the need of the conjugation of drugs into the targeting ligand which might affect negatively both the target molecule and the drug (Backer et. al. 2002). However, if the receptor/ligand recognition is disrupted it automatically results in the complete loss of activity of the drug (Backer et al., 2004) (Khan et al., 2008).

In this study liposomes were prepared using the modified thin film hydration technique (Szoka & Papahadjopoulos, 1980). This technique is commonly used in laboratory preparations and implicates three major steps during the production such as vesicle formation, vesicle size reduction and purification. This technique consists in the lipid dissolution in an organic solvent as chloroform, where afterwards this solvent is removed under small pressure vacuum evaporation leading to the formation of a thin film. The composition of the liposomes used is mainly based in phosphatidylcholine (PC) and cholesterol (Drummond et al., 1999). The incorporation of hydrophobic cholecalciferol is through dissolution with the lipids (Wagner & Vorauer-Uhl, 2011). After, the thin film is hydrated with phosphate buffer solution (PBS) where multilamellar vesicles (MLV) are formed. To obtain small unilamellar vesicles (SUV), extrusion of the hydrated lipid films is performed using different filter pore sizes under several cycles. This method is advantageous because allows to obtain high encapsulation percentage of lipophilic substance (Lasic, 1998). After that, the information about liposomes behavior in biological environment and also in storage conditions is effect of parameters such as the size, surface charge, and chemical composition, among others. Besides the surface charge will influence directly the physical stability since the fusion and/or aggregation of liposomes might happen if they are not characterized (Lasic, 1998). Thus in the present study liposomes were characterized in terms of particle size, surface charge, encapsulation efficiency and stability during storage time.

Currently, several preparation methods of lipid-based systems are under clinical trials for some cancer drugs (Cho et al., 2008). Those liposome formulations for chemotherapeutic delivery are already available for human use such as Doxil® which has successful result, that is normally used to treat cancer in AIDS-related Kaposi sarcoma and multiple myeloma, Mycet, DaunoXome, Depocyt (Malam et al., 2009)

(Torchilin, 2005). Another interesting point that has been under development in this particular system is the potential to overcome the multidrug resistance that is usually associated with cancers, whereas with that resistance the liposomes efficiency is reduced. While liposomes have showed good results with the Doxil®, new formulations are been explored (Malam et al., 2009). Drug loading efficiency for water-soluble antibiotics such as gentamicin has low values, meaning low drug-lipid ratio. On the other hand, the use of hydrophobic drugs might induce some delay in the drug release rate (Xu et. al. 2007).

Several chemotherapeutic drugs have been use in liposomes either *in vitro* or *in vivo* as well as paclitaxel, methotrexate, PALA, vincristine, vinblastine, AraC, topotecan, vinorelbine, daunomycin, doxorubicin and cisplatin (Drummond et al., 1999).

Moreover, in the work of Frankenberger *et. al.* (1997) was tested the possibility of entrap 1.25-vitamin D₃ compounds in liposomes to target myeloid leukaemia cells. That possibility was due to the development that liposomes were taken by myelomonocytic cells in various organs. They show that the use of liposomes to deliver 1.25-vitamin D₃ compounds might be a real possibility as consequence of the *in vitro* results obtained (Frankenberger et al., 1997).

3 Materials and Methods

3.1 Chemicals

Poly (D,L-lactide-co-glycolide) (PLGA, Resomer® RG 503 H lactide:glycolide 50:50, acid terminated MW 24-38 kDa), Pluronic® F127 and ethyl acetate were purchased from Sigma-Aldrich (St. Louis, USA). Cholecalciferol (MW 394,65 kDa) was purchased from Alfa Aesar (Karlsruhe, Germany). Uranyl Acetate (dehydrate, MW 424,146 kDa).

1-palmitoyl-2-oleoyl-sn-glycero-phosphocholine (POPC, MW 760,1 g/mol) was purchased from Avanti Polar Lipids, Inc (USA). Cholesterol (C₂₇H₄₆O, 99%), Phosphate buffer solution (PBS, pH 7.4) and EPC-Rhodamine (MW 1249,65 g/mol) were purchased from Sigma-Aldrich (St. Louis, USA). Chloroform (MW 119,38 g/mol) was purchased from Merck (Germany).

All aqueous solutions were prepared in deionized and filtered ultrapure Milli-Q water (Milli-Q Academic, Millipore, France).

3.2 Formulation of PLGA Nanoparticles

The technique for the preparation of PLGA nanoparticles was the emulsification-solvent evaporation method (Danhier et al., 2012). The masses of PLGA (10 mg) without and with cholecalciferol (1.0 mg) were dissolved in ethyl acetate solution (0.100 mL). An aqueous solution of 1% (w/v) Pluronic® F127 (0.200 mL) was added dropwise to the organic phase. The emulsion was then sonicated under ice bath. The mixture was placed into 2.5 mL of 0.1% (w/v) Pluronic® F127. The next step comprises magnetic stirring at 800 rpm for 3 hours at room temperature to fully solubilize the oil phase with the aqueous phase. After the evaporation of the organic solvent, the emulsion is filtered (0.2 µm) to exclude aggregates. The emulsion is stored

at 4 °C during overnight at final concentration of 1 mg/mL, to help in the consolidation of the physical structure of the nanoparticles. The bare and the cholecalciferol-loaded nanoparticles formed a pellet after the centrifugation (14.500 rpm, 30 min) and were re-suspended in ultrapure water. The same procedure is followed for the unloaded nanoparticles preparation.

3.3 Preparation of Liposomes

Liposomes were prepared by the thin lipid film hydration method. In this study, POPC was used for conventional liposome preparation (Szoka & Papahadjopoulos, 1980). Liposomes were prepared with the mixed lipid content of POPC, Cholesterol, rhodamine with molar ratios of 52:45:3 mixed in organic solvent chloroform to assure a homogeneous mixture. Rhodamine is selected because its emission wavelength is higher than for vitamin D and for its fluorescence. In order to prepare liposomes with cholecalciferol, a stock solution was prepared. Stock concentration was 0.63 mM. To evaporate the organic solvent is used a dry nitrogen stream forming a lipid film. Lipid solution was kept overnight through vacuum desiccation pump until a thin lipid film was observed and all of organic solvent was evaporated. The dried lipid film was hydrated with 2 mL phosphate buffer solution (PBS). The suspension was vortexed (15 min) and kept in a sonicator bath for 15 minutes. The vesicle suspension was then extruded through Whatman filters of decreasing size (up to 50 nm pore size membranes) using a pressure extruder apparatus running in discontinuous mode. The suspension is then extruded by multiple cycles. Desalting column (PD-10 Sephadex) was used to change buffers and remove the free cholecalciferol from the liposomes. The total lipid concentration was 0.01 M.

3.4 Physicochemical Characterization

The size, polydispersity index, zeta potential and morphological appearance were the parameters used to characterize the produced nanoparticles and liposomes.

3.4.1 Particle Size Distribution

Dynamic light scattering (DLS) is a very useful technique for the measuring of size, size distributions and in some cases the shapes of nanoparticles in liquids. This technique is targeted to measure hydrodynamic properties such as the translational and/or rotational diffusion coefficients, which are then related to sizes and shapes via theoretical relations. This technique is based on the intensity of a light scattered into a dispersion of particles with a given scattering angle. The particles in a solution are in Brownian motion, and when the light is sent through causes a fluctuation in the intensity. Thus, this intensity fluctuation is measured due to the real-time calculation of the scattered intensity time correlation function. For spherical monodisperse particles, the Stokes-Einstein relation relates the translational self-diffusion coefficient to the particle radius R ;

$$D = \frac{k_B T}{6\pi\eta R} \quad (1)$$

where k_B is the Boltzmann constant, T the absolute temperature and η the viscosity of the suspending medium.

Also, the nanoparticle dispersions are usually polydisperse, in the measure cell. It is possible to assume that may be a distribution of particles with several sizes and shapes (Pecora, 2000).

The particle size and polydispersity index (Pdl) were measured by DLS with a ZetaSizer Nano ZS (Malvern Instruments, Worcesterchire, UK), using a clear disposable zeta cell at approximately 25 °C and an angle of the laser incidence 173°. The results obtained from measurements of each batch were expressed in triplicate.

3.4.2 Zeta Potential

All particles in suspension exhibit a zeta potential, or surface charge which is result of the ionization of the surface groups or adsorption of charged species. Besides size and concentration of particles are key parameters to the measurements. In a solution, the movement of the particles through solution due to an applied voltage,

where at same distance from every particle exists a boundary, which the ions do not move with the particle. This potential is defined as zeta potential (ζ). The zeta potential is crucial to understand the stability of a colloidal suspension. The electrophoretic mobility is the used technique to measure the zeta potential, where an electric field is applied which induces charged particles to move. By measuring the electrophoretic mobility of a particle, the zeta potential may be calculated using the Henry equation;

$$U_E = \frac{2\varepsilon\zeta f(ka)}{3\eta} \quad (2)$$

Where U_E is the electrophoretic mobility, ε is the dielectric constant, ζ the zeta potential, η the viscosity and $f(ka)$ the Henry's function, which for aqueous medium and moderate electrolyte concentration is considered 1.5. In that case, the zeta potential can be determined from the electrophoretic mobility measured by the Smoluchowski equation;

$$\zeta = \frac{\eta U_E}{\varepsilon} \quad (3)$$

The suspension is considered stable if it presents a zeta potential higher than 30 mV in modulus. If this value is between -30 and 30 mV, the system is considered unstable because when it is weakly charged there is no force to prevent the particles to aggregate (Sze et. al. 2003).

The zeta potential of the nanoparticles is assessed using the dynamic light scattering (DLS) with ZetaSizer Nano ZS (Malvern Instruments, Worcesterchire, UK). All the determinations were performed in a clear disposable zeta cell at approximately 25 °C and a light scattered at an angle of 17°. The results were obtained in triplicate for each measurement.

3.4.3 Morphologic Analysis

Transmission Electron Microscopy (TEM) is a microscopy technique that uses a beam of electrons instead of light to observe the specimen. When the beam of electrons

interacts with the atoms in a sample, two types of scattering resulted from the incidence, as elastic and inelastic scattering. Then, the electrons are emitted by a source and are focused and magnified by a system of magnetic lens. Also, the electrons that are elastically scattered are the transmitted beams, which are the ones that pass through the objective lens. The objective lenses are the responsible to produce the image for the microscope (Voutou & Stefanaki, 2008).

The morphology of the nanoparticles is observed by TEM. For the following experiment, a sample of 10 μL of the solution PLGA/vitamin D₃ is placed into a copper TEM grid (Formvar/Carbon on 400 mesh Cu (50) from Agar Scientific). After drain out the excess fluid at room temperature, the grid is stained with 2% (v/v) uranyl acetate. The samples were air-dried and examined using a transmission electron microscope at an accelerating voltage of 80kV. The images are observed on a TEM Yeol Yem 1400 (80kV) (Tokyo, Japan) with a Digital Camera Orious 1100 (Japan).

3.5 Cholecalciferol Loading/Encapsulation Efficiency

Encapsulation efficiency (EE) is defined as the percentage of the mass of drug encapsulated in the polymeric carrier relative to the initial amount of drug added during the preparation (Li et al., 2011). The vitamin D encapsulation efficiency (EE) of PLGA nanoparticles was calculated by the equation 4.

$$\text{EE (\%)} = \frac{\text{total amount vitamin} - \text{unloaded amount vitamin}}{\text{total amount vitamin}} \times 100\% \quad (4)$$

Due to the modification of the initial protocol to produce the nanoparticles by the solvent-evaporation method, the samples are suspended from the agitation and then they were centrifuged at 14,500 rpm for 30 minutes using a MiniSpin Plus (Eppendorf). The samples were centrifuged to remove the extra PLGA and the loaded vitamin D. Then the absorbance of the supernatant was measured, using a dilution factor (5x). On the other hand, the measurement of cholecalciferol content in liposomes was performed by UV – 1700 PharmaSpec UV-Vis spectrophotometer from Shimadzu (Japan). Firstly,

the unloaded cholecalciferol was separated from liposomes through Sephadex PD-10 desalting column. In order to verify the location of vitamin, several aliquot of liposomal suspensions were diluted with phosphate buffer solution (PBS) at pH 7.4. Then, to apply the dilution ratio 1:4 for liposomes and ethanol to achieve the disruption of the liposome structure, enabling the release the drug into the solution. The solution is used to measure the absorbance at 265 nm. Drug content was estimated according to the following equation (Hazra et. al. 2005).

$$EE (\%) = \frac{\text{Amount of drug in liposomal suspension after filtration through column}}{\text{Amount of drug in liposomal suspension before filtration through column}} \times 100\% \quad (5)$$

3.6 Stability of Liposomes

Generally, for the lipids used to manufacture liposomes is necessary to establish the storage conditions and the shelf life time (uniformity of size distribution and minimal structural degradation) (Lasic, 1998). Furthermore, the physical stability of liposome is due to the integrity and the size distribution of the lipids. Besides, liposomes are susceptible to fusion, aggregation, and leakage of the drug substance during storage (Stark et al., 2010). To evaluate the physical stability of liposomes with regard to the retention of the loaded content, daily observation of size, polydispersity index, and zeta potential were assessed for the integrity and size of the liposomes and vitamin D loaded. The liposomal systems were maintained at 4 °C during the storage period (du Plessis et. al. 1996).

3.7 Cytotoxicity Studies

Immortalized human pancreatic duct epithelial cells (hTERT-HPNE) were maintained in DMEM with 10% fetal bovine serum (FBS) under 5% CO₂ humidified atmosphere at 37 °C. The hTERT-HPNE cells were seeded in 96-well plates at a density of 10⁴ cells/mL, for a period of incubation of 24 hours. The cells were then treated for

48 hours with vitamin D and PLGA/vitamin D nanoparticles at the concentrations ranging between 0.001 and 100 nM. After the incubation period (48 hours) for the samples of nanoparticles and free vitamin D, cell viability was determined by PB and SRB assays. In order to study the cytotoxic effect of the incubations, the following assays PrestoBlue™ (PB) cell viability reagent (Invitrogen, USA) and protein-binding dye sulforhodamine B (SRB) were used. The PB reagent, when added to the cells is modified by the reduced environment of the viable cells turning red in colour to become highly fluorescent, where it can be measured by fluorescence measurement at 560/590 nm. As the SRB assay is used to estimate the cell density and the drug-induced toxicity by the measurement of cellular protein content. For PB assay, 50 µL of PB reagent, diluted 1:10 in DMEM, was incubated in each well for 45 minutes, at 37°C in humidified atmosphere with 5% CO₂, followed by the fluorescence reading (excitation at 560 nm and emission at 590 nm) using a microplate reader (PowerWave HT Microplate Spectrophotometer, BioTek). On the SRB method, the cells were fixed *in situ* with 50 µL of PBS and 25 µL of TCA for 1 h, in refrigerator. After the incubation period, cell monolayers were washed and stained with 50 µL of SRB dye for 30 minutes. After washed repeatedly with 1% (v/v) of acetic acid to remove the excess dye and dried, the protein-bound dye is dissolved in 10 mM Tris base solution for absorbance determination at 560 nm using a microplate reader (PowerWave HT Microplate Spectrophotometer, BioTek). Concentration-response curves using non-linear regression analysis were obtained and the inhibiting cell survival by 50% (IC₅₀) was determined. IC₅₀ is defined as the drug concentration needed to kill 50% of the incubated cells in a designated time period (Skehan et al., 1990; Vichai & Kirtikara, 2006).

3.8 Statistical Analysis

Data were calculated with the mean and standard deviation. Student's two-sided t-tests were performed to compare data sets using p-values ($P < 0.05$) to determine statistical significance (McDonald, 2009).

4 Results and Discussion

4.1 PLGA/cholecalciferol system

A detailed study was developed in order to optimize the adequate conditions of the physicochemical characteristics of the bare PLGA and PLGA/cholecalciferol nanoparticles prepared by the emulsion-solvent evaporation technique. Consequently, the influence of the sonication time a critical step in the emulsion procedure to define the size of the nanoparticles was analyzed. The methodology for the encapsulation of cholecalciferol in the PLGA nanoparticles was also optimized.

The *in vitro* cytotoxic effects on a human normal pancreatic cell line upon its treatment with PLGA/cholecalciferol nanoparticles were assessed, comparatively to the free cholecalciferol.

4.1.1 Characterization of PLGA nanoparticles

The nature and volume of the organic and the aqueous phase, as well as the properties and employed surfactant and polymer concentrations provide important consequences in the size distribution of nanoparticles. These parameters were already optimized by our group for the PLGA nanoparticles (Frasco et. al. 2012).

Likewise, the intensity of the shear rate of the homogenization step helps to determine the final mean diameter size of the nanoparticles (Moinard-Chécot et al., 2008).

PLGA nanosized particles were induced by sonication. The sonication time was evaluated for 1 and 5 min. In these conditions, the physicochemical properties of the PLGA nanoparticles are summarized in Table 1. The sonication time is important to accomplish a good emulsion and also the formation of smaller sized particles (Mainardes & Evangelista, 2005). This step was performed in a sonication bath, with ice to avoid suspension overheating because excessive temperature heating can cause

substantial liquid evaporation resulting in changes to the sonication volume, or the degradation of the formulation components (Jwala et al., 2011). Thus, the effectiveness of the applied sonication conditions can be mainly evaluated by the results attained in the particle size distribution. The slightly reduction of the average particle size and polydispersity are indicative of a more effective dispersion. Although the statistical results had shown there for all parameters the difference was not significant ($P > 0.05$). It was decided to use a sonication time of 5 minutes for the following experiments. The mean PLGA nanoparticles sizes obtained were concordant when compared to values found in the literature are approximately 200 nm (Moinard-Chécot et al., 2008). Additionally, zeta potential measurements confirmed the negative values due to the use of non-ionic surfactant Pluronic® F127 and the presence of polymer terminal carboxylic groups. Zeta potential negative values are expected for PLGA nanoparticles due to the existence of the polymer terminal carboxylic groups. Moreover, when the range values are between -25 mV and -30 mV it is normally to expect good colloidal stability. This is explained by the high energy barrier that exists between the particles in suspension (Mora-Huertas et al., 2010).

Table 1 – Physicochemical characterization of the bare PLGA nanoparticles, with different sonication time
Data represented as mean $\pm$ SD (n=3).

Sonication Time	PLGA NPs	Mean Diameter (nm)	PdI	Zeta Potential (mV)
	1 min	176 $\pm$ 2	0.10 $\pm$ 0.09	-34 $\pm$ 4
	5 min	172 $\pm$ 4	0.06 $\pm$ 0.04	-38 $\pm$ 3

The single emulsion solvent evaporation method (Danhier et al., 2012) allowed the encapsulation of vitamin D. The size, polydispersity index and zeta potential values for bare and cholecalciferol-loaded PLGA nanoparticles are presented in Table 2.

Table 2 – Physicochemical characterization of the bare PLGA and cholecalciferol loaded nanoparticles, with 5 min sonication time. Data represented as mean $\pm$ SD (n=3).

PLGA NPs	Mean Diameter (nm)	PdI	Zeta Potential (mV)
Bare/unloaded	176 $\pm$ 4	0.04 $\pm$ 0.01	-40 $\pm$ 0.3
Cholecalciferol-loaded	185 $\pm$ 6	0.08 $\pm$ 0.04	-29 $\pm$ 4.0

The size and the polydispersity index increased for the cholecalciferol loaded nanoparticles when compared with the bare PLGA nanoparticles, but the differences are not significantly different ($P > 0.05$). It is assumed that the slightly increase on mean diameter value was due to the inclusion of cholecalciferol. The single emulsion solvent evaporation method had already shown high efficiency for the loading of lipophilic drugs (Dinarvand et. al. 2011). When cholecalciferol is added to the nanoparticles it can be adsorbed in the surface and cause a small decrease in the zeta potential value. However, the values for zeta potential are concordant with literature leading to a stable colloidal dispersion (Mukherjee et. al. 2008). The same explanation is valid for the slight increase of mean diameter. These parameters play an important role for determining, the future drug release behaviour, specially their small size to avoid the capture from phagocytes retaining their presence in blood circulation (Dinarvand et. al. 2011). Moreover, PLGA possess negative charges and is stabilized in the process with the emulsifying agent Pluronic® F127 as already demonstrated (Mura et al., 2011). These stabilization mechanisms are function of DLVO interactions and also due to the role of hydration forces of the surfactant (Santander-Ortega et al., 2006). Pluronic® F127 amphiphilic character has the capacity to establish interactions with hydrophobic surfaces and even with biological membranes. Although it cause negatively charged nanoparticles confirmed by their value bringing stabilization to the colloidal system. Also steric repulsion by adsorption at the nanoparticles surface and the existence of the polymer terminal carboxylic groups helps to maintain the negatively charged surface (Mura et al., 2011). This relationship between PLGA nanoparticles and Pluronic® F127 his advantageous because may neutralize the acidic environment resulted from the polymer degradation and might help in the drug interaction with the PLGA (Santander-Ortega et al., 2006). Also, these results are in agreement with the transmission electron microscopic (TEM) images (Figure 3).

4.1.2 Vitamin D encapsulation efficiency

Drug encapsulation efficiency analysis was carried out in triplicate and expressed as percentage of drug in the produced nanoparticles with respect to initial amount (mg) used for the formulation of PLGA nanoparticles. The vitamin D entrapment efficiency was found to be $74 \pm 8\%$.

Regarding the values for encapsulation efficiency for emulsion-solvent evaporation technique are approximately 80%, the range values presented are similar to those founded in literature (Mora-Huertas et al., 2010). Moreover, in the work of Almouazen *et. al.* PLA nanoparticles for cholecalciferol entrapment were prepared with the nanoprecipitation technique, and high values of encapsulation efficiency were achieved (Almouazen et al., 2013).

It can be observed that higher mean diameter may result in high quantity of cholecalciferol entrapped. However due to the experimental procedure, some polymer loss can be assumed resulting in the variation of the mean size diameter registered. This is due to the loss of organic solvent or even some polymer. Also the higher mean diameter observed in the table 2 is probably due to the slightly change in the diffusion of the organic solvent caused by removal of Pluronic® F127 and then originating larger particles. On the other hand, if the Pluronic® F127 volume is in the suspension that could be one of the reasons to decrease the nanoparticless mean diameter size making them more stable. The reason is that smaller particles have higher surface area/volume ratio which becomes an advantage for drug encapsulation (Zhang & Feng, 2006). Moreover, higher absolute values of zeta potential indicate strong repulsive forces among the particles to prevent aggregation in the medium (Mu & Feng, 2002). Besides, EE might increase due to the effect of the Pluronic® F127 which can help to accumulate the drug in the hydrophobic core (Lin et. al. 2005).

It can be assumed that the formation of the nanoparticles with the diffusion method depends on several parameters. Namely, the concentration of the liposoluble compound in the organic phase which is capable of alters the mean diameter of the nanoparticles. The polymer concentration can also modify the thickness of the

nanoparticle membrane. Still, the volume used can also influence directly in the final mean diameter size of the NPs (Guinebretière et. al. 2002). Therefore is crucial to control the key aspects of the experimental process (Y. Liu et al., 2010).

4.1.3 Morphologic Analysis

The morphologic examination of the nanoparticles was performed by TEM. The obtained images for formulations of bare and cholecalciferol-loaded PLGA nanoparticles are shown in Figure 3.

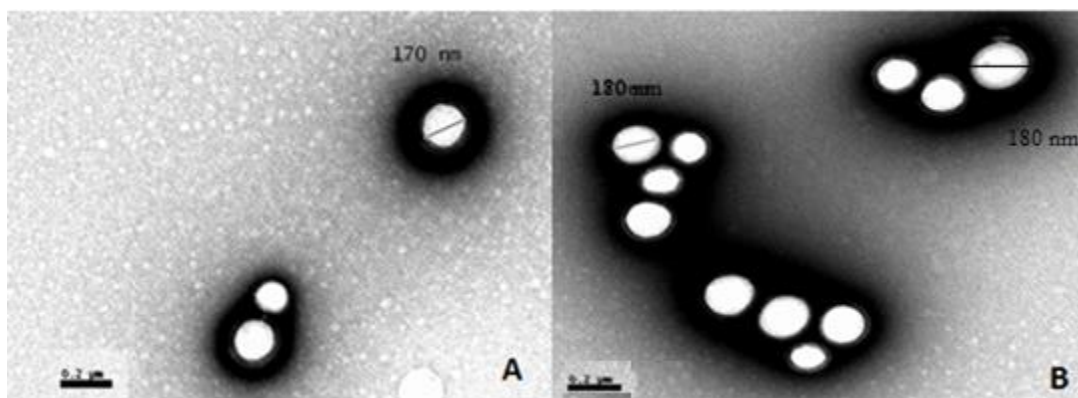


Figure 3 - TEM micrograph of free (A) bare PLGA and (B) PLGA/cholecalciferol nanoparticles.

The PLGA nanoparticles were found to be spherical in shape with nano-sized range. The dark shadow in the image is probably due to some Pluronic® F127 residues that are still present from the separation of the pellet from the supernatant. Besides, the diameter determined by this method is ranged between 170 and 180 nm, which is supported by the size measurement performed by DLS.

4.1.4 Cytotoxicity Studies

The *in vitro* cytotoxic studies of PLGA/vitamin D formulations were performed on immortalized human pancreatic duct cells (HPNE) cells. The nanoparticles were tested and compared to free vitamin D using PB and SRB assays. The attained results

are presented in figure 4 and they show the decrease effect in cell survival after 48 h of exposure. This study was appreciated to evaluate the behavior of the interaction of vitamin D and PLGA/vitamin D nanoparticles with the cells viability. The results obtained by PB method are in figure 4A and the results by SRB method are in figure 4B.

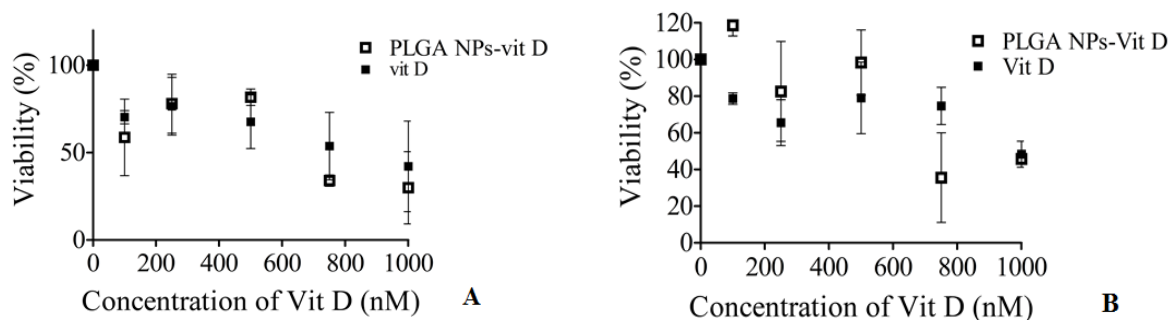


Figure 4 – Viability of HPNE exposed to vitamin D free and PLGA/vitamin D: (A) determined by PrestoBlue™ assay; (B) determined by Sulforhodamine B assay.

The results show the relationship between the vitamin D and PLGA/vitamin D nanoparticles concentration (nM) and cell viability (%). Several studies had shown the higher drug cytotoxicity due to the nano-encapsulation technique which is able to increase the cellular uptake (Wong et. al. 2007). The results also reveal that both PLGA/vitamin D and vitamin D alone had substantial toxic impact to cells after 48h exposure. It is observed the similar behavior for both assays and they are no significantly different from each other ($P > 0.05$). Although it is noted a different behavior for the concentration 500 nM since the drug is encapsulated and the values are higher when compared to the vitamin D alone. This substantial toxic impact is important because non-tumoral cells are used in this study. However to better understand the role of NP uptake by the cells, further fluorescent microscopy studies needed to be performed. Nonetheless, the goal of this study was to evaluate the cytotoxic behavior of the vitamin D in *in vitro* studies.

The obtained IC_{50} values by both methodologies (PB and SRB) are concordant with each other and presented on the table 3.

Table 3 - Concentration of PLGA/vitamin D and vitamin D free required killing 50% of HPNE cells. Data represented as mean $\pm$ SD (n=3).

Nanoparticles	IC ₅₀ (nM)	
	PB	SRB
Vitamin D	631 $\pm$ 43	851 $\pm$ 16
PLGA/vitamin D	661 $\pm$ 31	512 $\pm$ 27

These complementary quantitative assays reveal that these nanoparticles inhibited the cell viability. While a major difference is obtained by SRB method, where higher values of cell survival are registered causing a higher value of IC₅₀, these results might be explained by the controlled release of the PLGA nanoparticles after cellular uptake related with the delayed therapeutic effect of the drug. In the PB method lower vitamin D free value is observed when compared with the presence of PLGA/vitamin D nanoparticles. To obtain lower cytotoxicity, higher concentration of vitamin D to encapsulate is needed to report a cell death rate of 50%. Moreover, for HPNE cells it is advantageous that less cytotoxicity is achieved. The IC₅₀ values of both methodologies are not significantly different ($P > 0.05$) meaning they are concordant with each other. In accordance with literature, in medicine the cytotoxicity only should be revealed in low concentration in the range of 10^{-4} and 10^{-5} M. In the same order if the value of IC₅₀ is higher than 10^{-4} M is assumed that the toxic effect is increased to tissues and healthy organs (Bojat V., 2011). Several studies agreed that the *in vitro* active substance release behavior depends on a group of parameters such as the concentration and physicochemical characteristics of the active substance, solubility, the nature degradability, molecular weight and concentration of the polymer solid matrix when re-precipitated, the nature of oil compound, nanoparticle size. Likewise depends on the conditions of the *in vitro* release test as medium pH, temperature, contact time and conditions of the preparation method (Mora-Huertas et al., 2010). However, further studies need to be performed to elucidate the *in vitro* and *in vivo* cytotoxicity of this system. So far, *in vivo* studies for delivery of vitamin D were not found in literature.

4.2 Liposomes/cholecalciferol System

A different encapsulation system, based on liposomes as carrier vehicle was tested to find favourable formulations for cholecalciferol. It is well studied that several anticancer drugs had already been incorporated into liposomes, for example liposomal doxorubicin (Drummond et al., 1999). This system contributes for higher therapeutic efficacy at low dosage, higher biological half-life and also provides stability against enzymatic degradation (Elbayoumi & Torchilin, 2010). The physicochemical properties as size and lipid composition are the essential factors of stability and *in vivo* behavioural of liposomes (Harashima et. al. 1995). Hence was performed a characterization study to comprehend the interaction that vitamin D might cause when is incorporated into liposomes. For this work liposomes were prepared by modified thin film hydration technique. Further size homogenization was achieved by extrusion technique which avoids the application of high temperatures and shear stress forces that could damage the liposomal structure (Stark et al., 2010).

4.2.1 Characterization of liposomes and Vitamin D encapsulation efficiency

The results of the sizes, polydispersity index and zeta potential of the unloaded and cholecalciferol-loaded liposomes are presented in the table 4.

Table 4 – Physicochemical characterization of unloaded and cholecalciferol-loaded liposomes. Data represented as mean $\pm$ SD (n=3).

Liposomes	Mean diameter (nm)	PdI	Zeta Potential (mV)
Unloaded	77.1 $\pm$ 0.6	0.09 $\pm$ 0.01	-6 $\pm$ 2
Cholecalciferol-loaded	78.4 $\pm$ 0.2	0.11 $\pm$ 0.01	0.4 $\pm$ 0.2

The size distribution and zeta potential value of liposomes unloaded and cholecalciferol-loaded are significantly not different ($P > 0.05$). However, in the case of the cholecalciferol-loaded liposomes, the mean diameter value has the tendency to be higher due to the location of the cholecalciferol in the hydrophobic area of the bilayer that increases the liposome packing. The size distribution is an important parameter for liposomes due to the drug accommodation and also for the recognition in the blood circulation enabling the uptake by the mononuclear phagocytic system (MPS) (Hupfeld et. al. 2006). The mean size of liposomes is determined by the selected pores of the extrusion technique which does not degrade the phospholipids and even can increase the encapsulation efficiency (Olson et. al. 1979). The presented measurements were obtained after the decreasing filter pore sizes, and as expected with the application of the smaller pore size (50 nm) resulted in a liposomal formulation with smaller mean diameters (77-78 nm).

The zeta potential of the liposomes is approximately zero, thus confirming the neutrality of POPC (Cohen & Khorosheva, 2001). Although some minimal change in the value is detected that may be attributed to the aggregation or fusion of some liposomal particles. Also with the application of potential, the liposomes are more susceptible to destabilization. In the opposite, the values for the polydispersity index are not significantly different ($P > 0.05$). Thus knowing the physical characterization of liposomes such as size and zeta potential is important and can help to understand the fate of the liposomes when undergoing *in vivo* experiments (Brandl, 2001).

The encapsulation efficiency achieved for this system was 67%. The values were measured by UV-Vis and showed the ability of the liposomes to entrap cholecalciferol, since the value is satisfactory. In literature, several other studies were carried out where they show the positive effect of the presence of cholesterol in the drug encapsulation (Ramana et. al. 2010). As it is believed that cholecalciferol is incorporated entirely in the bilayer, so the encapsulation efficiency is related to the values founded in the literature for water-insoluble drugs trapped in the hydrophobic region. Unlike the water soluble compounds, the hydrophobic ones are more resistant to loss by diffusion resulting in superior values of encapsulation efficiency (Nii & Ishii, 2005). Furthermore, Also it is assumed that hydrophobic substances are efficiently incorporated between the bilayer in the preparation process for multilamellar vesicles

(Kulkarni & Vargha-Butler, 1995). The results suggest the affinity of the cholecalciferol for the liposome membrane. Normally trial and error tests are performed to optimize the liposomal encapsulation instead of the investigation of the factors. Although it is necessary to take into account the initial conditions both for liposomes and for the drug (Kulkarni & Vargha-Butler, 1995).

4.2.2 Stability test

For their successful application as drug carriers, liposomal formulations need to be controlled regarding their stability. Liposomes tend to aggregate and fuse which results in the loss of the entrapped compound during storage or even during administration where its ability is diminished. Another problem that might appear during storage for a long period of time is the oxidation of phospholipids that will modify the liposome structure (Takeuchi et al., 1998). The physical stability of liposomes on storage can be studied by monitoring the size and eventually the zeta potential of liposomes. The test was performed to evaluate the physical stability of liposomes alone and regarding to the retention of the entrapped content during 3 weeks. The physical stability of the liposome was evaluated by measuring the change in particle size and zeta potential. The values obtained are shown in the table 5.

Table 5 – Stability test study of unloaded and cholecalciferol-loaded liposomes. Data represented as mean $\pm$ SD (n=10).

Liposomes	Mean diameter (nm)	PdI	Zeta Potential (mV)
Unloaded	78.5 $\pm$ 0.9	0.09 $\pm$ 0.01	-4 $\pm$ 1.6
Cholecalciferol-loaded	78.8 $\pm$ 0.6	0.12 $\pm$ 0.01	-1 $\pm$ 0.9

The results obtained for size diameter was approximately 79 $\pm$ 0.6 nm in average for both formulations and as zeta potential values -4 mV for liposomes alone and approximately -1 mV for liposomes loaded with vitamin D. The mean diameter and the

PdI values were not statistically significant ($P > 0.05$), however, the zeta values had shown to be significantly different ($P < 0.05$). Generally, the shelf-life is determined by the physical stability where the uniformity of size and minimal degradation of the compounds is observed (Sabin et. al. 2006). Besides, the presence of cholesterol in liposomes structure is responsible to enhance overall stability, however non-coated liposomes are still susceptible to destabilization more easily than the coated ones. The size is an important aspect for stability, where aggregation and fusion are the main sources to install instability (Takeuchi et al., 1998). Moreover, the formation of liposome aggregates is a natural and is difficult to avoid for neutral liposomes. Besides, those physical processes can result in the loss of the cholecalciferol loaded and changes in the size. However, liposomes containing phosphatidylcholine (PC) are considered the more stable than other type of phospholipids due to their neutral charge (Ulrich, 2002). The particle sizes are maintained during the stability control time period which express is stability after 3 weeks days in storage and also shows the ability of liposomes to incorporate cholecalciferol. It was already observed that liposome vesicles containing phosphatidylcholine (PC) with cholesterol exhibit better ability to deal with shear stress. Likewise, shown the increase of retention capacity by decreasing the membrane permeability which can work as positive or negative feature for drug delivery application (Tseng et. al., 2007). Also it is observed change in the zeta potential however it remains minimal. A possible explanation is the adsorption of some layer at the surface or even the nature composition of the medium in which liposomes are suspended during the storage time can cause that minimum change. Usually the zeta potential measurement does not play an important role for evaluate the liposomal stability except if was possible to control the effects of the applied charge by an electronic equipment. In this case the zeta potential is supplementary with the size diameter measurements, and should be regarded to support the discussion (du Plessis et al., 1996).

5 Conclusion and Future Perspectives

5.1 Conclusion

Vitamins have been the subject of interest in several research fields to investigate their physiological benefits and methods to preserve their chemical integrity. Encapsulation has appeared as a promising approach to protect and take advantage of vitamin properties over time. In that order the encapsulation process should provide particles with a high encapsulation rate, good polydispersity index, high shelf life, avoid possible side effects and providing control delivery into the target. Here vitamin D a liposoluble compound was studied which among others has particular anticancer properties and it was verified that some limitation exists associated with vitamin D encapsulation in the literature. Nanotechnology has been regarded as one of the most promising techniques to deal with cancer. Likewise, to provide a solution polymeric nanoparticles and liposomes are the two primary choices for nano-carriers for anticancer drug delivery. Polymeric nanoparticles has favourable characteristics like small size, long circulation half-life, ease of fabrication conditions, controlled release manageable and are able to encapsulate drug of poor solubility. The biodegradable polymer selected for this study was PLGA, which is already well-known to be used as a drug delivery vehicle. On the other hand, liposomes are widely used due to the high biocompatibility registered, ease of surface modification and liposomal formulations are already in use as anticancer drug for human use.

In this work, PLGA-based nanoparticles were prepared by the solvent-emulsion method. Also was possible to perform the physicochemical and morphologic characterization, shown by TEM images, and to verify their behaviour in *in vitro* cytotoxic studies. Cholecalciferol-loaded nanoparticles were produced with a mean diameter of approximately 185 nm, as well as a zeta potential value of -29 mV and with a encapsulation efficiency near 80%. The concentration-response studies on HPNE cells

shown a reduced the cytotoxic effect of the PLGA/vitamin D nanoparticles compared with the free vitamin.

In the other proposed system, liposomes were prepared by the modified thin film method. Cholecalciferol-loaded liposomes reveal a mean diameter of 78 nm and the values of zeta potential tending to neutral charge. The encapsulation efficiency obtained was 67%, revealing the satisfactory uptake of vitamin D by liposomes. Stability studies were performed to evaluate the bioavailability and shelf life of the liposomes and vitamin D-loaded liposomes. The physical stability was maintained over 3 weeks presenting their ability as carrier system.

5.2 Future Perspectives

There are several experimental tests that after the conclusion of this work could had been interesting to perform, but were not possible due to time restrictions.

The developed work was important to understand the behavioural conditions that influence the vitamin D entrapment efficiency for both PLGA nanoparticles and liposomes. However, further experimental studies are required to support the knowledge of these systems as well as drug loading or process yield. Some other studies may be accomplished to evaluate the results such as the change of the polymer ratio or even the possibility of coating the particles since some authors defend the coating of PLGA nanoparticles. The body recognizes the particles with hydrophobic surfaces as foreign and to overcome that limitation the nanoparticles can be coated to have a hydrophilic surface. Also is important to study the release profile of the vitamin D nanoparticles by dialyses to further application in cancer cells, as well as stability study to fully characterize their conditions for long periods of time.

Stability tests with the analysis of vitamin D absorbance would be helpful to understand if the vitamin is still active inside the liposomes. Moreover, for liposomes a morphologic characterization by TEM observation should be performed as well as stability tests using differential scanning calorimetry (DSC) and also cytotoxic *in vitro* studies to fully characterize this system.

6 References

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7 Appendix

A – Cholecalciferol calibration curves

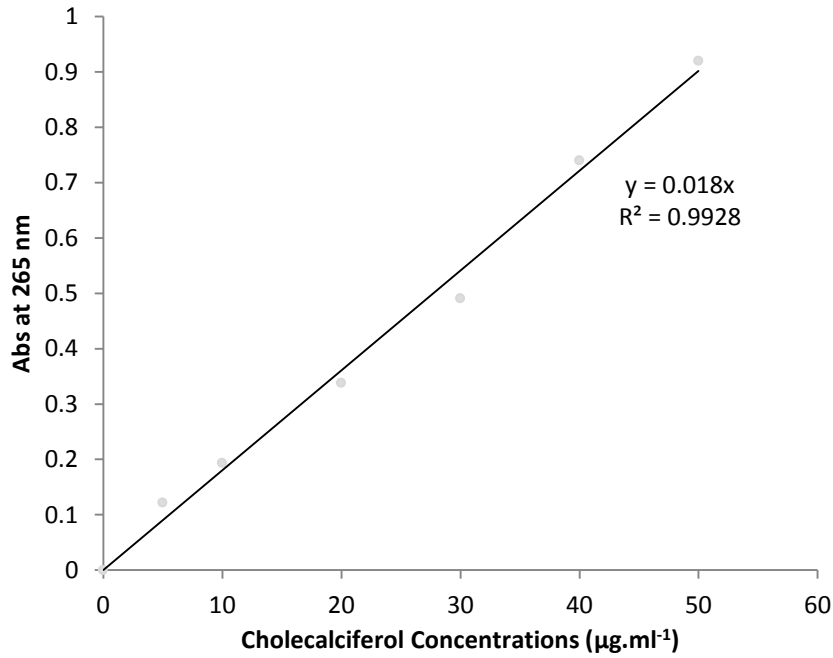


Figure 5 - Cholecalciferol calibration curve in ethyl acetate and 0.1% Pluronic F127. Data represented as mean (n=3).

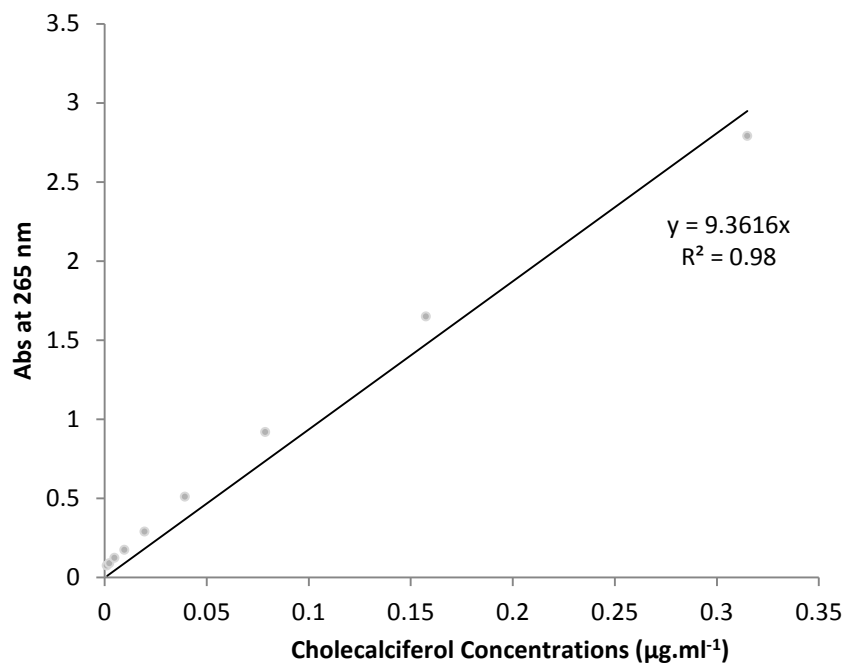


Figure 6 - Cholecalciferol calibration curve in ethanol. Data represented as mean (n=3).

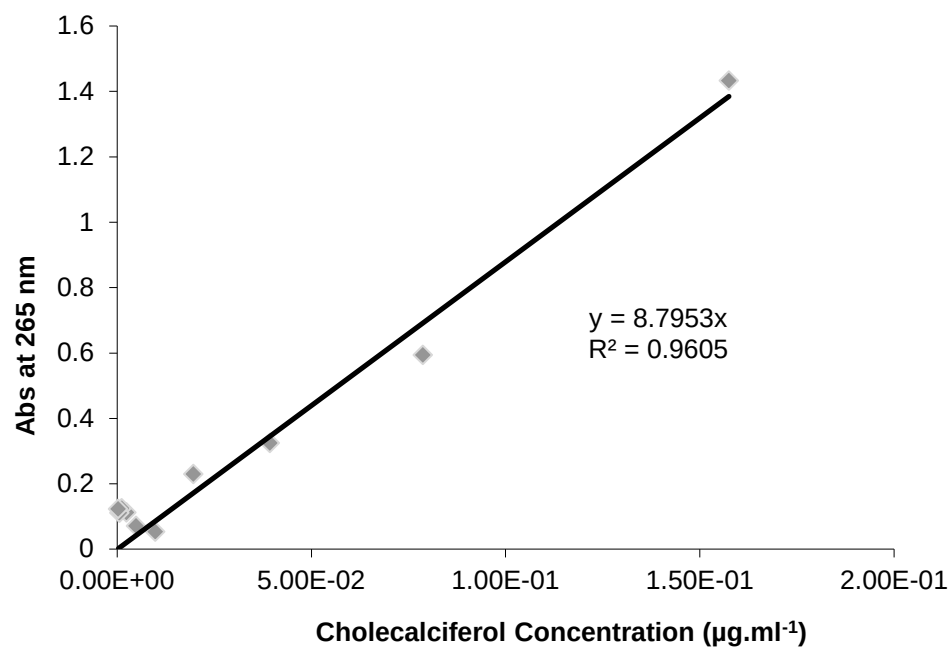


Figure 7 - Cholecalciferol calibration curve in PBS and ethanol (in ratio 1:4). Data represented as mean (n=3).