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DESIGN, DEVELOPMENT AND CHARACTERIZATION OF INNOVATIVE LIPID NANOCARRIER-BASED EPIGALLOCATECHIN GALLATE DELIVERY SYSTEM FOR PREVENTIVE AND THERAPEUTIC SUPPLEMENTATION

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ABSTRACT

Nowadays the society is facing a large health problem with the rising of new diseases like heart diseases, diabetes, neurodegenerative diseases, and obesity. For many years, these diseases were only found in the elders and with low incidence, but nowadays they are emerging in a more aggressive way, in a larger and younger group of people. Thus, it is important to invest in substances that enhance the health of the population. In this context, epigallocatechin gallate (EGCG) is a flavanol naturally found in many plants, especially in tea, being a molecular modulator of various inflammatory and metabolic pathways and possessing high antioxidant activity. Several studies support that EGCG shown several benefits in fighting cancer, diabetes and heart diseases, among others. Nevertheless, the intestine poorly absorbs EGCG, which is the main drawback to be used as a supplement or as a therapeutic drug.

In this work, solid lipid nanoparticles and nanostructured lipid carriers were developed and studied as transporters and bioavailability enhancers of EGCG. The mean diameter of the nanoparticles was found to be around 325 nm, which is suitable to be used by oral route. Moreover, EGCG was effectively encapsulated and with a remarkable efficiency of encapsulation of 80% and 90% for SLN and NLC, respectively. In addition, high storage stability for the formulations is expected since they maintain the initial characteristics for 10 weeks. These nanoparticles present high stability in simulated gastric medium with minimal EGCG release and an moderate stability in simulated intestinal fluid with controlled released of EGCG. The viability (MTT) and cytotoxicity (LDH) assays demonstrated that the nanoformulations possess a low toxicity, having potential to be used *in vivo*.

KEYWORDS: bioavailability, epigallocatechin gallate (EGCG), lipid nanoparticles, nanocarrier system, nutraceutical, supplementation.

RESUMO

Atualmente a sociedade está a enfrentar um grande problema de saúde com o surgimento de novas doenças, como doenças cardíacas, diabetes, doenças neurodegenerativas e obesidade. Durante muitos anos, estas doenças eram encontradas com baixa incidência e surgiam maioritariamente em pessoas idosas, mas hoje em dia elas estão a surgir de uma forma mais agressiva, num grupo maior e mais jovem de pessoas. Assim, é importante estudar substâncias que melhoram a saúde geral da população. Neste contexto, o galhato de epigalhocatequina (EGCG) é um flavanol encontrado naturalmente em muitas plantas, especialmente no chá, sendo um modulador molecular de várias vias inflamatórias e metabólicas e tem uma elevada atividade antioxidante. Vários estudos suportam que o EGCG demonstra vários benefícios no combate ao cancro, diabetes e doenças cardiovasculares, entre outras. No entanto, a absorção intestinal de EGCG é muito baixa, sendo a principal dificuldade para ser utilizada como um suplemento ou como uma substância terapêutica.

Neste trabalho, nanopartículas lipídicas sólidas (SLN) e carregadores lipídicos nanoestruturados (NLC) foram desenvolvidos e estudados como possíveis transportadores e sistemas que aumentem a biodisponibilidade do EGCG. O diâmetro médio das nanopartículas foi de aproximadamente 325 nm, o que é adequado para serem utilizadas por via oral. Além disso, EGCG foi encapsulado com uma eficiência de encapsulação elevada de 80% e 90% para as SLN e NLC, respetivamente. Além disso, as formulações apresentam elevada estabilidade, mantendo as características iniciais ao longo de 10 semanas a 25 ºC. Estas nanopartículas apresentam uma elevada estabilidade em meio gástrico simulado, apresentando uma libertação mínima de EGCG e uma estabilidade moderada, apresentando uma libertação controlada em meio intestinal simulado. Os ensaios de viabilidade (MTT) e citotoxicidade (LDH) demonstraram que as nanoformulações possuem uma baixa toxicidade e como tal poderão ter potencial para serem utilizadas *in vivo*.

PALAVRAS-CHAVE: biodisponibilidade, epigalocatequina galato (EGCG), nanopartículas lipídicas, nanosistemas de transporte, nutracêutico, suplementação.

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ABBREVIATIONS AND SYMBOLS

AD – Alzheimer's disease

BBB – blood brain barrier

COX-2 – cyclooxygenase-2

DLS – dynamic light scattering

DMEM – Dulbecco's modified Eagle's medium

DMSO – dimethyl sulphate

EC – epicatechin

ECG – epicatechin gallate

EE – encapsulation efficiency

EGC – epigallocatechin

EGCG – epigallocatechin gallate

FBS – fetal bovine serum

GRAS – Generally Recognized as Safe

HIF-1α – hypoxia-inducible factor 1-α

IC50 – half maximum inhibitory concentration

IL-1β – interleukin-1β

LC – loading capacity

LDH – lactase dehydrogenase

MMPs – matrix metalloproteinase

MTT – thiazolyl blue tetrazolium

NF-kB – nuclear factor kappa-light-chain-enhancer of activated B cells

NLC – nanostructured lipid carriers

P_{app} – apparent permeability

PBS – phosphate buffered saline

PD – Parkinson disease

PEG – poly(ethylene glycol)

PhC – phosphatidylcholine

PLA – polylatic acid

PLGA – poly (lactide-co-glycolide) acid

p53 – tumour suppressor p53

SD – standard deviation

SEM – Scanning Electron Microscopy

SLN – solid lipid nanoparticles

TEM – transmission Electron Microscopy

TNFα – tumour necrosis factor α

VEGF – vascular endothelial growth factor

D_{enc} – mass of EGCG encapsulated

D_{lost} – mass of EGCG non encapsulated

D_{used} – mass of EGCG

L_{mass} – lipid and surfactant mass combined

1
INTRODUCTION

1.1. MOTIVATION

In the last years, the modern society is facing a large health problem with the rising of new diseases like heart diseases, diabetes, neurodegenerative diseases and obesity. For many years, these diseases were only found in the elders and with low incidence, but nowadays they era emerging in a more aggressive way, in a larger and younger group of people. For the above-mentioned reason, it is important to invest in a healthier and more holistic life style.

Green tea is an infusion of the tea plant, which is consumed for centuries in China, associated with health benefits. This beverage is highly concentrated in antioxidants, namely polyphenols, such as epigallocatechin (EGC), epicatechin (EC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) (1). Recent studies found that these polyphenols have numerous benefits in the prevention and treatment of cancer, vascular and degenerative diseases, diabetes, obesity and other health concerns. Of the former compounds, EGCG is the most abundant and therapeutically active(2, 3).

The worldwide population consumption of EGCG would be of high interest, being the use of a nutraceutical or supplement EGCG-based since it may prevent the appearance of severe health concerns. Notwithstanding, EGCG has an extremely low intestinal absorbance and a high degradation rate in the intestinal environment(4, 5). For the former reasons, the use of dietary natural sources of EGCG in the dietary nutrition seems to be insufficient to reach therapeutic concentrations of EGCG, and consequently health benefits.

The use of nanotechnology in the medicine, more specifically the use of nanocarrier systems is well known, and currently several nanoformulations are already in the market. More specifically, nanoparticles are used to enhance permeability, transport and in some cases to target a specific tissue for the treatment of a certain illness(6).

In this context, in this work two nanocarrier systems for the delivery of EGCG were synthetized, developed and characterized. The chosen nanocarriers were lipid nanoparticles, more specifically solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC). The nanocarriers were designed to be biocompatible, and to improve the stability and bioavailability of EGCG. In the future, the developed nanocarrier-based EGCG delivery systems may be exploited *in vivo* for preventive and therapeutic supplementation of EGCG.

1.2. NATURAL SOURCES OF EGCG

Catechins are compounds widely distributed in nature, being well dispersed through the plant kingdom. Specifically, EGCG has been identified in a large number of plants, being found in a variety of edible vegetables. In the human diet, most of the source of catechins is obtained by the consumption of tea, and more specifically green tea. A list of plants containing EGCG is presented in the Table 1.

Table 1 - Natural sources of EGCG

Plant	Scientific name	Concentration of EGCG (mg/100g)	Organ	Ref.
Strawberry	*Fragaria X ananassa*	0.11	Fruit	(7)
Apple	*Malus domestica*	0.11 – 1.93	Fruit	(7)
Avocado	*Persea amaricana*	0.15	Fruit	(7)
Pear	*Pyrus communis*	0.17	Fruit	(7)
Onion	*Allium cepa*	0.20	Bulb	(7)
Peach	*Prunus persica*	0.30	Fruit	(7)
Pistachio nut	*Pistacia vera*	0.40	Seed	(7)
Plum	*Prunus, spp.*	0.40 – 0.48	Fruit	(7)
Raspberry	*Rubus, spp.*	0.54	Fruit	(7)
Kiwifruit	*Actinidia deliciosa*	0.55	Fruit	(7)
Blackberry	*Rubus, spp.*	0.68	Fruit	(7)
Cranberry	*Vaccinium macrocarpum*	0.97	Fruit	(7)
Hazelnut	*Corylus, spp.*	1.06	Seed	(7)
Pecan	*Carya illinonensis*	2.30	Seed	(7)
Tea	*Camellia sinensis*	70.20	Leafs	(7)
Carob	*Ceratonia siliqua*	109.46	Seed pod with seeds	(7)
N/A	*Salacia reticulata*	N/A	Leafs	(8)

1.2.1. TEA

Tea is an infusion made from the leaves of the tea plant (*Camelia sinensis*) originated in China. Depending of the process used to dry the leaves, the tea will gain different aspects, tastes, chemical compositions and therapeutically properties. There are three principal types of tea: green tea, black tea and oolong. Green tea is prepared by steaming the tea leaves after the harvest. This process will prevent the enzymatic oxidation of the compounds found in the green leaf, mainly tea catechins, preserving the fresh state. After the initial process, leafs are dried and stored. By opposition, the black

tea is prepared by allowing the fresh picked leafs to ferment in a humid place at the room temperature for a day after drying. This allows the natural enzymes present in the leaf to oxidize the catechins in to complex polymeric products: thearubigins and theaflavins. These compounds change the taste, color and therapeutic properties of the tea. The oolong tea is an intermediate between green and black tea, where the leaves are allowed to partially ferment and dried. The composition of oolong is like the process itself, an intermediate between the two others, comprising a mixture of monomeric polyphenols and higher weight theaflavins(1).

Tea is a very popular beverage in Asia for centuries. Nowadays, tea is the second most consumed beverage after water and this beverage has gained a large interest recently for its promising health benefits in several fields, including weight loss, diabetes and cancer prevention. The health benefits of tea are mainly attributed to green tea and its polyphenol's content, in particular, tea catechins(2, 3).

Traditionally, tea is consumed in the form of infusion. This ancient way of consuming tea provides a substantial source of polyphenols, but for achieve the target benefits for the treatment or prevention of a particular pathology, several cups of tea would be needed to ingest the necessary amount of EGCG. In another perspective, would be interesting to use the most therapeutic compound of the green tea for supplementing food or in the form of a pharmaceutical supplement to be orally taken and surpass the low bioavailability issue. With this approach a higher amount of EGCG may be administered in a convenient way. Indeed, the bioavailability of tea catechins is very low, being in order of 1-2% (9).

Therefore, technologies that enhance the stability and bioavailability of the catechin would be of high interest, providing a more effective use and cost of EGCG.

1.3. CHEMICAL STRUCTURE OF CATECHINS (EGCG)

Green tea has a great amount of polyphenols divided in three major groups: flavanols, flavones and flavonols. Flavanols comprises the majority of these content, being about one third alone of the dry mass of green tea leafs, and are mainly distributed by four molecules: (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC) and (-)-epigallocatechin gallate (EGCG). The chemical structures of these compounds are illustrated in Figure 1. The last one, EGCG is the most abundant catechin present in green tea, being 65% of the total flavanols and one-third of the tea dry mass.

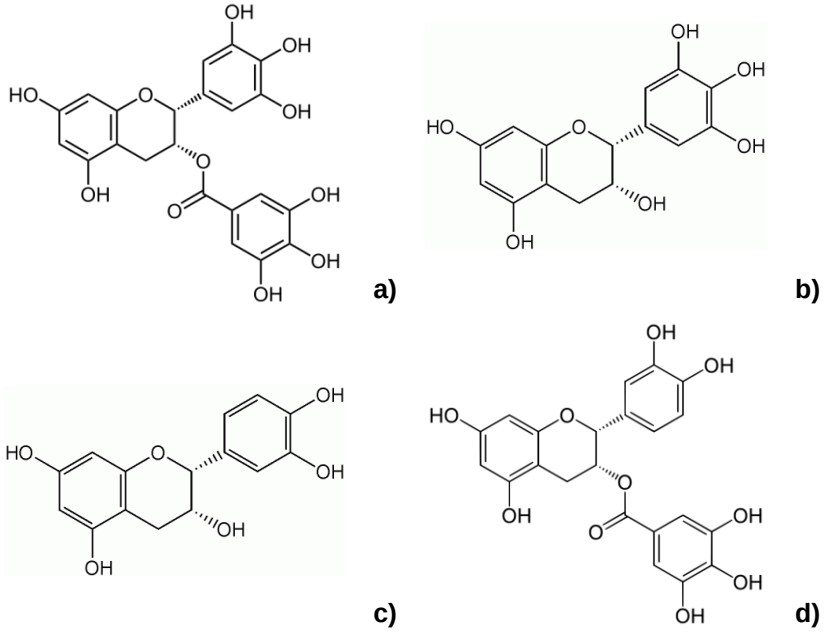

Figure 1 - Chemical structure of the four major catechins present in tea. a) (-)-epigallocatechin gallate (EGCG); b) (-)-epigallocatechin; c) (-)-epicatechin; d) (-)-epicatechin gallate.

Epigallocatechin-3-gallate (EGCG) is a complex molecule formed by a flavanol core (flavan-3-ols) structure with a gallocatechol group and a gallate ester. These two gallocatechol rings confer the potent antioxidant and chelating properties to EGCG. Each of the gallocatechol rings is capable of directly capture free radicals from the environment with high efficiency(10). Previous studies, have shown, that EGCG possesses the stronger antioxidant capacity comparing with the others green tea catechins, and it is also demonstrated that EGCG is more efficient in radical scavenging than vitamin E and C(11).

In the human body, catechins are capable of reducing the amount of free radicals by chelating metal ions, specially the iron ion. These metal ions are known to produce free radicals by the Fenton's reaction. They accomplish this sequestration by biding the ion to the catechol or the galloyl groups found in the structrure of the catechins. The numbers of these groups that are present in the catechin strongly influence the ion binding capacity. The catechins with only one of the group's, epicatechin and epigallocatechin, are only capable to bind one ion per identity. In the other hand, the two catechol rings in epicatechin gallate and the two galloyl rings in EGCG are spatially distant from one another, which allows then to independently chelate two ions per molecule(12).

1.4. PHARMACOKINETICS

EGCG has various health benefits. However, EGCG has very low bioavailability and undergoes through extensive degradation in the intestine. For above-mentioned reasons, a daily supplementation of the diet with this molecule would be of high interest for the society health care.

1.4.1. ABSORPTION

The absorption of a drug is a key component to achieving good bioavailability and ensuring that the drug is able to reach the systemic circulation. For oral drugs, the majority of drug absorption occurs in the small intestine where the presence of villi and microvilli greatly increase the surface area for optimal absorption. Drug absorption in the small intestine is greatly influenced by multiple interacting factors, including drug properties (*e.g.*, solubility, formulation, and amount), gastrointestinal properties (*e.g.*, pH, food intake, region of the small intestine), metabolism, permeability, and active transport across the intestinal epithelial membrane(13).

Permeability studies demonstrated that EGCG has a very low apparent permeability (P_{app}) of $3{,}5x10^{-7}cm^2/s$ (4). Comparing the apparent permeability of EGCG with manitol, which is a poorly permeable compound with an apparent permeability of 6 to $10x10^{-6}$ cm^2/s is clear that the intestinal mucosa is a highly impermeable barrier to EGCG. Therefore, the P_{app} of the catechin is almost ten times lower in comparison with manitol (14). One of the major reasons of the lower permeation through the intestinal barrier is the high hydrophility of the catechin, associated with a lack of transporters to help the uptake and the presence of efflux transporter that actively excretes EGCG to the intestinal lumen(13)

Although the low bioavailability, EGCG is rapidly absorbed after oral administration, being readily detected in the blood circulatory system. The maximum blood concentration of EGCG is accomplished after 1 to 2 hours after a single oral dose administration (15, 16).

To achieve therapeutic concentrations of EGCG, a larger dose or alternatively the use of a delivery system can be exploited to overcome the low bioavailability of this catechin

1.4.2. DISTRIBUTION

After the absorption across the gastrointestinal tract, the drug will be distributed through the entire body. This process can be influenced by several factors, such as the solubility of the drug, the protein binding properties, the bioaccumulation in non-target organs and the clearance rate. EGCG has the capacity to bind to plasma proteins, especially to fibronectin, fibrinogen and histidine-rich glycoprotein (17). In addition, EGCG shows affinity to some tissues of the body, being accumulated in several organs, such as the lung, intestine, liver and prostate gland (9, 18).

1.4.3. METABOLIZATION

Metabolization comprises the life-sustaining chemical processes that occur within the cells of living organisms. Humans have several metabolization pathways that can be allocated in two major categories; the phase I reactions and the phase II reactions. In the phase I reactions, typically the xenobiotic is activated by a sort of enzymes like the cytochrome P-450, modifying the molecule into a more reactive intermediate. This allows the chemical reactions with the phase II enzymes. The phase II enzymes bind the activated molecule to a functional group including among others, sulphate and acetate. These conjugates are in general less active and less toxic than the original molecules and are more easily eliminated(19).

EGCG like other molecules can be metabolized. There are three major metabolites of EGCG that can be found in plasma namely, the glucuronide, the methylated and the sulphated forms (16). These metabolites are present at lower concentration in the serum. Moreover, these metabolites are inert, with no biological relevance with the exception of the methylated EGCG. The methylated EGCG

seems to accumulate in the prostate tissues and could be helpful in the treatment of prostate cancer(20).

Another metabolization pathway of the EGCG in the human saliva was described recently (16). Chung and co-workers described the presence of a degalloylate esterase that hydrolysed the ester bound between the epigallocatechin core and gallate group(21).

Another mechanism is the metabolization of EGCG by the gut flora in the intestine, which seems to be also responsible for the low bioavailability of EGCG(5). A study performed by Kohri *et al* using radioactive EGCG found a spike radioactivity in the mouse urine 24 hours after the initial oral dosecontrairlly to the found in literature. So the presence of radioactivity was confirmed to be resultant of by-products of intestine flora metabolization. They found that mouse pre-treated with antibiotics show no significative clearance of radioactivity trough mouse urine. Based in this results they conclude that EGCG is metabolised by the gut flora and the resultant metabolites reabsorbed by the intestine to the blood current(22). This could be one major cause of the poor bioavailability of EGCG.

1.4.4. ELIMINATION

In the human body, there are two major elimination mechanisms. The most common is the elimination of EGCG metabolites through the urine. The kidney is an organ specialised in the filtration of the blood, eliminating board spectrum of unwanted substances and excreting then in the urine. According previous studies, only small amounts of EGCG are detected in the urine (18, 23).

The second most common elimination pathway in the human body is the elimination through faeces. In this case, the xenobiotic is captured by the liver and excreted to the bile, being posteriorly released in intestinal tract and excreted in the faecal matter. This elimination pathway is the principal elimination route of EGCG (9, 18). In lesser extent, the clearance of some substances can be helped by mechanisms of active transport in the intestinal mucosa through efflux transporters. This is more relevant when the drug is first absorbed by the epithelial cell and then is actively transported to the intestinal lumen again. With lesser extent, EGCG can be cleared by the last mechanism via efflux transporters bellowing to the P-glycoprotein family. The sum of the eliminations mechanisms leads to a rapid clearance of EGCG (24).

In summary, the conjugation of a low absorption in the gut, and high rate of degradation in the gut environment, the high extension of the metabolization and elimination processes makes the overall bioavailability of EGCG extremely low.

1.4.5. TOXICITY

Green tea is an ancient beverage, being consumed worldwide and never was reported any case of toxicity. However, the same cannot be extrapolated to the larger dosages founded in EGCG supplements. These new supplements possess doses of EGCG that are virtually not possible to obtain with only the tea consumption. EGCG seems to be well-tolerated by the human body and to the date, none adverse effects are described. Nonetheless, no studies that analysed the long-term use of high doses of EGCG in humans were founded (25). In France, a green tea supplement was withdrawn from the marked, with suspicions of liver toxicity. A fulminant liver hepatitis was reported after the consumption of a green tea extract in the form of weight loss supplement (26). This emphasizes the necessity of long-term studies to help to understand the risks of the supplementation with EGCG.

Another interesting property of EGCG is the ability to block the folate transporter in the intestine. The inhibition of the uptake of folate can lead to depletion of the folate levels in the human body, which may result in permanent brain damages(27).

1.5. THERAPEUTIC AND PREVENTION USE OF EGCG

For many years, the consumption of green tea was associated with numerous health benefits. These properties can be directly linked with the polyphenol content of tea, more specifically with EGCG. For these reason, the study of EGCG is of high interest because this compound seems to prevent and also be useful in the treatment of numerous diseases like cancer, cardiovascular and neurodegenerative diseases and also obesity. EGCG is a powerful antioxidant, anti-inflammatory, antibacterial, antiviral and is capable to modulate some metabolic pathways like the metabolism of lipids(2, 3).

1.5.1. CANCER

Cancer is the end of several steps of cellular growth lesions, namely hyperplasia, metaplasia, dysplasia and neoplasia. Each of the presented conditions is a progression in the cancer formation, culminating in the neoplasia malignant known as cancer(28). The development of cancer can be induced by several factors, including among others, diet, drugs and smoking. In the disease process, the cells evade the immune system and have the capacity to undergo through successful mitosis without external repression. Nowadays, most modern therapies currently available for treating cancer are very expensive, toxic, and have low effectiveness in treating the disease. Therefore, is necessary investigate natural compounds like EGCG derived from green tea, described traditionally, for the prevention and treatment of cancer and other diseases(28).

According to previous studies, EGCG is a promising molecule in the prevention and treatment of cancer. Some anti-cancer properties of EGCG are attributed to its free radical scavenging properties, avoiding the damage of the cell structures induced by the free radicals. Besides being antioxidant, EGCG has the ability to bind and modulate the activity of several signalling molecules related with mitosis, survival and cellular death, moderating the cellular responses present in cancer. Previous works demonstrated that EGCG is able to inhibit all of the processes involved in carcinogenesis: initiation, promotion and progression (20, 29).

EGCG has the ability to bind with some proteins associated in molecular pathways that are miss-regulated in cancerous cells. Indeed, EGCG modulates the suppression of two important transcription factors, tumour suppressor p53 (p53) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), leading to a regression of the tumours (20, 29, 30).

To assist the growth of the tumour, new capillaries are needed to ensure the requirements of the cells in oxygen and nutrients. The grow process of new blood vessels is called angiogenesis. To promote formation of new capillaries, the tumour secrete to the surrounding tissues signalling molecules, especially vascular endothelial growth factor (VEGF). VEGF is directly influence by the activity of hypoxia-inducible factor 1-α (HIF-1α) and NF-kB factors, which are modulate by the presence of EGCG. For these reason, EGCG is capable of diminishing the tumour angiogenesis and stall growth (31).

In addition, there is strong evidence that EGCG is capable of diminishing migration and metastasis formation of tumours. Previous studies report that EGCG promotes a reduction in the migration and

metastasis formation of tumoral cells with tumour size reduction, accomplishing a more reliable and efficient chemotherapy(32).

Although the single use of EGCG in chemotherapy is unlikely due its inefficacy in eliminating the disease completely, it would be very interesting using EGCG as adjuvant of the cytostatic drugs. This synergism may be useful to reduce the amount of the necessary cytostatic drugs, which will reduce the side-effects. Moreover, the health benefits of EGCG would be advantageous in enhancing the overall condition of the patients (28, 30).

1.5.2. CARDIOVASCULAR DISEASES

Cardiovascular diseases have a high incidence, mainly in the developed world due to a sedentary life style, poor nutrition and ambient factors. A diet rich in cholesterol, fat and sugar causes can lead to coronary diseases like arteriosclerosis and ischemia. These diseases are characterised by a deposition of cholesterol in the vascular vessels forming platelets, which can lead to thrombosis. These depositions can interfere and obstruct the normal flow of blood, and in some cases can lead to serious complications like strokes and myocardial infarctions(33, 34).

Recent studies show that EGCG can enhance the capillary circulation dilating the capillaries, diminishing inflammation and interfere with the lipid absorption and digestion(34-36).

On the other hand, EGCG interferes directly with the lipid emulsion process in the lipid digestion. This is achieved by directly interference in the micelle formation and by inhibiting the phospholipase A2, being this enzyme of high importance in the lipid digestion (37). The junction of the two processes can limit the absorption of lipids and consequently, lowering the amount of plasmatic lipids and cholesterol (37). In addition, EGCG can lower cholesterol even more, stimulating his excretion through the bile. Moreover, EGCG will further improve the lipid profile by enhancing the lipid metabolism. This catechin can also modulate the process of the platelets formation, from the macrophage recruiting to the macrophage up take of cholesterol. This effect is internally modulated in the macrophage and externally helped by the anti-inflammatory response caused by EGCG. Previous studies demonstrated that the administration of EGCG is capable of prevent the growth and also to reduce the size of existing platelets. The action mechanism responsible for the anti-inflammatory property of EGCG is the direct inhibition of the phospholipase A2(35).

1.5.3. NEURODEGENERATIVE DISEASES

The cause of neurodegenerative diseases like Parkinson (PD) and Alzheimer's (AD) diseases are still unknown, being various theories proposed. Both diseases present clinic features, like the oxidative damage to neurons and accumulation of iron in specific brain areas. Another relevant aspect is the accumulation of missfolded proteins in deposits, such as the β-amyloid peptide in AD that interfere with the survival of the neurons, leading to a premature apoptosis(38).

Special interest has been assigned to the therapeutic role of antioxidants in such neurodegenerative diseases. The neuroprotective properties of EGCG agent are related with its properties as antioxidant, anti-inflammatory and iron chelating. In addition, the blood brain barrier (BBB) is permeable to EGCG (39). The mechanism behind the passage of this hydrophilic compound through the BBA remains unknown.

In the literature it is described that EGCG is more efficient in radical scavenging than vitamin C and E, being its iron chelating ability useful to significantly improve the symptoms of these neurodegenerative diseases(11, 40).

According to the above-mentioned, EGCG is also a cellular modulator that interacts with various pathways. In neuronal cells, this catechin promotes cell survival responses and the inhibition of cell death signals, leading to an enhancement of neuronal health. The modifications in the cell signalling also promote the non-amyloid α-secretase pathway, diminishing the production of β-amyloid peptides(40).

Several research studies confirm that EGCG has neuroprotective properties in humans, being the degree of cognition enhanced after the oral administration of EGCG. These studies confirm that EGCG promotes an overall increase in the cerebral activity and calmness. All of these aspects are important in neurodegenerative diseases, being the administration of EGCG proven to help in maintaining the normal levels of cognition, diminishing agitated stages and mod shifts(41).

1.5.4. INFECTIOUS DISEASES

Viruses are small infectious agents that replicates only in living cells. They are composed by nucleic acid (DNA or RNA) and some enzymes encapsulated in a proteic sell and may have a lipid membrane covering the structure. To multiplied, they enter a living cell and redirect the cell bioquemistry to start producing virus components. Generally, viruses are dificul to treat due to its strong intricacy with cellular host mechanisms to replicate(42).

Nowadays, the main strategy to fight viruses is the immunization. Unfortunately, several viral infections lack one efficient vaccine, being the most mediatic the HIV infection. In the last viral infection, several studies were performed with some encouraging results. Fassina *et al*, shown a strong HIV inhibition caused by EGCG in cell cultures in a dose dependent manner(43). Later, Li S. *et al* also proven that EGCG inhibit the reverse transcriptase, and act synergistically with another reverse transcriptase inhibitor, namely azidothymidine(44). Some studies also described that EGCG is capable to bind to the CD4, preventing the virus to anchor and enter the cells(43).

EGCG is also useful in the inhibition of other viruses, such as *Enterovirus* 71, *Hepatitis* C, *Adenovirus, Herpes simplex* and *Influenza*. One of the molecular targets that seem to be deregulated by the viral infection is the NF-kB and the MAP-kinases pathway(45-49).

1.5.5. OBESITY

Obesity is a medical condition characterized by an excess accumulation of fat in the body in an extension that may have negative effects in the overall health condition and may lead to the development of diseases, such as diabetes and arteriosclerosis, among others. Obesity is mainly attributed to the diet and lifestyle (50). Sedentary habits and high caloric intake commonly lead to fat accumulation in the adipose tissue. The fat percentage can alter significantly the biochemistry of the human body, leading to hormonal problems, high oxidative stress, circulatory complications and other conditions. The main treatment of obesity is the lifestyle re-education, including diet modification. However, sometimes it is difficult to achieve the desirable results and in some cases, drugs and supplements are needed to help the losing weight process(50).

As previously stated, EGCG interferes directly with the lipid digestion by the inhibition of the phospholipase A2, and interfering with the lipid/cholesterol emulsion in gut (37, 51). The lipid blocking capacity of EGCG can be highly relevant in the loss of weight and weight managing protocols. In addition, EGCG is capable to enhance the lipid metabolism, leading to a more caloric burn and consequent fat loss. EGCG can also interfere with the digestion of starch by inhibition of α-amilase(52).

Besides the above-mentioned, the ingestion of EGCG during a weight loss programme is very useful because its administration is strong linked with the circulation improvement, free radical scavenging and mod enhancer (10, 34, 41, 49).

1.5.6. CHRONIC INFLAMMATORY DISORDER

The inflammation is a body response to foreign structures to the human body and damage in the tissues. This response is highly important to the normal health of an individual due to the capacity to combat diseases and assisting heals of damage tissues like cuts and bruises. The inflammatory process is characterised by an increase of the capillary diameter lead to an increase in swelling, redness and pain. The inflammation response also recruit immune cell to eliminate any foreign body and damage tissue. However in chronic inflammatory disorders, this inflammatory response is continuously active leading to the destruction of healthy tissues causing all the above mentioned symptoms. These conditions can be incurable and cause major discomfort to the patients(53).

Rheumatoid arthritis is one chronic inflammatory disorder characterized by cellular infiltration and proliferation of the synovium, leading to the progressive destruction of the joints through the interaction between infiltrating cells and mediators(54, 55). These injuries lead to chronic pain affecting the life quality of the patients. In this disease, the cartilage cells (*i.e.*, chondrocytes) enter in apoptosis in response to inflammatory cytokines interleukin (IL-1β) and tumour necrosis factor α (TNFα) and oxidative stress. The same cytokines also lead to the increase of bone resorption and the differentiation of osteoclasts(54).

IL-1β is an inflammatory cytokine that is over expressed in arthritis promoting imbalance between excessive cartilage destruction and repair processes. In addition, IL-1β is also capable of increasing the amounts of reactive oxygen species via overexpression of inducible nitric oxide synthase and increases the inflammation by an overexpression of cyclooxygenase-2 (COX-2)(56). The presence of IL-1β can also activate the expression of matrix metalloproteinases (MMPs) responsible for the matrix degradation. TNFα also plays an important role in the bone turn over. In arthritis, there is an overexpression of TNFα who is responsible the differentiation and activity of osteoclasts. The long term activation of this cells lead to the bone erosion and fragility(57).

The normal treatment in arthritis is the administration of methotrexate, which combines a good efficacy and low toxicity. This drug is widely used, however in some patients' it is ineffective and recent studies shown that the drug tends to lose efficacy over time. To attenuate the symptoms is also used analgesics and nonsteroidal anti-inflammatory drugs (58).

Although the efficacy of the current treatment, the complete remission of the disease is often not achieved and for this reason new therapies are needed. EGCG has high antioxidant activity and also capacity to decrease the inflammation response in the body. In cartilage cell cultures, EGCG showed a marked inhibition of IL-1β inducible nitric oxide synthase COX-2 expression and activity. The

expressions of both enzymes are mediated by the NF-kB, which is supressed in the presence of EGCG (59).

2
NANOTECHNOLOGY

Nanotechnology is the science and technology of small things and particularly materials with dimensions between 1 and 1000 nanometers(6). Nanoparticles are structures that have at least one of the dimensions in the nanoscale range. Due to their high surface area to volume ratio, nanoparticles present chemical, physic and biological properties distinct from conventional materials(60). The size and the different properties of the nanoparticles can be used for medical purposes. The small scale of nanoparticles makes then excellent drug carriers, and because they can be modified in various factors like size, chemical composition, outer layer and others they are very versatile. The physical properties can also be useful in diagnostic techniques, such as tomography imaging. In addition, nanoparticles can modify the pharmacokinetics and the stability of some drugs(60). This is specially truth in the case of EGCG, where the nanotechnology can be used to increase the bioavailability of this catechin (61). Another interesting characteristic of the nanoparticles is the possibility of enhance the cellular uptake by modifying the outer layer with different ligands to assign specificity to certain cells and/or structures. This technology may increase the bioavailability and stability of EGCG, enhancing the health potential of this compound. To the date, no studies are described related with the use of nanoparticle-based formulations with EGCG in humans(62).

There are several nanosystems used in drug delivery, including lipid nanoparticles liposomes, polymeric nanoparticles, nanocrystals, gold nanoparticles, nanotubes and magnetic nanoparticles.

2.1. LIPID NANOPARTICLES

Lipid nanoparticles were introduced in the early 1990s, being one of the most currently used nanosystems to delivery drugs. These nanoparticles are prepared from a lipid matrix, with final particle size ranging between 1 and 1000 nm(6). The combination of particle size, use of non-toxic materials, physical stability, controlled release properties, high drug load and excellent tolerability make them important colloidal carriers (63). Their synthesis requires three components, namely the hydrophobic lipid phase, one emulsifier and the hydrophilic aqueous phase. If their final destination use is for oral and topical administration, all components used need to hold a Generally Recognized as Safe (GRAS) status. The lipids used, normally have a melting point above the body temperature and can be mono, di and triglycerides alone or in mixtures, waxes and other types of lipids(64).

There are two main types of lipid nanoparticles, namely solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC). These two nanoparticles differed in the type of lipid used, being

in case of SLN used only solid lipids and NLC are composed of a mixture of both solid and liquid lipids. Due to their distinctive lipid crystalline structure, these nanoparticles have different encapsulation and release behaviours. The SLN lipid matrix fully crystallised tend to have lower drug loads, since there are fewer gaps in which the dug can enter. It also tends to expulse the drug content during long storage due to crystalline rearrangement. Instead, NLC have a higher drug loading and storage stability due to the larger number of imperfection in the lipid core(64) The structure and drug interaction is schematised in Figure 3.

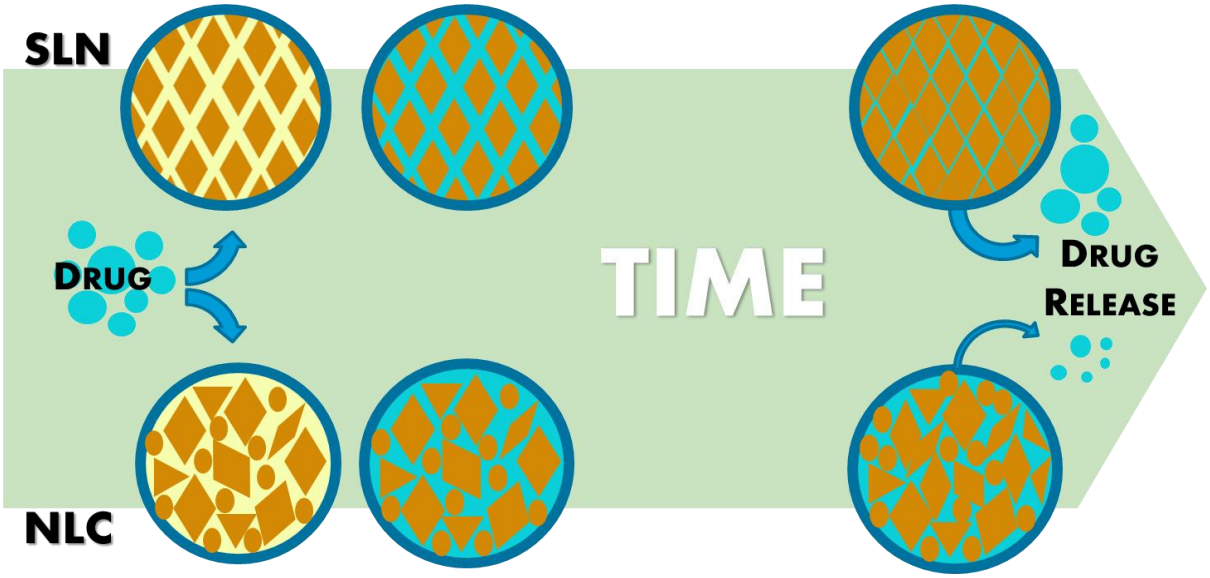

Figure 2 - Schematic representation of the matrix for SLN and NLC and drug release

Lipid nanoparticles have been used to enhance oral bioavailability of various drugs, by enhancing the gastrointestinal stability and/or by increasing the permeability. Only one study was found related with the encapsulation of EGCG in lipid nanoparticles. In this context, Zhang *et al* synthetized NLCs with a mixture of two lipids (glyceryl tridecanoate and glyceryl tripalmitate) and two surfactants (soy lecithin and Kolliphor® HS15). The formulation was designed to enhance bioavailability of EGCG by oral administration and safely transport the catechin through the blood stream, targeting THP-1-drived macrophages. THP-1-drived macrophages are responsible for the accumulation of cholesterol in arteries and therefore the primarily target for EGCG. In addition, the authors synthetized the lipid nanoparticles coated with a layer of chitosan to enhance the nanoparticles permeability and targeting to the macrophages. The nanoparticles exhibited a low release (*i.e.*, 4.43%) of the EGCG content after 9 hours of incubation in phosphate buffered solution (PBS) at pH 5.0, being the degradation rate of encapsulated EGCG in neutral pH significantly lower that the non-encapsulated EGCG. At 37ºC, the EGCG was completely degraded in the course of three hours; meanwhile 65% of the encapsulated EGCG was still unchanged. Cell toxicity was found to be very low, leading to a 9-fold decrease in the accumulation of cholesterol in macrophage cultures in combination with a diminishing secretion of inflammatory factors. Despite the promising results presented by Zhang *et al*., the study lacks the permeation study in caco-2 monolayer to estimate the permeation of EGCG (65).

Table 2 - Lipid nanoparticles used as EGCG carriers

Nanoparticles composition	Loading capacity (%)	Loading efficiency (%)	Size (nm)	Administration route	*In vitro*/*in vivo* results	Ref.
Glyceryl tridecanoate, glyceryl tripalmitate, soy lecithin and Kolliphor® HS15, chitosan	3%	99%	50	Oral	High stability in both acidic and neutral environments. *In vitro* studies performed in THP-1-drived macrophages showed decrease in inflammation and accumulation of cholesterol.	(65)

2.2. POLYMERIC NANOPARTICLES

Polymeric nanoparticles are made from polymers that can be natural or synthetic. These kinds of nanoparticles have been extensively studied for drug transport due to their high biocompatibility and easy synthesis. In polymeric nanoparticles, the drug is transported in the polymer matrix, being protected from the outside conditions. To enhance the pharmacokinetics profile of the drug, or in some cases, to target some specific tissue, the outer layer of the polymer can be functionalized with molecular markers. Some reports of EGCG enhancement uptake have been proposed and tested *in vitro* and *in vivo* with promising results(66).

Chitosan is a popular choice to encapsulate EGCG regarding its biocompatibility, biodegradability and low toxicity. In addition, chitosan is mucoadhesive and can enhance the permeability of the intestine by opening the tight junctions(67). Dube *et al* used chitosan nanoparticles to enhance the intestinal absorption of EGCG *in vitro*. Moreover, the same authors proved that chitosan nanoparticles also enhance the EGCG uptake *in vivo* (14, 68).

Khan *et al* used EGCG loaded chitosan nanoparticles in athymic mice implanted with prostate cancer cells and found a reduction in the growth of the tumour comparing to the two controls, one that receive void nanoparticles and the other that received the free EGCG. The results also indicate a dose dependent relation between the amount of encapsulated EGCG and the size of the tumour (69).

Hu *et al* synthetized chitosan cross-linked with casein phosphopeptides. The developed nanoparticles enhance the stability of EGCG through the digestive system (70). In addition, the permeability of EGCG was increased in caco-2 cells monolayers (71).

Polylatic acid (PLA) and poly (lactide-co-glycolide) acid (PLGA) are two of the most extensively studied polymers to create nanoparticles because of their versatility and high biocompatibility. The outer layer of these nanoparticles can be easily modified with functional groups to specifically target certain cells and/or structures. In addition, the degradation time of these nanoparticles can also be manipulated and they can be have high encapsulation rates and controlled release properties(66, 72).

Sanna *et al* synthetized PLGA-based nanoparticles coated with poly(ethylene glycol) (PEG) and they modified their surface with a pseudomimeticdipeptideN-[N-[(S)-1,3-dicarboxypropyl]carbamoyl]-(S)-lysine to act as a molecular target to a prostate cancer membrane protein (*i.e.*, prostate-specific membrane antigen), which is overexpressed. The nanoparticles were tested in prostate cancer cells

lines with promising results (73). Siddiqui *et al*, also have encouraging results but instead of PLGA-PEG they used PLA-PEG nanoparticles (74). The drawback is that both PLA and PLGA polymers are sensitive to the gastric pH and the majority of the studies focus in an intravenous administration method, which is an invasive route of administration (73, 74).

Srivastava *et al* used a PLGA EGCG loaded nanoparticle to prevent DNA damage caused by 7,12-dimethylbenzanthracene (DMBA). In this study, mouse was treated topically with EGCG and PLGA EGCG nanoparticles prior to topical of DMBA. The pre-treatment with the EGCG resulted in a protection of DNA damage in the order of 28%. However, the pre-treatment with the loaded nanoparticles result in a 63% protection in DNA. Additionally, EGCG loaded nanoparticles, showed significant induction of DNA repair genes and inhibiting inflammatory genes.(75). Other types of polymers have been studied; in this context, Li *et al* used ovalbumin-dextran conjugates nanoparticles to enhance the stability and absorption of EGCG through the digestive system. The results revealed that the ovalbumin-dextran nanoparticles were stable and the EGCG absorption profile was improved (76).

Table 3 - Polymeric nanoparticles used as EGCG carriers.

Nanoparticles composition	Loading capacity (%)	Loading efficiency (%)	Size (nm)	Administration route	*In vitro*/*in vivo* results	Ref.
Chitosan	0.4	N/A	440±37	Oral	Enhancement of the gastrointestinal permeation of EGCG in mice's.	(14, 68)
Chitosan	N/A	~10	~150	Oral	Reduction of human prostate tumors in mice.	(69)
Chitosan, casein and peptides	N/A	N/A	150	oral	Bioavailability of EGCG increment in caco-2 monolayers.	(70)
Chitosan casein phosphopeptides	N/A	N/A	150±4.3	oral	Enhancement of the intestinal permeation of EGCG using caco-2 monolayers.	(71)
PLGA	5.76	26	127	topical	Inhibition of DNA damage.	(75)
PLGA and PEG	~0.4	~9.5	80±15.0	Intravenous	Inhibition of the growth of cultured cancerous cells.	(73)
PLA PEG	N/A	N/A	N/A	Intravenous	Reduction in the size of the implanted tumor in mice.	(74)
Ovalbumin and dextran	20.9	30.0	339	oral	Enhancement of the intestinal stability and improvement of apparent permeability in Caco-2 models.	(76)

2.3. GOLD NANOPARTICLES

Gold nanoparticles constitute a recent approach to the enhancement of EGCG permeability and cytotoxicity to the cancer cells. Gold nanoparticles are made from a gold core covered with a film of functional molecules, which are capable to transport complex cargos like drugs and targeting molecules. It is possible to synthetize gold nanoparticles covered with EGCG due to the capacity of this catechin to combine with metals to form complexes by the gallate ring (77). Although gold EGCG complexes present high stability in acidic pH conditions, they start decomposing in more pH basic conditions. Shukla *et al* and Chen *et al* solved this disadvantage by using an intratumoural injection (78, 79). Shukla *et al* synthetized radioactive gold nanoparticles coated with EGCG, to evaluate the chemotherapy efficacy. In this case, EGCG serves as chemoadjuvant and targeting molecule simultaneously, due to its high affinity to prostate cancer cells and anti-cancer properties(20, 29). The results obtained were very promising and a high efficacy of the formulation was achieved in reducing the tumour size (78). On the other hand, Chen *et al.* synthetized nonradioactive gold nanoparticles coated with EGCG to test only the capacity of EGCG as a chemoadjuvant or chemotherapeutical in cancer therapies(79). These authors found a promising cytotoxic effect both *in vitro* and *in vivo* using a murine melanoma cancer model.

Although the promising results, the two gold nanoformulations above-mentioned were administered by intratumoural injection, which is an invasive route of administration.

Hseig *et al* synthetized gold nanoparticles with the main goal to enhance gastrointestinal stability, increasing the release time in the intestine. The nanosystems obtained, revealed to be stable in the gastrointestinal environment with a sustained release of EGCG over 2 hours. The authors also demonstrated that the formulation shown a preferential toxicity to cancerous cells and an *in vivo* efficiency in reducing the growth of implanted tumours in mice. The sustained release and the high cytotoxicity to cancerous cells make these nanoparticles promising future nanoformulations to use in cancer therapy (80).

Table 4 - Gold nanoparticles used as EGCG carriers.

Nanoparticles composition	Loading capacity (%)	Loading efficiency (%)	Size (nm)	Administration route	*In vitro*/*in vivo* results	Ref.
Gold	N/A	N/A	20-1200	Intratumoural	Noticeable reduction in bladder tumor in mice.	(77)
Gold	2%	27%	68	Intratumoural	High cytotoxicity in melanoma cell culture and in mice.	(79)
Gold	N/A	27%	50	oral	Nanoformulation stable in neutral pH with a sustained release of 2 hours. The *in vitro* and *in vivo* experiments revealed that the nanoformulation is highly effective in the cancer therapy.	(80)
Radioactive gold	N/A	N/A	N/A	Intratumoural	Noticeable reduction of tumor size achieved after 28 days with a single administration of the formulation and with minor radioactive leakage to other organs.	(78)

2.4. LIPOSOMES

Liposomes are artificial vesicles composed by a lipid bilayer and an aqueous milieu. Liposomes are versatile nanocarriers because of their unique structure with both hydrophilic and hydrophobic environments, which enable the transport of all type of drugs. The size of liposomes can be finely adjusted and their surface can be chemically modified to target specific tissues or evading the immune system. The intrinsic stability of liposomes can also be modified by the lipid content. External factors, such as temperature, pH, light and enzyme presence can trigger an abrupt release of their content by interfering with the membrane stability, causing the lipid layer to collapse(81, 82). Although the promising results of liposomes as drug carriers, only two studies are described using liposomes as EGCG carriers. Both studies are focused in enhancing the bioavailability of EGCG. Luo *et al* synthetized phosphatidylcholine (PhC)/cholesterol liposomes to enhance the stability of EGCG in the gut. The liposomes obtained present a high stability in gastric conditions with small cargo loss around

20%, which increases slightly in the intestinal fluid to 40%. Although, cellular uptake studies are lacking, the results of the intestinal release by itself are promising in a future use as an EGCG formulation (83).

Song *et al*. synthetized liposomes with non-ionic surfactants and cholesterol to improve the stability of EGCG in the gut and enhance cellular uptake. Liposomes shown to be stable at pH=7.4 with minimal loss of the catechin. The authors also investigated the permeability of caco-2 cell monolayers to the EGCG entrapped liposomes in comparison with free EGCG. The liposomes were capable of significantly enhance the apparent permeability of EGCG (84). These promising results stimulate the investigation of a nanoformulation liposome-based to enhance the bioavailability of EGCG.

Table 5 - Liposomes used as EGCG carriers.

Particle	Loading capacity	Loading efficiency	Size (nm)	Administration rout	In vitro/in vivo results	Ref.
PhC and cholesterol	N/A	85.8±1.65	180	Oral	Favorable release profile in the gastrointestinal fluids.	(83)
Sorbitan monostearate and cholesterol	N/A	40	100	Oral	The nanoformulation presents a high stability in neutral pH and enhances the cellular permeability in caco-2 cell monolayer.	(84)

3
MATERIALS AND METHODS

3.1. MATERIALS

The lipid Precirol® ATO 5 was a gift from Gattefossé (Gattefossé, France). The lipid Miglyol® 812 was purchased from Acofarma® (Terrassa, Spain). Tween® 60, TritonTM X-100, Thiazolyl Blue Tetrazolium (MTT), Trypan Blue powder, dimethyl sulfoxide ≥99.9%, acetic acid ≥99.8%, potassium phosphate monobasic, and Dulbecco's Phosphate Buffered Saline pH 7.4 (PBS) and (-)-epigallocatechin gallate (EGCG) ≥80% (HPLC) from green tea were obtained from Sigma-Aldrich®. Sodium phosphate monobasic monohydrated was acquired from Fluka® (Germany). Sodium chloride was purchased from Panreac® (Spain). Sodium hydroxide was obtained from VWR International (Belgium). Acetonitrile (Lichrosolv®) and Stearic acid were obtained from Merck (Germany). SIF Powder was obtained from Biorelevant.com (Croydon, Surrey, UK). LDH Cytotoxicity Detection Kit was from Takara Bio Inc. (Shiga, Japan). Dulbecco's Modified Eagle's Medium (DMEM) + GlutaMAXTM-I, 0.25% Trypsin-EDTA (1X), Penicillin-Streptomycin and Heat Inactivated Fetal Bovine Serum (FBS) (origin: South America) were purchased from Gibco® by Life TechnologiesTM (UK).

All the weighting measurements were performed using a Kern ACS-80-4 digital analytical balance (Kern & Sohn; Balingen, Germany). The pH measurements were executed using a Crison pH meter GLP22 with a Crison 52-02 tip (Crison; Barcelona, Spain). Particle size and seta potential was measured using a Dynamic Light Scattering (DLS) (Brookhaven Instruments Corporation; Software: Particle Sizing v.5 Brookhaven Instruments; Holtsville, NY, USA and PALS Zeta Potential Analyser v.5, Brookhaven Instruments; Holtsville, NY, USA). Microplate measurement were performed in a microplate reader BioTek Instruments Inc., Synergy HT, Software: Gen5 v1.08.4, BioTek Instruments Inc.; Winooski, USA. The Spectrophotometer used was (Jasco Corporation, Software: Spectra Manager v.2, Jasco Corporation; Easton, MD, USA) (Jasco V-660).

Ultrapure water was purified by an Ultra-pure water system (Milli-Q, Sartorius, Arium® pro, Sartorius Weighing Technology; Gettingen, Germany, Filters: Sartorius Arium® Cartige 1 and 2, Sartorius Stedin Biotech; Gettingen, Germany) by a reverse osmosis process.

Seringe filters Ministart®, pore size 0.8 µm, Satorius Stedin Biotech; Goettingen, Germany. Centrifugal filters Amicom® Ultra Centrifugal Filter Devices 50kD pore size (50000 NMWL) (Merk Milipore, Ltd; Cork, Ireland). Dialysis was performed using Cellu.Sep®T1, 3500 NMWCO,

Membrane Filtration Products, Inc.; (Seguin, TX, USA). UV-Vis microplate (UV flat bottom Microliter® plates, Thermo Electron Corporation; Vantaa, Finland).

The Cryo-SEM was performed in X-Ray Microanalysis and Cryo-SEM experimental facilities with (JEOL JSM 6301F/ Oxford INCA Energy 350/ Gatan Alto 2500). TEM imaging was performed with JEM-1400 Transmission Electron Microscope (JEOL Ltd., USA).

Cell cultures were incubated in a Unitherm CO_2 Incubator 3503 (Uniequip; Planegg, Germany). Cell counting were performed using an Improved Neubauer Bright-line (Boeco; Germany). Cells were cultured in Tissue culture flasks 250 mL (75 cm^2), 0.2 μm vented plug seal cap, Falcon® (Becton Dickson; England).

MTT and LDH were performed in Tissue Culture OrPlates, surface treated flat bottom, Orange Scientific; Belgium) 96 well microplates

Microplates were centrifuged in a 3k-2 Microplates Centrifuge (Sigma). Sonication's were performed using an sonication probe (Sonics Vibra-cell, with CV18 probe).

To simulate the gastric fluid was used Fasted State Simulated Gastric Fluid (FaSSGF: NaCl/HCl solution, pH 1.2 with SIF® Powder). To simulate the intestinal fluid (FaSSIF: buffer solution containing potassium dihydrogen phosphate, pH 6.5 with SIF® Powder).

3.2. INITIAL FORMULATION

NLCs were initially prepared with stearic acid ($C_{18}H_{36}O_2$; M=284.48 g/mol) as the solid lipid, and Mygliol® 812 as the liquid lipid. Tween® 60 ($C_{64}H_{126}O_{26}$; M=1310 g/mol) was used as a surfactant, to stabilize the emulsion. In SLN preparation, mygliol was not used in the formulation and his mass was substituted by the solid lipid. Quantities used of each component are descried in Table 6.

Table 6 – Quantitative composition of stearic acid lipid NP prepared.

	Stearic acid (mg)	Mygliol® (mg)	Tween® 60 (mg)
SLN	290	-	60
NLC	200	90	60

The method used in this work to produce the lipid nanoparticles was an ultra-sonication method. In detail, the lipids and the surfactant were heated in a water bath up to 70ºC, temperature at all the lipids and the surfactants are in the liquid state. When the solid lipid was fully melted, 4.4 mL of ultrapure water heated at same temperature was added to the lipid. Mixture then went through ultraturrax (Ystral X10/20 E3) at 7000 rpm for 30 s to produce an emulsion, followed by a sonication at 70% power for 5 min to reduce the diameter of the lipid particles. The nanoformulation was left to cool at room temperature and stored.

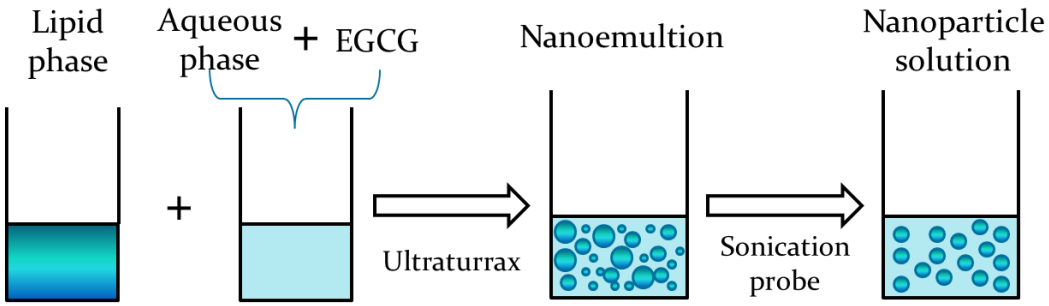

Figure 3 - A schematic representation of the steps to produce lipid nanoparticles

3.3. DRUG LOADING

The aim of this thesis was to successfully encapsulate EGCG ($C_{22}H_{18}O_{11}$; M=458.372 g/mol) in lipid nanoparticles.

A total of 10mg of EGCG was dissolved in the aqueous phase and then added to melted lipid followed by ultraturrax and sonication, as previously described. The equivalent mass of the EGCG was subtracted to the solid lipid mass.

3.4. CHOICE OF SOLID LIPID FOR IMPROVEMENT OF NANOFORMULATION STABILITY

Due to a complete instability of the stearic acid nanoformulations with catechin, another solid lipid Precirol® ATO 5 was tested with the same amount of EGCG and a larger amount of lipid. The quantities are presented in the following table.

Table 7 - Quantitative composition of Precirol® ATO 5 lipid nanoparticles.

	SLN	NLC
Precirol® ATO 5	500	350
Mygliol®	-	150
Tween® 60	100	100

Although a notorious improvement of the formulations' stability, the SLN obtained were not completely stable. To solve this issue, the sonication time was reduced from the initial 5 min to only 30 s using the same sonication' potency.

In order to obtain the leader formulation, four different amounts of EGCG were added and a qualitative solubility test was performed. The quantities of the formulation constituents are presented in the following table.

Table 8 – Composition of the EGCG-loaded nanoformulations.

	Precirol® ATO 5 (mg)	Mygliol® (mg)	Tween® 60 (mg)	EGCG (mg)
NLC	350	150	100	10
	340	150	100	20
	330	150	100	30
	300	150	100	50
SLN	490	-	100	10
	480	-	100	20
	470	-	100	30
	450	-	100	50

3.5. CHARACTERIZATION

3.5.1. PARTICLE SIZE MEASUREMENT

Particle size was measured by Dynamic Light Scattering (DLS) also known photon correlation spectroscopy. This technique uses the relation between the diffraction of light by particles and the Brownian movement to calculate the diameter, identify the presence of different size populations in the sample, and also measure the polydispersity. When a laser is pointed to the suspension, the movement of the nanoparticles will change the light diffraction over time. The velocity of which the nanoparticles move is inversely relate to their hydrodynamic size, so the smaller the particle, the faster it will move and consequently less light will diffracted. To calculate the hydrodynamic diameter of the nanoparticles, the DLS correlates the differences in the light diffraction caused by the Brownian movements, temperature and viscosity of the medium(85).

The mean hydrodynamic diameter and size distribution (polydispersity index) of the nanoformulation were analysed in a Particle Size Analyser operating at a scattering angle of 90º, at 37ºC, with dust cut-off set to 30 and refractive index of the particles set to 1.33. Measurements were performed by 6 runs of 2 minutes each and the mean size and polydispersity of the formulations were obtained. Before the measurements the formulations were diluted (1:100) and filtered with a single use syringe filter 0.8 µm pore to eliminate possible aggregates.

3.5.2. PARTICLE ZETA POTENTIAL MEASUREMENT

Zeta potential is a physical characteristic of the nanoparticles interface. When in solution, the surface charge will develop an arrangement of the molecules around then, creating a layer. The stronger the nanoparticles charge the thicker, polarized and stable the layer will be. The zeta potential is the potential measured in the limit of this interaction layer (86).

Assuming that the identical charges repel one another and the intensity of the charge is directly related with the zeta potential is possible to relate the stability in solution and the zeta potential intensity. A nanoformulation is considered stable when the absolute zeta potential value is superior to |30| mV. The method used to calculate the zeta potential is similar to the DLS. The difference lies in the use of an

electric field to force the nanoparticles to move according to their intrinsic charge, instead of the Brownian movement. To measure the zeta potential, an electric field is created by an electrode placed in the solution, being the velocity of the particles measured by light deviation (86).

The particles zeta potential measurements were performed in a Zeta Potential Analyser operating at a scattering angle of 90º, at 37ºC. Measurements were performed with 10 cycles for one run in a total of 6 runs and the values of zeta potential and standard deviation (SD) of the formulations were obtained.

Before the measurements the formulations were diluted (1:100) and filtered with a syringe filter (0.8µm) to eliminate possible aggregates.

3.5.3. Encapsulation Efficiency (EE) and loading capacity (LC) of EGCG

The encapsulation efficiency (EE) evaluates the amount of active agent that is incorporate into the carrier matrix expressed as a percentage. It was calculated by the equation 1 were D_{used} is the total amount of EGCG used in the nanoformulation synthesis and D_{lost} is the amount of EGCG that was not encapsulated and is dissolved in the aqueous phase outside of the nanoparticles.

$$EE = \frac{D_{used} - D_{lost}}{D_{used}} * 100 \quad \text{(Equation 1)}$$

The loading capacity (LC) is the amount of EGCG that was encapsulated in relation to the total mass of the lipids present in the nanoparticles in the form of percentage as the equation 2, were D_{enc} is the EGCG encapsulated in the nanosystem and L_{mass} is the lipid and surfactant mass combined.

$$LC = \frac{D_{enc}}{D_{enc} + L_{mass}} * 100 \quad \text{(Equation 2)}$$

To determine the EGCG lost, the nanoformulation was diluted with ultra-pure water in a ratio of 1:40 (sample to water). To separate the nanoparticles form their aqueous medium, the dilutions were centrifuge in 50 kD Amicom®, at 3500 rpm and 25ºC for 10min or until complete separation. The supernate with the EGCG dissolved was collected and measured with a UV-Vis spectroscopy at 273 nm and the EGCG content was quantified by linear regression.

Prior to the sample measures, a calibration curve was prepared using solutions with known concentrations. The measurements were performed with using a V-660 spectrophotometer 21 in quarts cells. A spectre of absorbance was measured for several samples with increasing concentration, being the maximum of absorbance recorded at 273 nm used to calculate the linear equation.

3.5.4. Particle morphology

To evaluate the morphology of the nanoparticles Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) were used. In SEM and TEM, the sample is radiated with a ray of charge particles (electrons) of high energy and short wavelength. This provides an observation of objects in the nanometric scale, in opposition to the optic microscopy.

For the present work, the SEM analysis was performed using a high resolution Scanning Electron Microscope. The nanoformulation was diluted (1:100) on ultrapure water and filtered with a 0.8 µm pore syringe filter. A sample of diluted nanoformulation was rapidly cooled (plunging it into subcooled nitrogen – slush nitrogen) and transferred under vacuum to the cold stage of the preparation chamber. The frozen nanoformulation was fractured, sublimated ('etched') for 180 seconds at –90 °C, and coated with Au/Pd by sputtering for 35 sec. The sample was then transferred into the SEM chamber, and observed at a temperature of -150 °C.

In the TEM analysis, an identical dilution (1:100) and filtration (0.8 µm pore filter) was prepared and placed on a cooper-mesh grid and left to rest for 2 minutes at room temperature after which the water excess was removed. For enhance contrast, 10 µL of 0.75% uranyl acetate solution was placed on the grid surface and let to set for 30 seconds at room temperature. Solution excess was removed and the samples observed in a Transmission Electron Microscope with an accelerating voltage of 80 kV.

3.5.5. STABILITY STUDY

To evaluate the nanoformulation stability over time, measurements of particle size, polydispersity, zeta potential were performed at 1, 2 and 12 weeks after the production of the nanoparticles. The encapsulation efficiency of EGCG was also evaluated at 2 and 12 weeks after the synthesis of the nanoparticles. The nanoparticles were stored at room temperature (T=25 ºC) and in the dark.

3.5.6. RELEASE STUDY

The release study was used to evaluate the release of the EGCG encapsulated in the nanoformulation. To perform this study, two millilitres of the nanoformulation were placed in cellulose dialysis bag) and exposed to a sink medium, with sufficient volume to dissolve at least three times the amount of EGCG encapsulated in the nanoparticles completely.

To mimic the gastrointestinal tract, the samples were first incubated for 4 hours in 60 mL of fasted state simulated gastric fluid (pH 1.2) and then placed for 19 hours in 60 mL of fasted state simulated intestinal fluid (pH 6.5). Through all the release, the mediums were maintained at 37ºC with constant agitation at 100 rpm and protected from light. At regular intervals, 200 µL of the medium was collected and transferred to 96 UV wells plate for posterior analysis.

At regular intervals, 200 µL of the simulated medium were collected for a UV-Vis microplate and replaced by an equal volume of fresh medium. EGCG release was analysed by UV-Vis spectroscopy using a microplate reader at the wavelength of 273 nm.

Prior to the analysis, a calibration curve, similar at the previously referred for the spectrophotometer, was made in a plate reader at 273 nm.

The cumulative percentage of release compound was determined using the average of the triplicates.

3.6. CELL CULTURE

Caco-2 cells (P.23 to P.32) were cultured in an incubator at 37ºC at 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin. For every two to three days the old medium was discarded and substituted for fresh one. Cells were subculture at 80 to 90% confluence by enzymatically detachment with trypsin-EDTA and cells counted in a Neubauer chamber) with 25% (v/v) Trypan Blue solution (0.4% (w/v) in

PBS) to exclude non-viable cells. Cells were then seeded at a density of 10000 cells per cm^2 in T75 flasks in 10 mL of DMEM.

3.7. CELL VIABILITY ASSAY

To measure the cell viability in the presence of the nanoparticles, methylthiazolyldiphenyl-tetratozium bromide (MTT) assay was performed.

Caco-2 cells were seeded in 96-well tissue culture microplates at a density of 10000 cells per well in 100 µL of supplemented DMEM medium and incubated for 20 hours at 37ºC in 5% CO_2 atmosphere. After the incubation, were added to each well 100 µL of an solution of supplemented DMEM medium with different concentrations (5 µM, 10 µM, 50 µM, 100 µM, 500 µM and 1000 µM) of free EGCG, NLC with and without EGCG and SLN with and without EGCG, as well the viability positive control (supplemented DMEM) and the cytotoxicity positive control (Triton X-100 at 1% (v/v) in PBS). The plates were then incubated for 4 hours in the same cell growing conditions. The supernatant was transferred to a 96-well tissue culture test microplate and stored for the realization of the cell toxicity study. In addition, 200 µL of a MTT solution (0.5 mg/mL of MTT in culture medium) was added to each well and incubated for 3 hours in cell culture conditions to allow the formation of formazan crystals. The MTT solution was then rejected and 200 µL of Dimethyl sulfoxide (DMSO) were added and incubated at room temperature (T=25 ºC) protected from light to dissolve the formazan crystals. Absorbance was read using a microplate reader at 550 nm and at 690 nm as reference wavelength. Cell viability was determined according as follows:

$$Cell\ viability\ (\%) = \frac{Absorbance\ values}{Mean\ of\ the\ positive\ control\ values} \times 100 \qquad \text{(Equation 3)}$$

Half maximum inhibitory concentration (IC50) was calculated using Prism® GraphPad® (version 6) 30 day trial demo.

3.8. CELL TOXICITY ASSAY

The cell toxicity assay was performed by Lactase Dyhydrogenase (LDH) assay. LDH is a cytoplasmatic enzyme present in most cells, being released to the culture medium in cells with cytoplasmic membrane damages. Using a LDH Cytotoxicity Detection Kit, a reagent will react with the LDH forming a colourful formazan, which can de quantify by UV-Vis spectroscopy and directly correlated with the LDH present on the culture medium and hence assessing the cell death.

The first steps of the LDH assay are equal to those performed on the cell viability assay until the end of the incubation period of the samples with the cells. The LDH assay uses the recovered supernatant that resulted from the incubation. The recovered microplates were centrifuged in order to deposit the cellular remains for 10 minutes at 250g, at room temperature (T=25 ºC). After centrifugation, 100 µL of the supernatant were carefully transferred from the top of the well for another 96-well tissue culture test microplate and 100 µL of the LDH Cytotoxicity Detection Kit reaction mixture added. The microplates were left incubate in the dark for 15 minutes at room temperature (T=25 ºC). Absorbance was then read using a microplate reader at the wavelength of 490 nm and at 690 nm as reference wavelength. Cell cytotoxicity was determined according as follows:

$$Cell\ cytotoxicity\ (\%) = \frac{Absorbance\ values}{Mean\ of\ the\ negative\ control\ values} \times 100 \qquad \text{(Equation 4)}$$

IC50 was calculated using Prism® GraphPad® (version 6) 30 day trial demo.

3.9. STATISTICAL ANALYSIS

Statistical analyses were performed using IBM® SPSS® Statistics software (v.22.0.0.0; IBM, Armonk, NY, USA). Data were analysed using one-way analysis of variance (ANOVA) and differences between groups compared by Bonferroni and Tukey post-hoc tests with a P value of <0.05 considered statistically significant.

4
RESULTS AND DISCUSSION

4.1. CHOSEN FORMULATION

Two different solid lipids were used for the synthesis of the nanoparticles. The stearic acid was used initially, being however the obtained formulation unstable in the presence of EGCG. To improve the stability of the solution, a different solid lipid, Precirol® Ato 5 was used, being EGCG dissolved in the water phase instead of the lipid phase. The formulation obtained was completely stable and with no precipitate visible. Notwithstanding, the chosen amount of EGCG was responsible for SLN formulations unstable. To refine the formulation, the initial time of sonication was reduced from 5 minutes to 30 seconds.

4.2. NANOPARTICLE CHARACTERIZATION

To evaluate the success of the nanoparticles' synthesis it is necessary to characterise them. The first sign of correct lipid nanoparticles synthesis is the visualization of the resultant solution, which should resemble milk in appearance with a low viscosity. In the Figure 4 is shown the appearance of the nanoformulations without and with EGCG. The SNL formulations have higher viscosity in comparison to NLC, which appear to be more fluid. The viscosity of both formulations with EGCG seems to be macroscopically more similar. A faint pink colour was noted in the fresh prepared nanoformulations that slightly disappeared over time. None deposit was found on the nanoformulation even after three months of storage at room temperature (T=25ºC) in the dark.

The physical measurements of diameter, zeta potential and polydispersity are presented in Table 9.

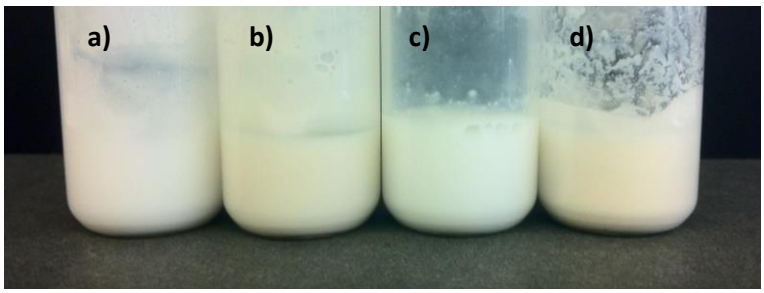

Figure 4 – Synthetized nanoformulations: a) SLN; b) SLN+EGCG; c) NLC; d) NLC+EGCG

Table 9 - Physicochemical properties of the synthetized nanoparticles

	SLN	SLN - 30mg of EGCG	NLC	NLC - 30mg of EGCG
Mean diameter (nm)	206±3	364±11	216±4	300±7
Polydispersity	0.18±0,01	0.19±0.03	0.16±0.02	0.15±0.02
Zeta potential (mV)	-28±1	-24±1	-30±1	-28±1
Encapsulation efficiency	-	83%±0.6	-	90%±0.2
Loading capacity	-	4.3%±0.04	-	4.6%±0.01
Mass encapsulated (mg)	-	26±0.3	-	27±0.1

The hydrodynamic particle size of the lipid nanoparticles (SLN and NLC) without catechin was around 200 nm. The encapsulation of EGCG alters the mean size of the nanoparticles to 364 nm and 300 nm for the SLN and NLC formulations, respectively. The polydispersity of the nanoparticles were all above the 0.1, meaning that the population of nanoparticles obtained are polydisperse (62). It is important to mention that the samples were filtrated before the experiment with a filter with a pore diameter of 800 nm to clean some possible aggregates.

Potential zeta of all the formulations was around -27 mV, which is close to the reference value of $|30\text{mV}|$, revealing that all the formulation are stable (86). The presence of the catechin in SLN slightly decreases the value of the potential zeta in module, being however the formulation also stable. In the case of NLC, the presence of catechin does not change the potential zeta of the formulation (inside the SD).

The encapsulation efficiency of EGCG was 83% and 90% for SLN and NLC, respectively. The loading capacity of EGCG was 4.3% and 4.6% for SLN and NLC, respectively. These encapsulation rates were far superior to the expected values for a high hydrophilic molecule, such as EGCG. These high values can be explained by the capacity of EGCG to complex with lipids. This also explains the high instability of the nanoformulation when the stearic acid was used instead of Precirol® ATO 5. Another possible explanation is that the catechin is interfering with the emulsion process leading to a formation of a double emulsion nanosystem. The loading capacity obtained was also high. Indeed, these results suggest that lipid nanoparticle can be exploited to encapsulate EGCG with a minimal waste of the compound, which is a desirable characteristic for one nanoformulation enters the market.

4.3. STABILITY STUDY

The nanoparticles seem to be presumably stable in the stored conditions (at T=25 ºC and protected from light) with no significant visual change. SLN and NLC formulations' stability monitored through time in terms of particles' diameter, polydispersion, zeta potential and EE. Visualizing the Figure 5 and Figure 6 is possible to see that the diameter of the nanoformulations appeared to shift gradually to a value of approximately 300 nm with no difference in polydispersity. No differences were distinguish from the zeta potential measurements, as observed in Figure 7 and Figure 8. Visualizing Figure 9 is

noticeable that EE remains unalterable during the stored time. These results are in good agreement with the high stability of the nanoformulation, indicating a long shelf time.

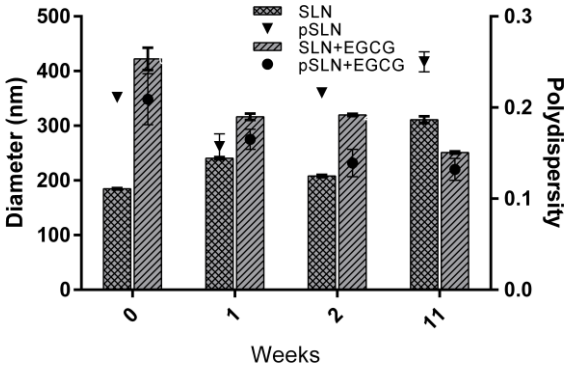

Figure 5 - Evolution of SLN mean diameter and polydispersity

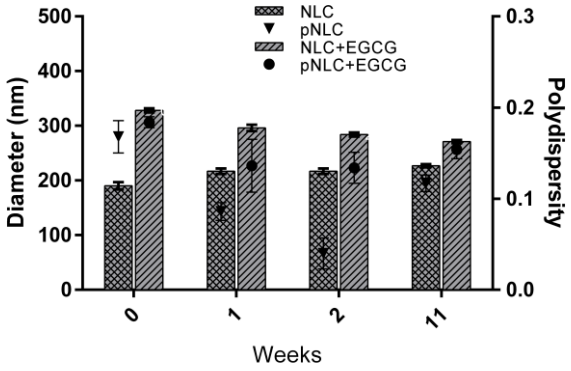

Figure 6 - Evolution of NLC mean diameter and polydispersity

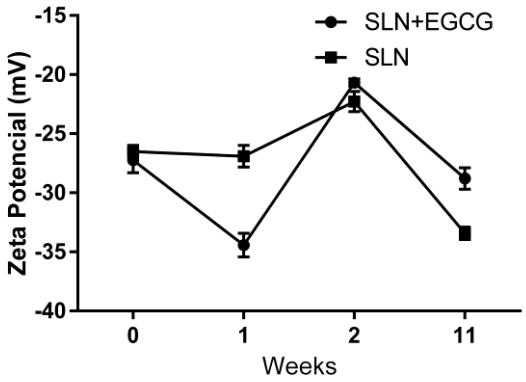

Figure 7 - Evolution of zeta potential of SLN

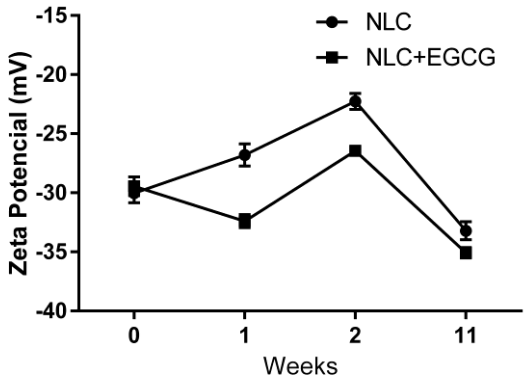

Figure 8 - Evolution of zeta potential of NLC

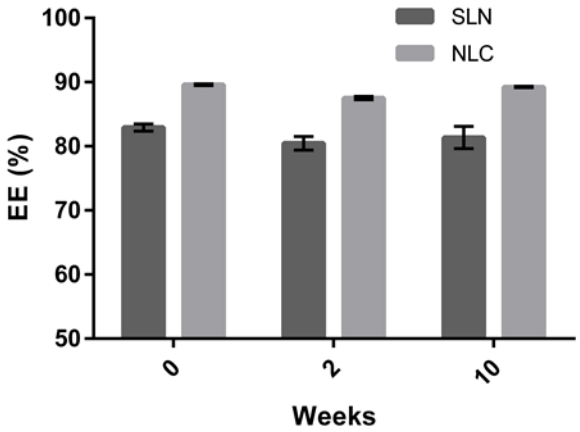

Figure 9 – Evolution of EE

4.4. PARTICLE MORPHOLOGY CHARACTERIZATION

SEM and TEM were used to evaluate the morphology of the nanoparticles. The SEM and TEM images of unloaded and EGCG-loaded SLN and NLC are shown in Figure 10 and Figure 11, respectively.

SEM imaging revealed almost spherical particles for all formulations. The presence of EGCG did not significantly alter the morphology of the nanoparticles. The size of the lipid nanoparticles seem to be divided in two diameter populations for each formulation of approximately 350 nm and 200 nm for SLN and 300 nm and 100 nm for the NLC which are different from the DLS results. The presence of the two populations explain the high polydispersity observed in the DLS results and explain the difference between the DLS and microscopy results, being the mean size calculated by DLS altered by the presence of the mean size populations. The presence of EGCG in the nanoformulations also alters the diameter of the SLN and NLC nanoparticles, leading to an increment of the diameter of around 100 nm.

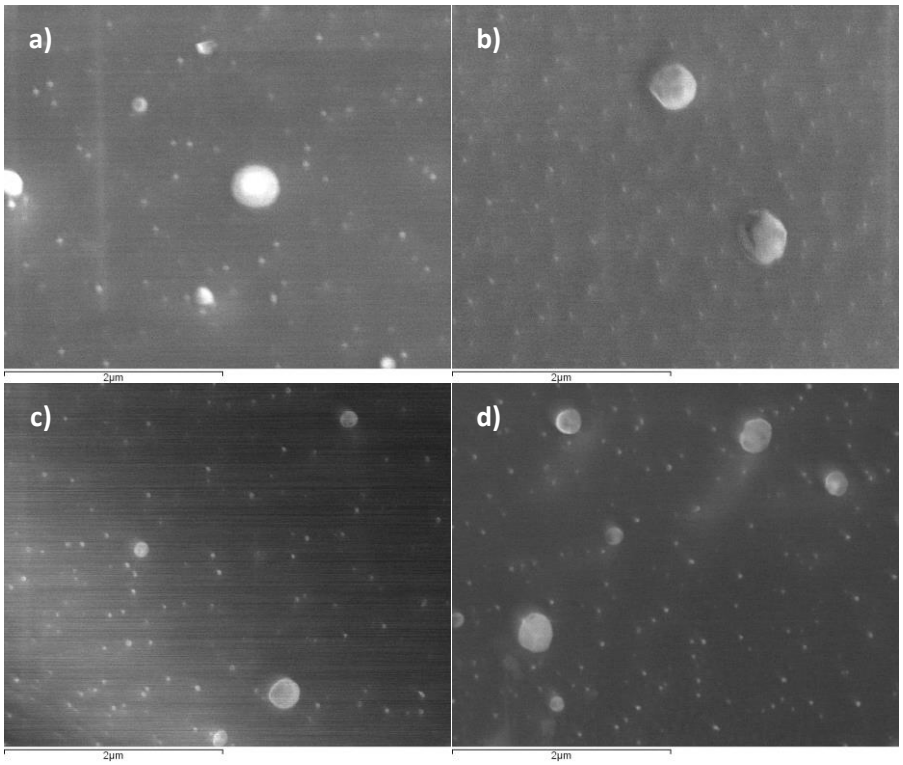

Figure 10 – Cryo-SEM images for: a) SLN; b) SLN+EGCG; c) NLC; d) NLC+EGCG. Scale bar of 2 µm

TEM images also revealed a spherical shape for both formulations in the absence of EGCG. The sizes of these formulations were similar to the sizes obtained using SEM. Notwithstanding, the presence of EGCG seems to alter the morphology of the SLN and NLC nanoparticles. In fact, it is visible that despite spherical nanoparticles can be identified a variety of other different shapes also coexist. Indeed, some nanoparticles with a rod-shaped and also some aggregates with a non-well-defined shape can be identified. The significative difference between SEM and TEM images may be explained by the high vacuum atmosphere, which may contribute to the alteration of the nanoparticles' morphology

In fact the high vacuum atmosphere the water present in the interior of the nanoparticles, was vaporized creating the lipid fragments and the empty concave lipid shells that are visible.

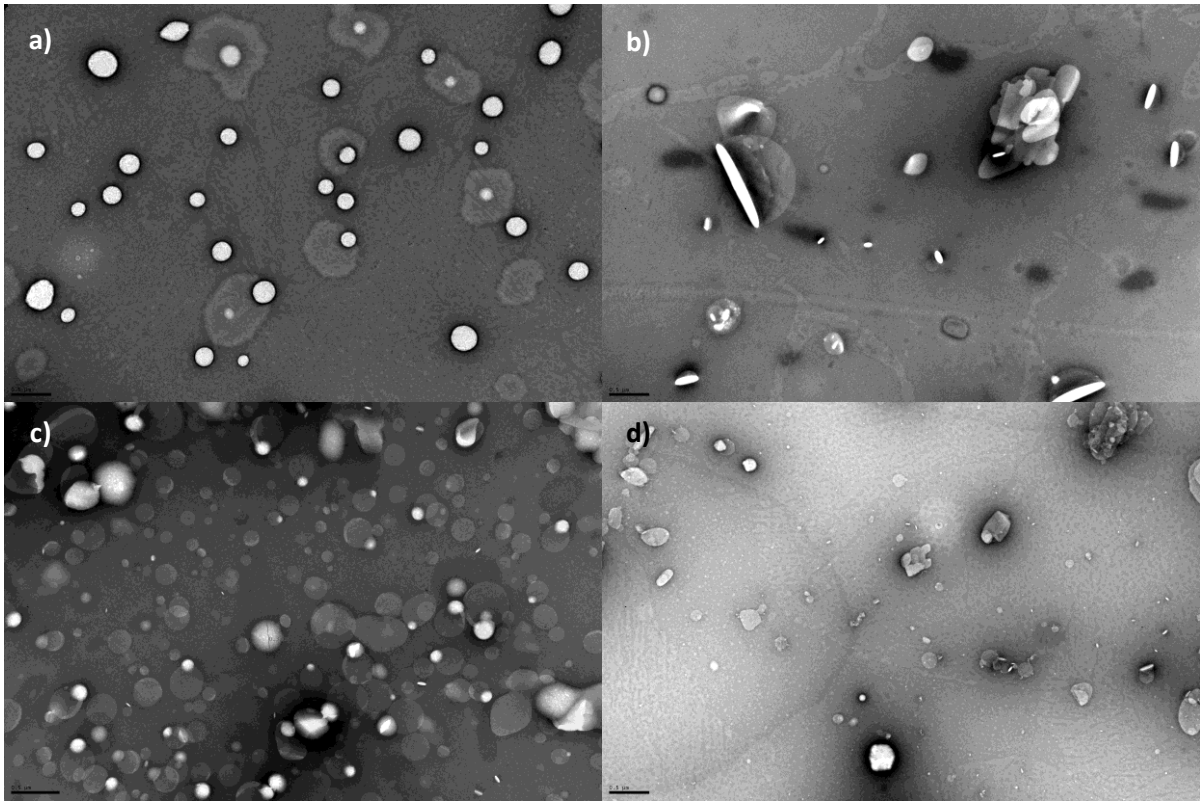

Figure 11 – TEM images for: a) SLN; b) SLN+EGCG; c) NLC; d) NLC+EGCG. Scale bar of 0.5 µm

4.5. RELEASE STUDY

To simulate the passage through the gastrointestinal tract, the *in vitro* release was designed to measure the stability in both gastric and intestinal environments. The nanoparticles were incubated for approximately four hours in gastric fluid to mimic the gastric digestion. After this incubation the nanoformulation is transferred to a simulated intestinal fluid like wood happened in the digestive process and stayed in the medium for another 19 hours to make up 24 hours of release. The release times were hover estimated to ensure the stability of the nanoparticles is guaranteed in the normal conditions.

The objective was to guarantee a low release in the gastric environment followed by a controlled release in the intestine and probably induce the uptake of EGCG still in the nanoparticles. These conditions are presented to minimize the quantity of EGCG destroyed in the digestive process and enhance the amount that is internalized.

The Figure 12 shows the release evolution of the EGCG in the formulations. In this gastrointestinal simulation, a very small amount of EGCG was liberated in the gastric fluid, about 10% in both nanoformulations, witch fulfill one of the desired aspects proposed. Is also visible the stabilization of the release profile after the 3 hours mark, meaning that the nanoparticles could reached an equilibrium in the gastric fluid diminishing the velocity of dissolution. In the following stage, the release of the

catechin was increased slightly. After 24 hours the cumulative release of the nanoformulations was around 40% remaining almost 20 mg of catechin in nanoparticles. Only a small difference can be seen between the two nanoformulations, were the SLN release about 13% more catechin tan the NLC in the end of the release time.

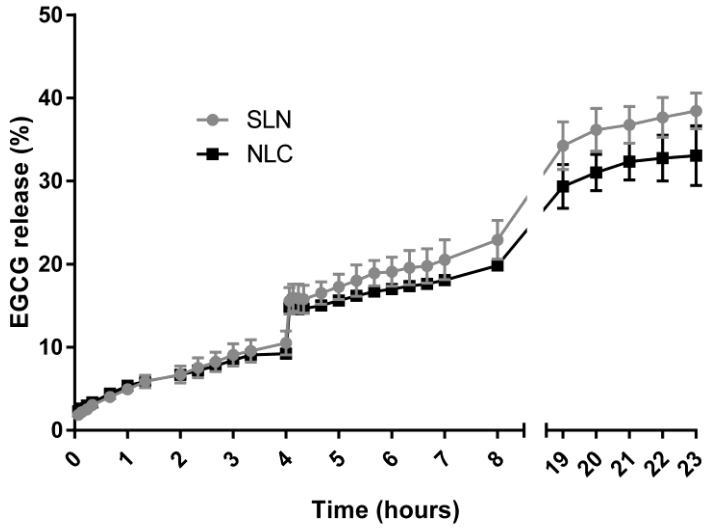

Figure 12 – EGCG release in simulated gastrointestinal.

These values are very interesting since the amount of drug that is leaked to the intestinal fluid is very low leading to a protection from the harsh conditions and ultimately enhance the bioavailability. If the nanoparticles are capable of permeate the cell membrane this could revealed a high efficient drug delivery system.

4.6. CELL VIABILITY AND CYTOTOXICITY ASSAY

To evaluate the potentially toxicity caused by the synthetized nanoparticles in the intestinal mucosa a MTT and LDH assays were performed in Caco-2 cell line observed in Figure 13.

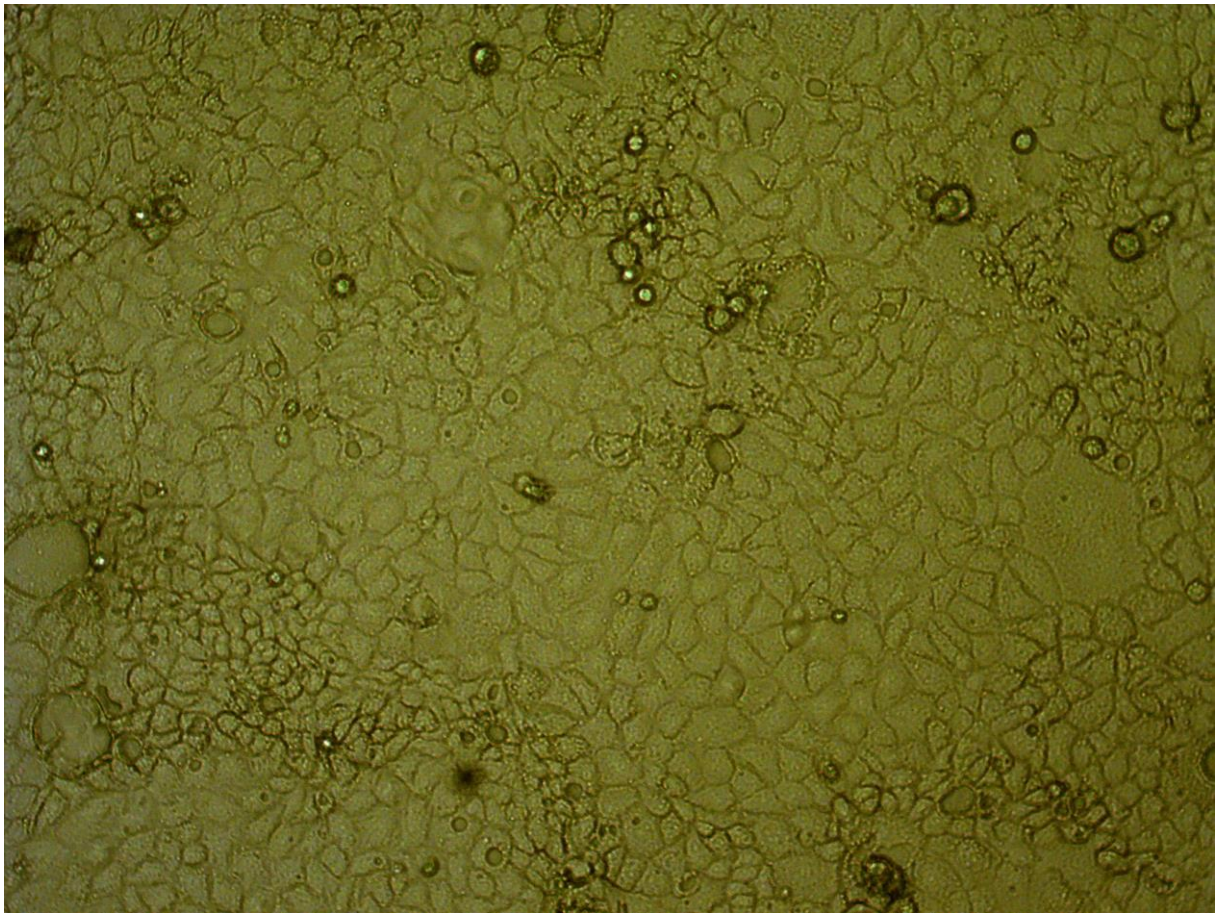

Figure 13 – Morphology of confluent Caco-2 cells by inverted microscope observation with 100X magnification

The viability of the cells in the presence of different concentrations of the nanoparticles, free catechin and positive control (culture medium) are presented in Figure 14 in relative percentage. In the figure is possible to observe that free EGCG in the concentrations used does not revealed any cytotoxic effect with the cell viability around 100%. Comparatively, the triton X-100 presents a viability closed to 0%, revealing high cytotoxicity, as expected. Observing the different nanoformulations is possible to see the placebo nanoparticles present a significative lower viability in comparison with the culture medium. This behaviour was also expected, because being well known that nanoparticles presents by then selves some level of toxicity. Interestingly, the presence of EGCG in the nanoparticles seems to diminish the cytotoxicity of the nanoparticles. Is possible to observe that only the higher concentration of SLN + EGCG (0.5 mM) present a significant difference comparatively to the culture medium.

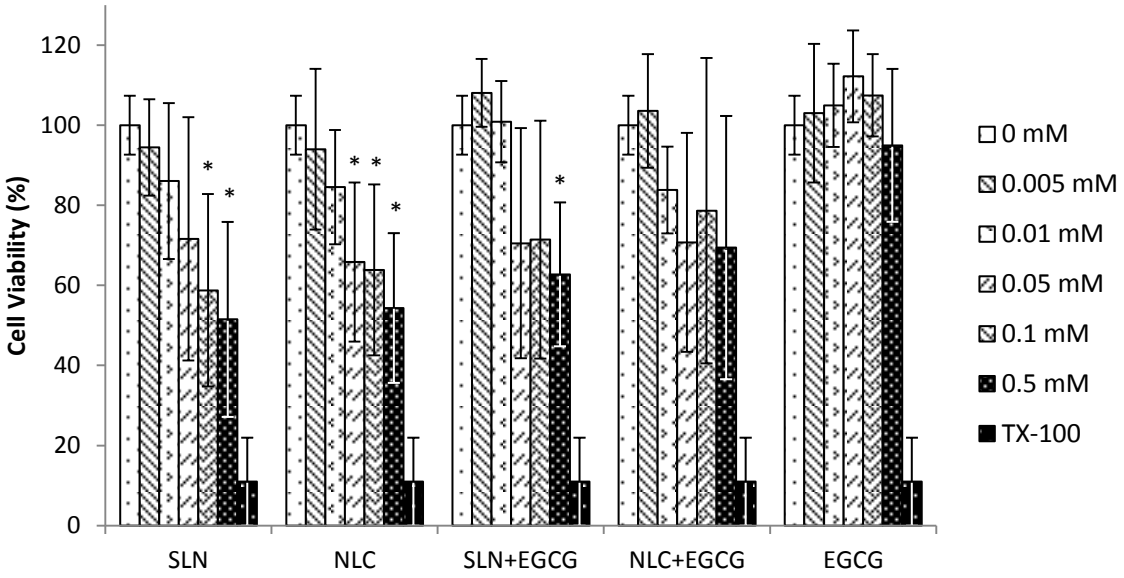

Figure 14 – MTT results for Caco-2 cell line (Mean ± SD)

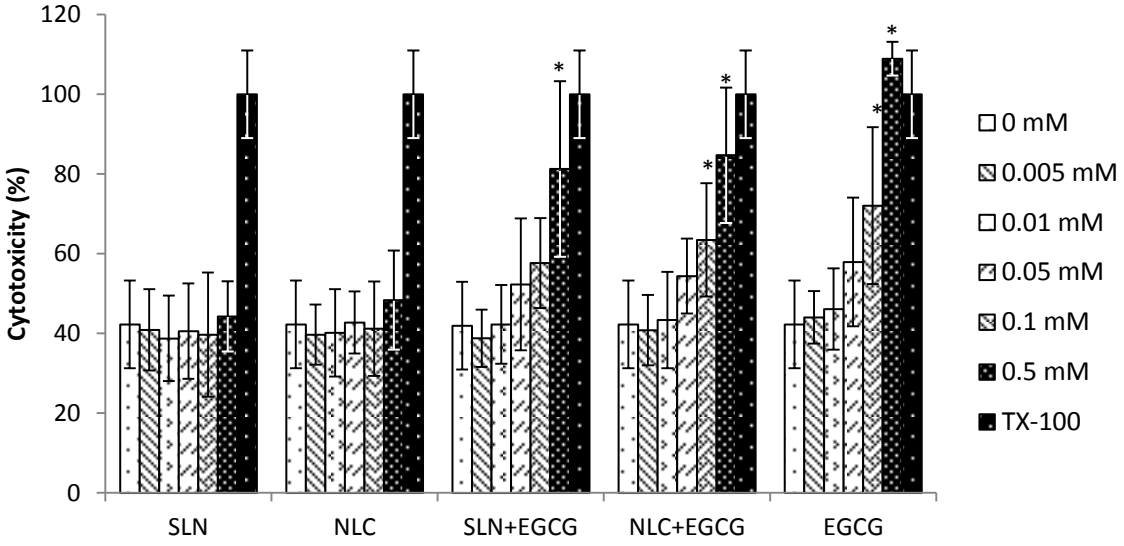

Figure 15 – LDH results for Caco-2 cell line (Mean ± SD)

In the other and, the LDH results presented in Figure 15, indicate no difference between the concentrations of placebo nanoparticles. The graph also shows an increase in cell cytotoxicity on the highest concentrations (0.5mM) for SLN+EGCG and (0.1 and 0.5 mM) for NLC+EGCG nanoparticles. Contrarily to the MTT assay, the free EGCG also seems to possess more cytotoxicity in concentrations above 0,1mM.

By analysing the two assays, EGCG appears to not have toxic effects in Caco-2 cells lines, although it appear to have an high membrane destabilization property due to the high LDH that was released. Both placebo nanoparticles diminished the cellular viability at concentrations superiors that 0.1mM and 0.05 mM for SLN and NLC formulations respectively. The placebo nanoformulations have not affected the LHD release, meaning that the nanoparticles do not interact with the cellular membrane.

The presence of EGCG in the formulation seems to enhance the viability of the cells in comparison with the placebos. Only the SLN+EGCG have shown a significative diminishing of the viability. The EGCG nanoformulations also appear to enhance the membrane stability, presenting some LHD reactivity. This could be due to the release of EGCG from the nanoparticles.

For further evaluate the toxicity of the nanoformulations, a half inhibitory concentration (IC50) was calculated for MTT and LDH assays and presented in Figure 16 and Figure 17 respectively. In the MTT assay, none of the formulations concentrations used reached the 50% of inhibition for Caco-2 cell lines.

In the LDH assay, was possible to determine the IC50 of 16, 55 and 35 μM for free EGCG, SLN+EGCG and NLC+EGCG respectively. The IC50 for the free EGCG is closed to the value found in previous reports which is around 20 μM. According to the literature, the value of EGCG reach in the blood after two or three cups of tea is around 1 μM which is far less than the minimum IC50 found in this work. The nanoformulations also appear to diminish the toxicity of EGCG. Based in these results is possible to infer that EGCG nanoparticles can be a safer way to administer EGCG.

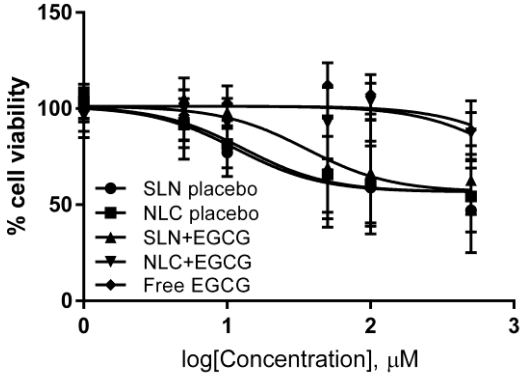

Figure 16 – IC50 graph for MTT assay

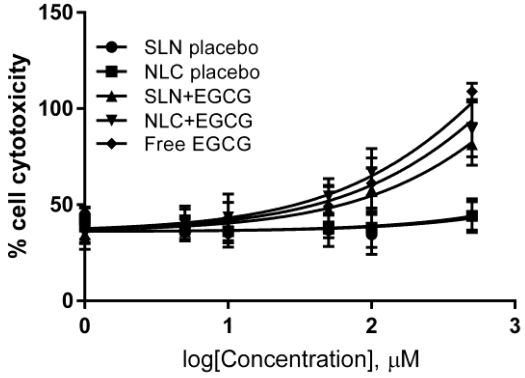

Figure 17 – IC50 graph for LDH assay

5
CONCLUSIONS

In the course of this work it was evident that the tea catechin, EGCG, can contribute to the life quality of overall population in the prevention and treatment of several health problems like: cancer, Parkinson, Alzheimer's, obesity and cardiovascular diseases. Additionally, the tea catechin is also very safe with no documented side effects, making an interesting molecule to research for the health care marked in the form of drug, supplement of nutraceutical.

However a systematic use of these polyphenol is affected by the low bioavailability. Is needed a large dose to accomplish the therapeutical objectives leading to a waste of the compound.

To overcome this problem, is proposed the use of nanoparticles as carriers of the catechin. Lipid nanoparticles are well known as drug transporters allowing high encapsulating rates associated with improve in molecule pharmacokinetics.

For these reasons was proposed a development of a lipid nanoformulation to transport and enhance the oral bioavailability of the poorly absorbed EGCG leading to the creation of a product capable to enter the marked as a supplement or nutraceutical.

The goal in this thesis was to create this nanosystem resulting in creation of a Precirol® ATO 5, Tween® 60 solid lipid nanoparticles, SLN, and a Precirol® ATO 5, Miglyol® 182, Tween® 60 nanostructured lipid carriers, NLC, successfully loaded with EGCG. Both formulations present themselves with high encapsulating efficiencies of 80% and 90% with a loading capacity of 4.3% and 4.6% for SLN and NLC respectively. SLN formulation presented a mean diameter of 360 nm with a polydispersity of 0.19. NLC particles were slightly smaller with a size of 300 nm and a narrower polydispersity of 0.16. The nanoparticles zeta potential was inferior to |30| mV, however the values were sufficiently high to not interfere significantly with the nanoformulation stability. Under microscopy, the particles seemed to be spherical with two close size populations in both nanoformulations with similar diameters to those obtained by DLS analysis.

The nanoformulation stability in storage conditions was high with no significant difference observed over a period of three months.

The *in vitro* release study revealed a high stability and small release of the catechin in the gastric environment with a small increase in intestinal medium. The cumulative release over the 24 hours of the release study, only 40% of the total catechin used was liberated to the sink medium. This was the

aimed profile, enabling a controlled release in the intestinal environment and the possibility of enhancing the cellular uptake of the EGCG loaded nanoparticles by epithelial cells in the intestine.

The developed nanoformulations and all the results described in this work exposed the promising potential to enhance the bioavailability of EGCG unlocking the restrains associated with this effective and multipurpose molecule leading to a broader use trough the population.

Notwithstanding more research needed to be done to accomplish the initial goal to create a health improving formulation to improve the overall healthiness population.

5.1. FUTURE PROSPECTS

The promising results of this work lead to the performance of a permeability study using caco-2 cell monolayers to evaluate the possible permeability enhancement induced by the lipid nanoparticles.

An *in vivo* study would be also very useful to further evaluate the pharmacokinetics and to determine if some toxicity is not detected in the *in vitro* studies.

Is also important develop a lyophilisation protocol that did not interfere with properties and stability of the nanoformulation.

Improvements of some characteristic of the nanoformulation are also possible for further refinement like modifying the lipid to enhance the EE.

At last and if the results of all the anterior studies reveal encouraging results is possible to pass to human studies to evaluate safety and pharmacokinetics profiles.

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