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Master Thesis for the degree Master of Pharmacy

Development of liposomal formulation for green tea catechins targeted for the treatment of vaginal inflammation

By

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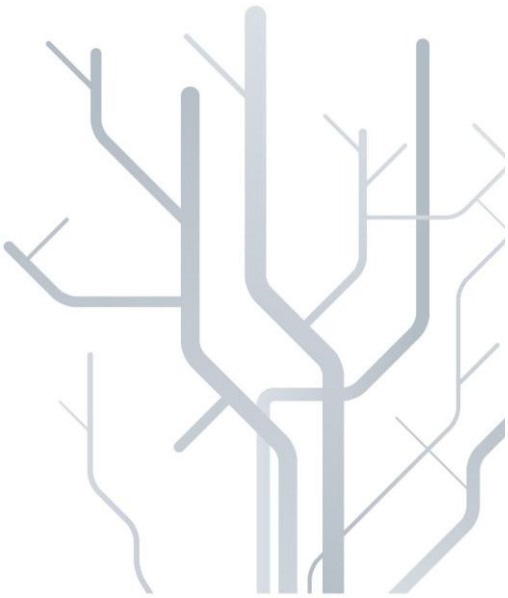

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Johanne Naper Trønnes

Table of contents

List of Figures

List of Tables

Abstract

Green tea catechins are well known for their antioxidative and anti-inflammatory properties. In this connection, catechins are thought to be potential candidates in prevention and treatment of diseases caused by oxidative damage. In addition, catechins have been shown to be effective in the treatment of genital warts. However, they exhibit low bioavailability and poor solubility, which limits their use. This project focused on development of liposomal delivery system containing catechin and epicatechin, respectively, as mean to circumvent their physiochemical limitations and to enhance their biological activity. The formulation was intended for treatment of genital warts/vaginal inflammation. To become closer to an actual *in vivo* application, drug-bearing liposomes were incorporated in a mucoadhesive hydrogel.

A strong antioxidative activity of both substances were confirmed, with epicatechin exhibiting significantly stronger antioxidant potential ($p<0.01$).

Phosphatidylcholine liposomes containing catechin were optimized with respect to vesicle size and entrapment efficiency. The optimized formulation was sonicated for 2 minutes and had an average diameter of 150 nm. These vesicles entrapped approximately 50 % of catechin. The corresponding liposomes prepared with epicatechin were found to have smaller size, however the entrapment was similar. Liposomes containing catechin were further incorporated in a hydrogel vehicle and the hydrogel was found to exhibit satisfactory adhesiveness and cohesiveness. *In vitro* release of catechin from liposomal preparations (both in a form of suspension and incorporated in hydrogel) was performed on Franz diffusion cells under condition simulating both the vaginal environment and physiological conditions. Liposomal hydrogels were found to provide sustained release of catechin. The release was more sustained at pH 4.0 than neutral pH.

In conclusion, we can confirm that encapsulation of catechins in liposomes and their subsequent incorporation in hydrogels provides potentials for development of novel drug delivery systems for vaginal therapy with catechin. The evaluation of anti-inflammatory potentials of developed delivery system is now in progress in our laboratory.

Keywords: green tea catechins; liposomes; hydrogels; vaginal therapy, genital warts

List of abbreviations

BCC	Basal cell carcinoma
(+)-C	Catechin
DD	Degree of deacetylation
DPPH	1,1-diphenyl-2-picrylhydrazyl
(-)-EC	Epicatechin
(-)-EGCG	Epigallocatechin-3-gallate
(-)-ECG	Epicatechin-3-gallate
(-)-EGC	Epigallocatechin
FDA	Food and Drug Administration
FDC	Franz diffusion cells
(+)-GC	Gallocatechin
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GTP	Green tea polyphenols
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
INF-α	Interferon alpha
iNOS	Inducible nitric oxide synthase
LUVs	Large unilamellar vesicles
MLVs	Multilamellar vesicles
Mw	Molecular weight
N-SL	Non-sonicated liposomes
PBS	Phosphate buffer saline
PC	Phosphatidylcholine
PI	Polydispersity index
SD	Standard deviation
SL-2	Liposomes sonicated for 2 minutes
SL-4	Liposomes sonicated for 4 minutes
SUVs	Small unilamellar vesicles
VFS	Vaginal fluid simulant

1 General introduction

Green tea has been consumed in China and other Asian countries since ancient times for the purpose of maintaining and improving health. Nowadays, green tea is considered one of the most promising nutraceuticals for the prevention and/or treatment of many diseases and is therefore being extensively studied worldwide (Cabrera et al., 2006).

Green tea contains high amount of polyphenols, mostly catechins, which are known to be strong antioxidants. Antioxidants normally protect the body against free radicals produced under physiological stress conditions. Catechins have a potential as preventive and therapeutic agents for various conditions caused by oxidative damage such as for example cancer, cardiovascular and neurodegenerative diseases. In addition, evidence also suggests that green tea is effective for treating genital warts (Graham, 1992; Schneider and Segre, 2009). This property of catechins was of particular interest for this project.

Landmark in development of natural origin drugs came in 2006, when a green tea extract was approved by the Food and Drug Administration (FDA, USA) for the treatment of genital warts. It is the first botanical extract ever approved by the FDA as a prescription drug, as up to then all natural origin substances were approved as single, defined substance (Schmidt et al., 2007). The product Veregen® is an ointment applied topically and its efficacy was shown to be superior in clinical studies, compared to conventional genital warts therapy (Gross, 2008).

However, catechins suffer from poor solubility and low bioavailability and thus, their pharmacological properties are not fully utilized. Their bioavailability and corresponding therapeutic efficacy can be improved by their incorporation in suitable delivery systems. The delivery system is also expected to reduce the dose needed for efficient drug therapy, as the carrier system enhances activity of associated active substance.

We aimed at development of nano delivery systems for improved anti-inflammatory properties of catechins destined for topical vaginal therapy.

Due to the physicochemical properties of catechins, liposomes were proposed as optimal carrier system for catechins. Liposomes have the ability to encapsulate a wide variety of drugs and enhance their biological activity (Basnet et al., 2012). However, liposomes are liquid in

nature and will not remain at the site of administration for a longer period of time when applied topically (Pavelic et al., 2001). To obtain a delivery system with an appropriate viscosity and to become closer to an actual *in vivo* application, we propose incorporation of liposomes in suitable hydrogels. Optimization of liposomal delivery system for catechins is described in this Thesis.

2 Introduction

2.1 Green tea

2.1.1 Origin, cultivation and classification of tea

Tea is a popular beverage, consumed worldwide. Tea, in its marketed form is obtained from the leaves of the plant *Camellia sinensis* (L.) Kuntze, Theaceae family. The plant is an evergreen shrub with elliptical, serrated leaves. In the wild it can grow up to 10 – 15 meters high, but when cultivated it is pruned to a height of 0.6 – 1.5 meters. It has white, fragrant flowers (Ross, 2005).

The plant originated from China and Southeast Asia and has been cultivated for more than 2000 years (Graham, 1992). It grows best in tropical and subtropical areas with adequate rainfall, good drainage, and slightly acidic soil. Today, the plant is cultivated in about 30 countries worldwide (Chan et al., 2007).

Figure 1: Schematic representation of different aerial part of Tea plant (*Camellia sinensis*). (www.plant-pictures.de)

The youngest leaves of the plant and the terminal buds are used in the production of tea, resulting in the finest quality of tea (Chan et al., 2007).

Based on the manufacturing process, tea can be classified into three major types, namely:

- Black tea
- Green tea
- Oolong tea (Graham, 1992)

Green tea is produced in such a way as to prevent the fermentation of the tea constituents, while in black tea production, the fermentation of these constituents is promoted. Oolong tea is an intermediate of green and black tea and is only partially fermented (Graham, 1992; Cabrera et al., 2003; Yang et al., 2008).

Approximately 76-78 % of the tea produced and consumed worldwide is black tea, while 20-22 % is green tea and less than 2 % is oolong tea (Cabrera et al., 2003).

Recently, green tea has attracted a lot of attention among scientists and the general public, due to its health beneficial potential (Dube et al., 2010).

2.1.2 Green tea components

The composition of green tea is quite complex and varies with the origin of the leaf and with manufacturing conditions. The chemical composition of green tea is similar to that of fresh tea leaves (Chan et al., 2007). Table 1 shows the major components of fresh green tea leaf according to Graham, (1992):

Table 1: Chemical composition of fresh green tea leaf, expressed in dry weight percentage.

Chemical constituents	(%)
Polyphenols	36
Carbohydrates	25
Protein	15
Lignin	6.5
Ash	5
Amino acids	4
Methyl xanthines	3.5
Lipids	2
Organic acids	1.5
Chlorophyll	0.5
Carotenoids	<0.1
Volatiles	<0.1

The polyphenols constitute the largest group of the chemical components in green tea, followed by carbohydrates and proteins. Caffeine is also found in green tea as methyl xanthenes, together with theobromine and theophylline. The carotenoids are present in very low quantities, but are important precursors of tea aroma (Graham, 1992).

Polyphenols have long been regarded as a pool of bioactive natural products with potential benefits for human health and are therefore the most interesting group of the green tea components (Quideau et al., 2011).

2.1.3 Polyphenols in green tea

Polyphenols constitute a large group of many different compounds. They are not only found in green tea, but also in other plants and foodstuff (Basnet and Skalko-Basnet, 2011).

Most of the polyphenols present in green tea are flavanols, commonly known as catechins (Huang et al., 2011). They are derived from a combination of the shikimate and acetate-malonate biosynthetic pathway and constitute up to 30 % of the dry weight of green tea leaves (Zaveri, 2006; Quideau et al., 2011). Catechins are colourless, slightly water-soluble compounds and contribute to the astringency and bitterness of tea infusion (Dewick, 2001; Fang and Bhandari, 2010).

The major catechins found in green tea are listed below:

- (-)-epigallocatechin-3-gallate (EGCG)
- (-)-epigallocatechin (EGC)
- (-)-epicatechin-3-gallate (ECG)
- (+)-gallocatechin (GC)
- (-)-epicatechin (EC)
- (+)-catechin (C) (Graham, 1992; Zaveri, 2006)

The structures of these compounds are summarized in Figure 2:

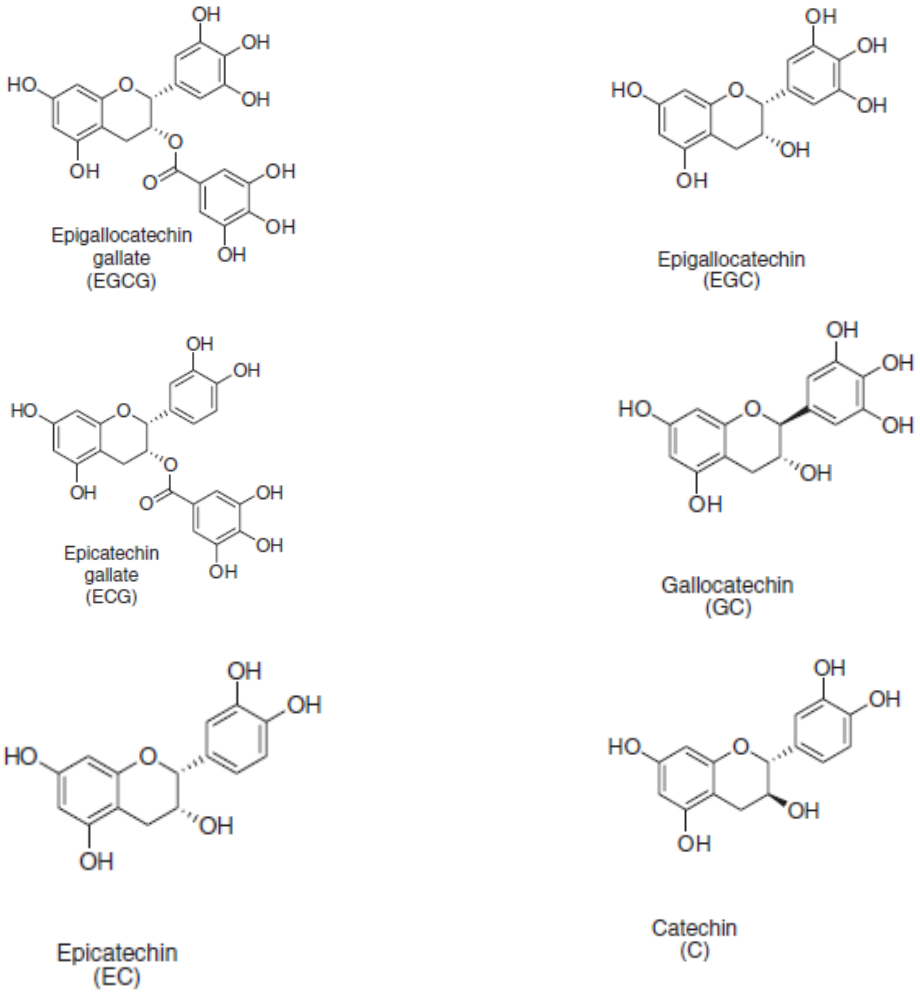

Figure 2: Structure of the major catechins in green tea (Elabbadi et al., 2011).

Epigallocatechin-3-gallate (EGCG) is the most abundant catechin in green tea (Huang et al., 2011).

Some of the catechins are stereoisomers, for instance catechin and epicatechin, which are epimers. They have the same absolute configuration, except at one designated stereocenter, where their configurations are opposite (Johnson, 1999).

All polyphenolic catechins are well-known strong antioxidants and it is this property that makes them interesting candidates for development into therapeutic agents (Dube et al., 2010).

2.1.4 Green tea polyphenols as antioxidants in prevention and treatment of diseases

A free radical is any atom or molecule that has an unpaired electron. Free radicals are highly reactive species (Halliwell, 1994). They are formed in the human body as a response to environmental radiation and/or physiological stress. At moderate concentrations they play an important role as regulatory mediators in cell signaling processes, whereas in high concentrations, they are hazardous and may cause cell damage (Dröge, 2002). Under oxidative stress conditions there is an overproduction of these radicals in the body and the endogenous cellular antioxidants are not able to subdue these reactive species (Quideau et al., 2011). Oxidative stress to biomolecules has been implicated in the pathology of many chronic inflammatory and degenerative disorders (Cabrera et al., 2003; Higdon and Frei, 2003). Since polyphenols have the capability to scavenge these radicals (antioxidative activity), they have a potential in disease prevention and treatment.

Two main radical scavenging mechanisms have been proposed by now (Quideau et al., 2011):

1. Hydrogen-atom transfer: This mechanism is based on the ability of a phenol function group to donate a hydrogen atom to a free radical (R·). The efficiency of the reaction relies on the rapidity of the H-atom transfer and the stability of the resulting phenoxy radical.

$$R\cdot + ArOH \rightarrow RH + ArO\cdot$$

2. Single electron transfer: The second mechanism is based on a single-electron transfer from ArOH to a free radical (R·), with formation of a stable radical cation ($ArOH\cdot^{+}$).

$$R\cdot + ArOH \rightarrow R^{-} + ArOH\cdot^{+}$$

It is through these mechanisms that polyphenols exhibit their main antioxidative activity. However, they can also exhibit protective roles by chelating metal ions and blocking the action of the enzymes responsible for reactive oxygen species production (xanthine oxidase and protein kinase) (Quideau et al., 2011).

Structural features of the catechins that appear to be important in conferring its antioxidative activity include (see Figure 2 and Figure 3):

- The presence of at least two hydroxyl groups on the B ring in an orthodiphenolic arrangement, which participates in the delocalization and stabilization of the radical form.
- A hydroxyl group (R1) or an esterified gallate moiety (R2) at the 3rd position in the C ring, adding more hydroxyl groups to the molecule.
- Additionally, the A ring for EGC and EGCG (Higdon and Frei, 2003).

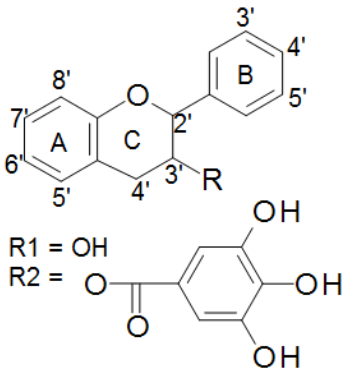

Figure 3: General structure and nomenclature of catechins (drawn).

In addition to its antioxidative capabilities, catechins have effects on several cellular and molecular targets in signal transduction pathways, which contribute to anti-inflammatory properties as well:

- Green tea catechins have been found to inhibit the activation of the transcription factor NF-κB, which regulates the expression of pro-inflammatory gene products.
- Catechins have been found to inhibit lipoxygenase and cyclooxygenase activity, enzymes which are capable of increasing oxidative stress or damage in some tissues.
- Catechins decrease the activity and protein levels of inducible nitric oxide synthase (iNOS) enzymes through reducing the expression of iNOS genes.
- Catechins have been found to increase the activity of the antioxidant enzymes normally present in the human body, such as glutathione peroxidase, catalase and superoxide dismutase (Higdon and Frei, 2003).

The above mentioned effects of green tea catechins show the potential of these agents in prevention and treatment of diseases linked to oxidative damage or inflammation. A number of studies have been conducted in order to investigate these potentials further.

2.1.5 Health beneficial potential of green tea

Green tea has been considered a medicine and a healthy beverage since ancient times. In traditional Chinese medicine, this beverage has been used for the treatment of headaches,

depressions, digestion problems, detoxification and in general to prolong life (Cabrera et al., 2006).

The antioxidative properties of green tea catechins have been examined in a number of *in vitro* systems. In a study, catechins were found to be more potent scavengers of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals than vitamin C and vitamin E. The relative activities in scavenging DPPH radicals were found to be in order of EGCG ≈ ECG > EGC > EC. The explanation for this finding is related to the presence of trihydroxyl groups on the B ring and the gallate moiety at the 3 position in the C ring of EGCG and ECG (Figure 2), which increases their activity (Higdon and Frei, 2003; Zaveri, 2006).

Oxidative damage and inflammation have been proven in preclinical studies as the root cause of cancer and chronic diseases. Studies have suggested that cancer could be prevented or significantly reduced by treatment with antioxidants and anti-inflammatory drugs (Basnet and Skalko-Basnet, 2011). Results from human studies investigating the effect of catechins on cancer treatment are however inconsistent, some studies have shown a reduced cancer incidence, whereas others have failed to show an effect. On the other hand, animal models provided more convincing data and have demonstrated an inhibition of tumorigenesis by tea extracts and tea polyphenols in organs such as the lung, oral cavity, esophagus, stomach, small intestine, colon, skin, prostate, mammary glands, liver, pancreas and bladder (Yang et al., 2008). An excellent review of recent studies is given by Yang et al. (2008). Researchers have linked the anticancer effects of catechins, and especially EGCG, to growth inhibition, regulation of cell cycle progression and induction of apoptosis (Lambert and Yang, 2003; Cooper et al., 2005). Additional information and detailed mechanisms of the chemopreventive and cytoprotective effects linked to the EGCG from green tea is provided by Na and Surh (2008) and Tachibana (2011).

Epidemiological studies have linked green tea consumption with a reduced risk of cardiovascular diseases. An example is a study conducted by Sano et al. (2004). The mechanism is proposed to be linked to antioxidative properties of catechins, which may prevent the oxidation of LDL-cholesterol, a molecule associated with an increased risk for atherosclerosis and heart disease (Sano et al., 2004; Cabrera et al., 2006).

Catechins are reported to have protective properties against neurodegenerative diseases such as Parkinson's and Alzheimer's disease. An *in vivo* study showed that catechins protected dopaminergic neurons against neurotoxins produced under oxidative stress (Parkinson's

disease) (Zaveri, 2006; Pan et al., 2010). A study by Choi et al. (2001) showed that EGCG protects against beta-amyloid-induced neurotoxicity in cultured hippocampal neurons (Alzheimers's).

Today's society is very focused on maintaining a healthy body image and a normal body weight. A study conducted by Sayama et al. (2000) investigated the anti-obesity effect of green tea in mice, by feeding female mice with 1-4 % of green tea powder for 4 months. The results showed that the mice fed with green tea in their diets had a significantly suppression of food intake, body weight gain and fat tissue accumulation. Also the serum leptin levels decreased, indicating that green tea may have a beneficial effect leading to weight loss (Sayama et al., 2000).

Green tea catechins are also reported to have antibacterial and antiviral activities (Cabrera et al., 2006)

A green tea extract of polyphenols were reported to have antimicrobial activity against oral bacteria, such as *Escherichia coli*, *Streptococcus salivarius* and *Streptococcus mutans* in an *in vitro* study conducted by Rasheed and Haider (1998). This indicates that catechins might have therapeutic and/or preventive applications in microbial dental diseases (Rasheed and Haider, 1998; Cooper et al., 2005).

EGCG is also reported to have a protective role against Human immunodeficiency virus (HIV) infection. A study conducted by Kawai et al. (2003) showed that EGCG prevents the attachment of the HIV-1 virion, gp120, to the CD4 molecules on T-helper cells, thus preventing the initial step in the HIV-1 infection process. However, further studies remain to be conducted in order to see if these effects can be confirmed in humans.

In 2006, a green tea extract was approved by the Food and Drug Administration (FDA) for the treatment of genital warts, caused by Human papilloma virus (HPV) infection. It is the first botanical extract ever approved by the FDA as a prescription drug (Schmidt et al., 2007). The product, named Veregen® (originally called Polyphenone E®), contains a mixture of catechins and other green tea components. Catechins constitute 85-95 % (by weight) of the total drug substance. Veregen® is an ointment with 15 % drug concentration, in a water free ointment base consisting of isopropyl myristate, white petrolatum, cera alba, propylene glycol, palmitostearate and oleyl alcohol (www.veregen.no). Veregen® ointment 15% has shown better efficacy in clinical studies compared to other conventional therapies, e.g.

imiquimod, for genital warts. The recurrence rate was relatively low (6.2 %), and the product was well tolerated among patients. Systemic side effects are expected to be very unlikely and local application site reactions occurred rarely (Gross, 2008; Stockfleth et al., 2008; Tatti et al., 2010).

Based on the demonstrated effects of green tea catechins, we wanted to further study their effect in topical vaginal therapy focusing on genital warts management and/or treatment of vaginal inflammation.

2.1.6 Delivery systems for catechins

Green tea catechins possess certain physiochemical limitations which are challenging their development into therapeutic agents. They are slightly water-soluble and unstable in solution. In addition, catechins have very low oral bioavailability, due to their poor absorption and rapid systemic elimination (Fang and Bhandari, 2010; Peres et al., 2011). To fully utilize the potentials of these agents, there is a need to overcome the limitations seen with free catechins, e.g. by their encapsulation. Up to now, several delivery systems have been proposed as suitable for catechins encapsulation.

In a study performed by Dube et al. (2010) catechin and EGCG, respectively, were encapsulated in chitosan nanoparticles of a mean diameter below 500 nm. The aim of this study was to evaluate whether the encapsulation in carrier system could enhance the *in vitro* intestinal absorption of catechins. The study concluded that encapsulation of catechins in chitosan nanoparticles enhanced *in vitro* intestinal absorption, probably due to increased stability of catechin and EGCG. Another study by Wisuitiprot et al. (2011) showed that encapsulation of catechins from a green tea extract in chitosan microparticles (mean diameter 1.88 μm) improved their skin penetration as compared to penetration of free substances. The chitosan particles also limited the degradation of catechins by skin enzymes (Wisuitiprot et al., 2011).

Poly(ε-caprolactone)/multi-walled carbon nanotubes (PCL/MWCNTs) composite nanofibers with various content of green tea polyphenols (GTP) were successfully fabricated in a study conducted by Shao et al. (2011). It was demonstrated that GTP loaded composite nanofibers had low cytotoxicity on normal osteoblast cells, but high inhibition effect to tumor cells, especially Hep G2 cells (Shao et al., 2011).

Liposomes have also been studied as a promising carrier system for catechins. A study conducted by Huang et al. (2011) investigated the effect of elastic liposomes containing catechin on its oral delivery and brain distribution. The results showed a significantly increased plasma concentration and 2.9 times higher accumulation of liposomal catechin in certain areas of the brain compared with the aqueous solution of catechin which served as control (Huang et al., 2011). The study by Fang et al. (2006) showed that EGCG encapsulated in liposomes increased drug deposition in basal cell carcinomas (BCC) by a 20-fold increase compared to the free form. Liposomal EGCG induced cell death of BCCs more effectively than did the control solution at lower EGCG doses. This was due to the ability of liposomes to maintain EGCG stability.

Up to be best of our knowledge, no liposomal catechins, destined for administration and evaluation in vaginal site were reported.

2.2 Liposomes

Liposomes have received a lot of attention during the past 30 years as drug carriers of great potential (Torchilin, 2005). They are spherical particles, consisting of one or several concentric membrane(s) which entirely enclose an aqueous volume. The membrane(s) consists of lipid molecules, usually phospholipids. The phospholipids form liposomal vesicles in bilayered structures which resemble the lipid portion of natural cell membranes (New, 1990; Torchilin, 2005). The lipids used in formation of liposomes can be derived from both natural and synthetic sources. The most commonly used phospholipid is phosphatidylcholine (PC). This is due to its neutral charge, chemical inertness and wide availability. However, other lipids, lipid-blends or substances such as cholesterol, may be used in liposome preparation to optimize their properties. A different lipid composition may alter the bilayer properties such as its permeability and fluidity (New, 1990; Brandl, 2001).

Liposomes have the ability to incorporate both hydrophilic and lipophilic drugs. The entrapment efficiency depends on the method of preparation, vesicle size, lipid composition and the physiochemical properties of the drug. In general, hydrophilic drugs will be entrapped in the aqueous compartments, while lipophilic drugs will be incorporated in the lipid bilayers (New, 1990).

Liposomes have a number of advantages as a drug carrier for substances of plant origin and plant extracts. These include enhanced substance solubility and bioavailability, enhancement of its biological activity, protection from physical and chemical degradation and enhancement of stability (Ajazuddin, 2010; Fang and Bhandari, 2010). It is also worth mentioning that liposomes are non-toxic, non-immunogenic, biodegradable and can be manufactured on a large scale (New, 1990). A study by Basnet et al. (2012) showed that liposomal formulation of curcumin, a well know natural antioxidant and anti-inflammatory agent from turmeric, were two to six fold more potent than corresponding curcuminoids when tested for anti-oxidative and anti-inflammatory properties.

2.2.1 Classification of liposomes

Liposomes are usually classified according to their lamellarity and size (Figure 4):

- Multilamellar vesicles (MLVs): These vesicles have multiple concentric lamellae and occur in a wide range of sizes (100-1000 nm).
- Large unilamellar vesicles (LUVs): The structure of these vesicles consists of a single lamellae and the size is normally up to 1000 nm.
- Small unilamellar vesicles (SUVs): These vesicles normally consist of a single lamellae and the diameter is below 100 nm (New, 1990).

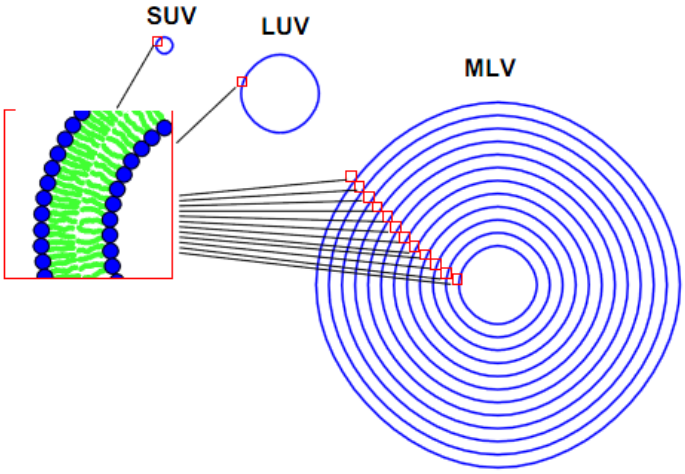

Figure 4: Schematic presentation of small unilamellar, large unilamellar and multilamellar vesicles (Brandl, 2001).

However, they can also be classified according to their lipid composition (Samad et al., 2007):

- Conventional liposomes: made of neutral or negatively charged phospholipids and/or cholesterol.
- Cationic liposomes: made of positively charged phospholipids.
- Immunoliposomes: have antibodies or antibody fragments on their surface, which aids and enhances target site binding.
- Long circulation liposomes: have attached surface molecules such as polyethylene glycol to avoid the reticuloendothelial system and obtain prolonged circulation time.

2.2.2 Methods of preparation

There are different methods to prepare liposomes. However, all preparation methods can be simplified as to involve three basic steps:

1) Hydration of lipids, resulting in liposome formation
2) Adaption of liposome size
3) Loading of the active ingredient into the liposomes (Brandl, 2001)

These three steps may be carried out in consecutive order or take place simultaneously (Brandl, 2001). The following is a brief description of methods used to prepare liposomes in this project:

Film-hydration-method:

Liposomes are made by dissolving lipids and the active ingredient in an organic solvent. The solvent is evaporated in a rotary evaporator under reduced pressure. The thin lipid film obtained on the round bottom flask is then hydrated by the addition of an appropriate aqueous medium, followed by shaking. Upon hydration, the lipids are allowed to swell and to form MLVs with a size around and over 1 μm incorporating the drug substance (New, 1990; Brandl, 2001).

Sonication:

Sonication is a method applied to reduce the size of liposomes and can be done by bath or probe sonication. Sizes (diameter) of the liposomes are dependent on the time and amplitude of sonication, respectively. Although probe sonication has an efficient transfer of energy to the liposomal dispersion, it is associated with metal particle shedding from its probe which often can result in contamination of the sample (New, 1990; Brandl, 2001).

For a more detailed description of available methods used in liposome preparation, refer to Brandl (2001) and Samad et al. (2007).

2.2.3 Liposomes as carrier system for topical administration

Liposomes are used for a wide range of therapeutic applications via different routes of administration. They are well established as drug carriers in topical treatment of diseases, especially in dermatology. The extensive overviews of liposomes as a topical drug delivery system and their development are provided by Egbaria and Weiner (1990) and de Leeuw et al. (2009).

The major advantages of topically applied liposomal drugs can be summarized as:

- Reduction of drug related side effects and incompatibilities that may arise from undesirable high systemic absorption of drug.
- Enhancement of the accumulation of drug at the site of administration (depot effect).
- Capability to incorporate a wide variety of hydrophilic and hydrophobic drugs (Egbaria and Weiner, 1990; de Leeuw et al., 2009).

Additionally, due to their ability to provide a prolonged release of incorporated material locally, they also have a potential to be applied vaginally (Pavelic et al., 2001).

2.2.3.1 Vaginal application

The vagina as a site for drug delivery offers certain unique features that can be exploited in order to improve therapeutic effects of various drugs (Vermani and Garg, 2000). Advantages of the vaginal route of drug administration include: avoidance of hepatic first pass metabolism, a reduction in the incidence and severity of gastrointestinal side effects, easiness of application and the complete privacy of the therapy for women (Hussain and Ahsan, 2005). However, the currently available vaginal drug dosage forms (e.g., foams, creams, gels and tablets) have some limitations, such as their leakage, messiness and low residence time, which contribute to poor patient compliance and limited therapy outcome. For that reasons, attempts were being made to develop novel vaginal drug delivery systems that can provide prolonged and/or controlled release of drugs (Pavelic et al., 2004).

A liposome formulation with interferon alpha (INF-α) was developed by Foldvari and Moreland (1997) as a topical drug delivery system for the treatment of genital HPV infections. INF-α shows activity against papillomaviruses and the aim of this study was to evaluate the potential liposomal encapsulation to deliver INF-α through the topical route. The formulation was tested on two patients who showed a decreased number of lesions at the end of the observation period. The authors concluded that liposomes are a promising delivery system for INF-α in the treatment of genital HPV infections, but that the assessment of its efficacy needs to be studied in a larger number of patients (Foldvari and Moreland, 1997).

Pavelic et al. (1999) studied the stability of liposomal formulations containing clotrimazole, metronidazole and chloramphenicol, drugs which are commonly used for treatment of vaginal infections. The authors concluded that liposomes retained 40, 28, 37 % respectively, of the entrapped drug after 6 hours of incubation in an environment that mimics the vaginal cavity of pre- and post-menopausal women. These stability studies confirmed the applicability of liposomes as carrier systems for vaginal delivery (Pavelic et al., 1999).

However, liposomal suspensions are liquid in nature and will not remain at the administration site over longer period of time. Therefore, attempts have been made regarding incorporation of liposomes in a suitable bioadhesive base, such as hydrogels, in order to obtain a suitable viscosity and application properties of the liposomal suspension (Pavelic et al., 2001).

2.3 Hydrogels

2.3.1 General properties

Hydrogels are water-swollen polymeric materials that maintain a distinct three-dimensional structure. Hydrogels can be used for a wide range of applications, especially as they have several characteristics that make them excellent vehicles for drug delivery systems. They are considered to be non-toxic and highly biocompatible, due to their high water content. Many polymers have mucoadhesive and bioadhesive characteristics that enhance drug residence time and tissue permeability. These properties can help improve site-specific binding to regions such as the colon, nose and vagina (Kopecek, 2009; Bhattarai et al., 2010).

Hydrogels are usually classified based on the origin of gelling material, which can be of natural or synthetic origin, but they can also be classified based on:

- the nature of the crosslinking: covalent or physical gels
- the nature of the polymer network: homopolymer, copolymer, interpenetrating or double networks
- their physical structure: homogeneous, microporous or macroporous hydrogels
- their degradability: degradable or non-degradable hydrogels (Kopecek, 2009)

The rheological properties of a gel govern important features such as its spreadability and retention characteristics. Generally, hydrogels composed of hydrophilic polymers exhibit non-Newtonian pseudo plastic behavior. If the degree of pseudo plasticity increases, the ability to spread augments (das Neves et al., 2009).

The highly porous structure of hydrogels enables incorporation of a relatively large amount of drugs (Jagur-Grodzinski, 2010). The release of drugs will be influenced by several factors e.g.:

- The hydration of the polymer by liquids, particularly water
- Swelling of the polymer to form a gel
- Diffusion of drug through the swollen gel
- Erosion of the gel (Boateng et al., 2008)

The mechanisms by which the drugs can be released are either: diffusion controlled, swelling controlled or chemically controlled release. Diffusion controlled release is the most applicable mechanism and is dependent on the mesh sizes within the matrix of the gel. In swelling controlled release, the diffusion of drug is considerably faster than the swelling of the gel. Chemically controlled release is dependent on chemical reactions occurring within the gel matrix (Hamidi et al., 2008).

There are limited numbers of studies reporting the use of hydrogels in genital warts therapy, which might be of interest. A study by Syed et al. (2000) reported development of vaginal hydrophilic gel (based on hydroxyethylcellulose 1 %; w/w) containing 5-fluorouracil for the treatment of intravaginal warts. The clinical results demonstrated that 5-fluorouracil in a vaginal hydrophilic gel is safe, tolerable and significantly more effective than placebo (Syed et al., 2000). In another study, investigators evaluated the capacity of several pharmaceutical

hydrophilic gels to deliver granulocyte-macrophage colony-stimulating factor (GM-CSF) on HPV-associated cervicovaginal pre-neoplastic lesions topically. A mouse xenograft model demonstrated that polycarbophil gels (1 % w/w) were suitable to deliver GM-CSF to the vaginal mucosa, with the advantage of stabilizing the protein. This stabilization was dependent on the gel pH: at pH 5.5 GM-CSF was stabilized, whereas at pH 6.9 a dramatic loss of bioactivity was observed (Hubert et al., 2004).

Different polymeric materials have been used in hydrogel formation. For vaginal application, polymers with a mucoadhesive property are of particular interest, since they have an ability to enhance site-specific binding and thus prolong drug residence time on vaginal site. An extensive overview of polymeric materials used in vaginal drug delivery is given by Valenta (2005). In brief, synthetic poly-acrylates are the most studied bioadhesive polymers for vaginal application, but natural polymers, such as chitosan, are expected to gain more significance in the future (Valenta, 2005).

2.3.2 Chitosan hydrogels

Chitosan is a hydrophilic polymer of natural origin, used as structural material in hydrogels. It is the deacetylated form of chitin, a polysaccharide found in the exoskeleton of insects, crustaceans and some fungi. Chitosan (Figure 5) is composed of β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine units (Bhattarai et al., 2010).

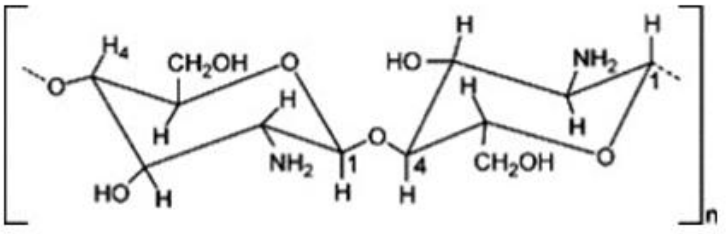

Figure 5: Structure of chitosan (George and Abraham, 2006).

Chitosan is commercially available in a range of molecular weights (Mw) and degrees of deacetylation (DD), representing the proportion of deacetylated units. These are the main parameters influencing the characteristics of chitosan and are determined during the preparation and characterization of the polymer (He et al., 1998; Berger et al., 2004).

Chitosan exhibits certain properties that make it an appropriate agent for use in drug delivery systems, such as its good biodegradability, low toxicity and bioadhesiveness.

If chitosan is given orally, it is thought to be degraded by bacterial and lysozymes present in the large intestine. When absorbed into the systemic circulation, it should have Mw suitable for renal elimination. If the Mw is too high, it should undergo further enzymatic degradation. Eight human chitinases have been identified, three of which have shown enzymatic activity. These enzymes will hydrolyze the glucosamine-glucosamine, glucosamine-N-acetyl-glucosamine and N-acetyl-glucosamine-N-acetyl-glucosamine linkages and degrade chitosan polymer into smaller fragments. The DD is also determining the rate and extent of chitosan biodegradability. If the DD is high, a decrease in the rate of degradation can be seen (Kean and Thanou, 2010).

Chitosan is generally regarded as non-toxic and therefore safe for use in pharmaceutical preparations. Its toxicity profile is dependent on the DD and Mw. When DD is high, the toxicity is related to the Mw and the chitosan concentration. When the DD is lower, the toxicity is less pronounced and less affected by the Mw (Kean and Thanou, 2010).

Chitosan is a cationic polymer, due to its positively charged amino groups, and can interact electrostatically with negatively charged surfaces, such as mucosa or skin. The interactions are strongest in an acidic environment, since the charge density of chitosan will be high. An increase in Mw and DD will make this effect more pronounced. This gives chitosan an ability to adhere and reside for a longer period of time at the site of administration (George and Abraham, 2006). The good bioadhesive properties of chitosan were confirmed in a study done by Hurler and Skalko-Basnet (2012).

Chitosan hydrogels have been studied for a wide range of medicinal application e.g. oral, ocular, subcutaneous and transdermal delivery. A review of this is given by Bhattarai et al. (2010). Chitosan has also received attention for its use in skin and burns therapy, particularly discussed by Hurler and Skalko-Basnet (2012).

With respect to vaginal therapy, limited numbers of studies reporting the use of chitosan hydrogels are available. However, chitosan or chitosan derivatives have been investigated for preparation of mucoadhesive vaginal tablets (Valenta, 2005). A study by El-Kamel et al (2002) reported the use of chitosan in mixtures with different ratios of anionic polymers for

the preparation of mucoadhesive tablets to be used as a vaginal delivery system for metronidazole (El-Kamel et al., 2002).

Hydrogels made of mucoadhesive polymers offer a potential of prolonged residence time at the administration site, which increases drug-contact time with infected tissue and thus provides improved therapy. This also means that the number of administrations can be reduced, resulting in increased patient compliance. If the retardation of drug release using hydrogels is not sufficient to slow the release rate for long-term applications, another release system may be incorporated into the hydrogel, such as liposomes containing drugs (Bhattarai et al., 2010).

2.3.3 Liposomal hydrogels for vaginal application

As we were aiming at vaginal therapy, vaginal application of liposomal hydrogels is the focus of this chapter.

Liposomal hydrogels offer the advantage of sustained release and longer residence time at the site of administration, as described above. Several research groups have studied the use of liposomal hydrogels in the treatment of vaginal disorders.

The study conducted by Jain et al. (1997) investigated liposomes containing progesterone for intravaginal administration. Liposomes were incorporated in a polyacrylamide gel and *in vitro* release studies showed that a zero order kinetics release was obtained. Progesterone is a potent contraceptive and the progestational activity of liposomal and control gel was tested in rats. Both formulations were found to inhibit the formation of *corpora lutea*, but the effect of liposomal preparation was found to be greater and prolonged as compared to the control gel (Jain et al., 1997).

The study conducted by Pavelic et al. (2004) investigated the *in vitro* release of chloramphenicol from a liposomal gel in a media simulating the vaginal environment. The gel was made of Carbopol, which is a synthetic polymer. Liposomes incorporated in the gel showed a prolonged release of chloramphenicol compared to the control gel. After 24 hours of incubation in the media simulating vaginal conditions, more than 40 % of the originally entrapped drug was still retained in the gel (Pavelic et al., 2004). A similar study was performed by the same group, for the drugs clotrimazole and metronidazole. The results

showed that after 24 hours of incubation the gel retained approximately 30 and 50 % of the originally entrapped drugs, respectively (Pavelic et al., 2005a).

Similar studies have also been performed for acyclovir and calcein (Pavelic et al., 2001; Pavelic et al., 2005b).

We propose a liposomal formulation of catechins as means to enhance their activity. To come closer to an actual *in vivo* application and to obtain suitable viscosity of the formulation, drug-bearing liposomes were incorporated in chitosan hydrogel. To the best of our knowledge, this is a pioneering work and we hope that the proposed formulation will show promise in topical vaginal inflammation/genital warts therapy.

3 Aims of the study

The main aim of this project was the development of liposomal delivery system for green tea catechins, as a mean to enhance their anti-inflammatory properties. Their anti-inflammatory properties are directly related to their antioxidative activities and the formulation is intended for treatment of vaginal inflammation/genital warts. To the best of our knowledge, this has not been previously reported. Two catechin epimers (catechin and epicatechin) were selected for optimization of liposomal characteristics. Liposomes are expected to enhance the activity of associated substance, therefore, it is necessary to examine the antioxidative activities of catechin and epicatechin before optimization of liposomes. To come closer to an actual *in vivo* application, liposomes were incorporated in a chitosan hydrogel. The hydrogel is expected to add a suitable viscosity to the formulation and additionally provide sustained release of incorporated active substance.

Specific aims, in more details, were:

- Evaluation of antioxidative activities of catechin and epicatechin by using a DPPH radical scavenging method.
- Development of liposomal formulation for catechin through optimization of vesicle size and entrapment efficiency.
- Preparation of liposomes containing epicatechin in order to compare their liposomal characteristics with liposomes containing catechin.
- Evaluation of stability of selected liposomal formulations containing catechin or epicatechin by using accelerated stability testing.
- Development of liposomes-in-hydrogel delivery system for catechin and evaluation of its textural properties.
- Evaluation of the *in vitro* release profile of catechin from liposomal preparations (both suspension and gel) by using a Franz diffusion cell system.

4 Materials and methods

4.1.1 Materials

Acetic acid (glacial) GR for analysis, Merck, Darmstadt, Germany

Ascorbic acid, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany

Calcium hydroxide, Merck, Darmstadt, Germany

(+)-Catechin hydrate, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany

Chitosan, high molecular weight, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany

Dialysis tubing, Size 1 Inf Dia 8/32" – 6.3 mm: 30 M (Approx.), MWCO 12 – 14000 Daltons, Medicell International Ltd, London, UK

Dialysis tubing, size 9 Inf Dia 36/32" – 28.6 mm: 30 M (Approx.), MWCO 12 – 14000 Daltons, Medicell International Ltd, London, UK

di-Potassium hydrogenphosphate, Merck, Darmstadt, Germany

Distilled water

1,1-Diphenyl-2-picrylhydrazyl (DPPH), Sigma-Aldrich, Oslo, Norway

Ethanol 96 % (vol/vol), Sigma-Aldrich, Chemie GmbH, Steinheim, Germany

(-)-Epicatechin, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany

Glucose, NMD, Oslo, Norway

Glycerol 86-88 %, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany

Hydrochloric acid, Merck, Darmstadt, Germany

Lactic acid, Apotekerproduksjon AS, Oslo, Norway

Lipoid S 100 (soybean lecithin, > 94 % % phosphatidylcholine), a generous gift from Lipoid GmbH, Ludwigshafen, Germany

Methanol CHROMASOLV® for HPLC, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany

Potassium dihydrogenphosphate, Merck, Darmstadt, Germany

Potassium hydroxide, NMD, Oslo, Norway

Sodium chloride, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany

Sodium hydroxide, Sigma-Aldrich, Laborchemikalien GmbH, Seelze, Germany

(±)-α-Tocopherol, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany

Urea, NMD, Oslo, Norway

4.1.2 Buffers

Vaginal fluid simulant (VFS) (Owen and Katz, 1999):

I	Sodium chloride	3.51 g
II	Potassium hydroxide	1.40 g
III	Calcium hydroxide	0.222 g
IV	Acetic acid	1.00 g
V	Lacetic acid	2.00 g
VI	Glycerol	0.16 g
VII	Urea	0.40 g
VIII	Glucose	5.00 g
IX	Distilled water	ad 1000.0 ml
X	Hydrochloric acid 1 M	ad pH 4.0

I-III were dissolved in a portion of IX. IV-VIII were added to the solution. Rest of IX was added up to total volume of 1000.0 ml. X was used to adjust pH to 4.0.

Phosphate buffer saline (PBS) pH 7.4

I	Potassium dihydrogenphosphate	3.40 g
II	di-Potassium hydrogenphosphate	4.35 g
III	Sodium chloride	16.00 g
IV	Distilled water	ad 2000.0 ml

I-III were dissolved in IV. The rest of IV was added to a final volume of 2000.0 ml. The pH was adjusted to 7.4.

4.1.3 Instruments

Agilent 8453 UV-Visible Spectrophotometer, Agilent Technologies, Santa Clara, USA

Büchi Waterbath B-480, Büchi Rotavapor R-124, Buchi Vaccum Controller B-721, Buchi Vac® M-500, Båuchi labortecknik, Flawil, Switzerland

Distillation unit Distinction D4000, Bibby Sterlin LDT, Straffordshire, UK

Franz diffusion cell 15 mm with 12 ml receptor volume, flat ground joint, clear glass, clamp and stir bar, PermeGear, Hellertown, USA

Julabo heating circulator F12-ED, JULABO Labortechnik GmbH, Seelbach, Germany

NICOMP Submicron particle sizer, model 370, Nicomp Particle Sizing Systems, Santa Barbara, USA

PermeGear V6A Stirrer, PermeGear, Inc., Hellertown, USA

Sonics High Intensity Ultrasonic Processor, 500 W model with temperature controller, probe tip 13 mm, Sonics & Materials Inc, Newtown, USA

SpectraMax 190, Microplate Spectrophotometer, Molecular Devices, Sunnyvale, USA

TA.TX.Plus Texture Analyser, Stable Microsystems, Surrey, UK, equipped with Backward Extrusion Rig A/BE, Stable Microsystems, Surrey, UK

4.2 Determination of antioxidative activities of catechin and epicatechin by DPPH radical scavenging assay

Free radical scavenging (antioxidative) activities of catechin and epicatechin were determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, as described by Basnet et al. (1997). DPPH is a relatively stable free radical and has been widely used to test the free radical scavenging activity of various samples (Basnet et al., 1997; Jun et al., 2011)

In brief, 0.5 ml of DPPH solution (60 μM) was mixed with 0.5 ml of solution of each sample at 0.5, 1, 2, 5 and 10 μg/ml as the final concentrations. The reaction mixture was shaken and left for 30 minutes at room temperature.

The antioxidative activities of catechin and epicatechin were expressed as the scavenging of DPPH radicals by measuring the absorbance at 518 nm spectrophotometrically, as compared with the control group (DPPH solution, 30 μM as the final concentration)

The antioxidative activities of catechin and epicatechin were compared with some well-known antioxidants such as vitamin C and vitamin E, under the same experimental conditions.

All experiments were performed in triplicates.

4.3 Liposome characterization

4.3.1 Preparation of empty liposomes

Lipoid S100 (200 mg) was dissolved in excess methanol in a round bottom flask. Methanol was allowed to evaporate in a rotary evaporator for 90 minutes at 50 mBar, on a water bath at 50 ºC and 95 rpm. The flask was then removed from the water bath and kept rotating in the air for 30 more minutes. The dry film was hydrated by the addition of 10 ml of distilled water and the resultant liposomal suspension manually shaken until homogeneous suspension was obtained. The liposomes were stored in the refrigerator (4-8 ºC) for at least 24 hours before further characterization and use.

4.3.2 Preparation of liposomes with catechin

Catechin (15, 20 and 25 mg, respectively) was dissolved together with Lipoid S 100 (200 mg) in excess methanol in a round bottom flask. The solvent was allowed to evaporate under the

same conditions as described for empty liposomes. The liposomes were stored in the refrigerator (4-8 ºC) for at least 24 hours before further characterization and use.

4.3.3 Preparation of liposomes with epicatechin

Epicatechin (20 mg) was dissolved together with Lipoid S 100 (200 mg) in excess methanol in a round bottom flask. The solvent was allowed to evaporate under the same conditions as described for empty liposomes. The liposomes were stored in the refrigerator (4-8 ºC) for at least 24 hours before further characterization and use.

4.3.4 Size reduction of liposomes

Four ml of each liposomal suspension was transferred separately to a beaker and placed on ice bath. The needle probe tip of sonicator was positioned in the center of the volume. The liposomal suspensions were sonicated in intervals of 2 minutes to obtain the desired particle size. The amplitude was set to 40 %. Between each interval, the probe and sample were allowed to cool down for 10 minutes.

4.3.5 Vesicle size analysis

The vesicle size and size distribution of the sonicated liposomes were determined by photon correlation spectroscopy. The measurements were performed on the NICOMP Submicron particle sizer model 370. Sample preparation was carried out in a clean area using particle free equipment to avoid any contamination with particles from the environment. All handling was performed in a laminar airflow bench. Test tubes were filled with distilled water and sonicated in an ultrasonic bath for 10 minutes and rinsed with filtered water (using 0.2 μm pore size syringe filter) prior to use. The samples were diluted empirically with freshly filtered distilled water until a particle intensity of 250-350 kHz was obtained (Hupfeld et al., 2006). Each sample was measured in 3 cycles of 10 minutes by using the intensity-weighted Nicomp distribution.

4.3.6 Entrapment efficiency determination

In order to determine the entrapment efficiency of catechin and epicatechin in liposomes, respectively, dialysis was applied. Dialysis was performed in dialysis tubing. One ml of liposomal suspension was dialyzed against 200 ml of distilled water. After 24 hours the samples were taken out. Fifty μl of the liposomal suspension were further dissolved in 1 ml of methanol (total volume) and used in spectrophotometrical analysis. The dialysate was used directly in spectrophometrical analysis, without any further dilution.

4.3.7 Spectrophotometrical analysis

A stock solution of catechin in ethanol was prepared in a concentration of 1 mg/ml. Working solutions of 2, 5, 10, 25, 50 and 100 μg/ml of catechin was made by diluting the stock solution with appropriate volumes of ethanol. Spectral analyses of the working solutions were performed on the UV-Vis spectrophotometer. Based on the spectral analysis a calibration curve was made at 280 nm wavelength.

The same procedure was also performed with epicatechin, in order to obtain a standard curve.

4.4 Stability testing of liposomal formulations

An accelerated stability test was performed in order to predict the stability of liposomal formulations. The liposomal suspensions were stored for a 30-days period in an airtight container at 40 ºC. Change in original vesicle size and loss of entrapped drug (where applicable) were determined.

The liposomal suspensions tested were:

- empty liposomes
- liposomes containing catechin
- liposomes containing epicatechin

4.5 Hydrogel preparation and characterization

4.5.1 Preparation of chitosan hydrogel

Chitosan forms gels when dispersed in a weak acid (Cao et al., 2009). The preparation method was based on methods reported by Alsarra (2009) and Cao et al. (2009). Ten percent of a glycerol 87 % solution was mixed with acetic acid (2.5%; w/w) and shaken manually until the blend was homogeneous. High molecular weight chitosan (2.5 %; w/w) was dispersed in the prepared glycerol/acetic acid mixture. The mixture was then manually stirred for approximately 10 minutes. The preparation was allowed to swell for 48 hours in a sealed container at room temperature before further use (Alsarra, 2009; Cao et al., 2009).

4.5.2 Preparation of liposomal hydrogel

Chitosan hydrogel was prepared as described under section 4.5.1. A defined amount of liposomal suspension (10%, w/w) was incorporated in the pre-prepared chitosan hydrogel by hand stirring, to fully disperse the liposomes within the hydrogel (Skalko et al., 1998). The liposomal preparations used were free from unentrapped catechin. The prepared hydrogel was allowed to set for 2 hours before further use.

4.5.3 Texture analysis

Texture analyser was used to determine the texture properties of the hydrogels prepared as described above (in section 4.5.1 and 4.5.2). The method used was developed in our laboratory by Hurler et al. (2012). A 40 mm (diameter) submerge probe disk was used and measurements conducted by backward extrusion.

Before starting the experiment, the height of the probe and the force applied were calibrated. Sixty grams of gel were used in the experiments. The disc was compressed into the gel and rested for 30 seconds 1 mm from the bottom of gel to relieve air-bobbles under the disc. The disc was then moved to 30 mm submerged and rested for 30 seconds. Five replicate measurements with 30 seconds resting interval between each run were performed.

The experimental conditions were as follows:

Pre-test speed:	4 mm/sec
Test speed:	4 mm/sec
Post-test speed:	4 mm/sec
Distance:	10 mm; return to the start point

Four parameters were measured (Figure 6), namely the maximum compressing force (Force 1), the cohesiveness (Area 1), the minimum retracting force (Force 2) and the adhesiveness (Area 2).

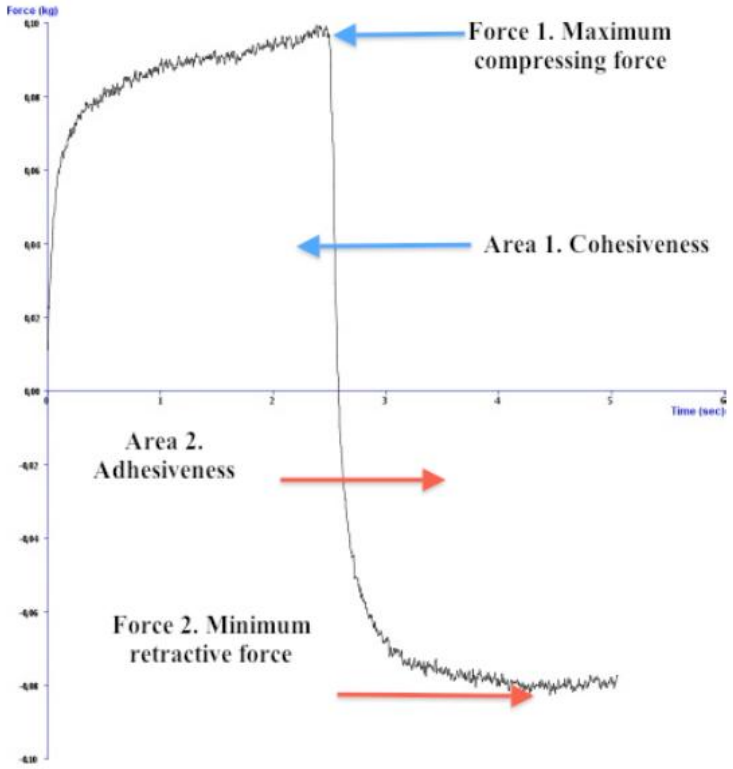

Figure 6: Parameters measured in texture analysis (Berg, 2011).

4.6 *In vitro* release studies

4.6.1 *In vitro* release of catechin

A Franz diffusion cell (FDC) system was used to determine the *in vitro* release of catechin from liposome preparations (both suspensions and gels). Prior to the experiments, the receptor and the donor chambers (Figure 7) were filled and washed with methanol, followed by

distilled water and deionized water for 30 minutes in each rinsing. The FDC were dried sufficiently before they were filled up with the receptor medium.

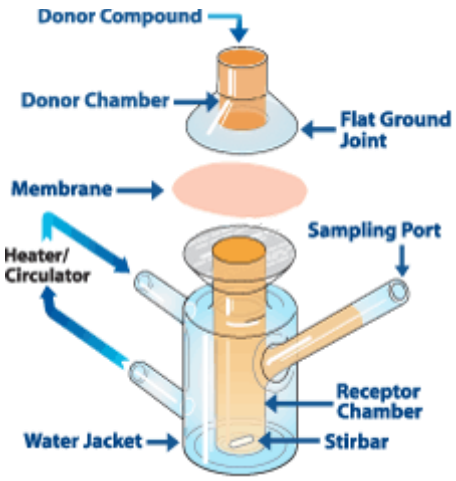

Figure 7: Schematic presentation of a Franz diffusion cell.
(www.permegear.com)

As the receptor medium (12 ml) we used either vaginal fluid simulant (VFS) with a pH of 4.0 or phosphate buffer saline (PBS) with a pH 7.4 (See Table 2). To assure uniform stirring during the dialysis process, automatic stirrers were applied.

The cells were connected to a heater circulator, in order to keep the temperature constant and corresponding to physiological temperature (37 ºC).

The membrane used in the experiments was dialysis tubing Size 9, Medicell International Ltd (MWCO 12 – 14.000 Daltons). Prior to use, they were evenly cut to fit the top of the receptor chamber (1.77 cm^2) and allowed to swell in distilled water for 15 min. The swollen membrane was then placed on top of the previously filled receptor chamber. The donor chamber was placed on top, with a joint packing in between. A metal clamp was used to hinder any interference of air in the FDC. If air bubbles were detected, the cell was flipped to manage release of entrapped air.

Samples (1000 µl aliquot of the gel preparations and 100 µl aliquot of liposomal suspension) of different formulations were put into the donor chamber with help of a plastic syringe. At different time intervals (1, 2, 4, 8 and 24 hours) sample aliquots of 250 µl were taken out with

a Hamilton microliter syringe. The withdrawn amount was replaced with fresh receptor medium after every sampling point. The sampling ports were covered with triple layers of Parafilm® and the donor chambers were closed with a rubber plug in order to prevent any contamination and evaporation of sample and receptor medium.

The taken samples were analyzed by using a microplate spectrophotometer. Two hundred µl of the samples were put in a well of a 96 well plate and the absorbance at 280 nm was measured. Two new calibration curves of catechin with standards in the receptor mediums were performed.

The following formulations were tested:

Table 2: Formulations tested in FDC experiment.

Donor chamber	Receptor chamber
Liposomal hydrogel	VFS pH 4.0
Liposomal suspension	PBS pH 7.4
Liposomal hydrogel	PBS pH 7.4

All experiments were performed in triplicates.

4.7 Statistical evaluations

When applicable, student t-test was used to determine the level of significance.

5 Results and discussion

The focus of this project was development of liposomal formulation as carrier system for green tea catechins destined for treatment of vaginal inflammation. Therefore, in the present work, two simple but important epimers, namely catechin and epicatechin, were selected for optimizing their liposomal formulation. It is well known fact that inflammation is induced by the free radicals produced by external pathogens or internal induction (Basnet and Skalko-Basnet, 2011). In this connection green tea catechins are widely discussed and clinically relevant substances due to their anti-inflammatory properties. The anti-inflammatory properties of catechins are directly related to their antioxidative activities (Quideau et al., 2011). Therefore, it was necessary to examine the antioxidative activity before optimization of liposomal formulation.

5.1 Antioxidative activities of catechin and epicatechin

A DPPH radical scavenging assay method was applied in order to evaluate and compare the antioxidative activities of catechin and epicatechin, together with well-established antioxidants such as vitamin C and vitamin E.

The antioxidative activities of catechin and epicatechin are expressed as the amount of DPPH radicals scavenged (Figure 8). DPPH radicals give concentration-dependent UV absorption at 518 nm and, as the number of free radicals decreases, the corresponding UV absorption is expected to be lowered. During the reaction between antioxidants with DPPH radicals, a decreased UV absorbance indicates the scavenging of free DPPH radicals by the antioxidant. Therefore, a stronger antioxidant will scavenge more DPPH radicals at its lowest concentrations, resulting in lower UV absorption.

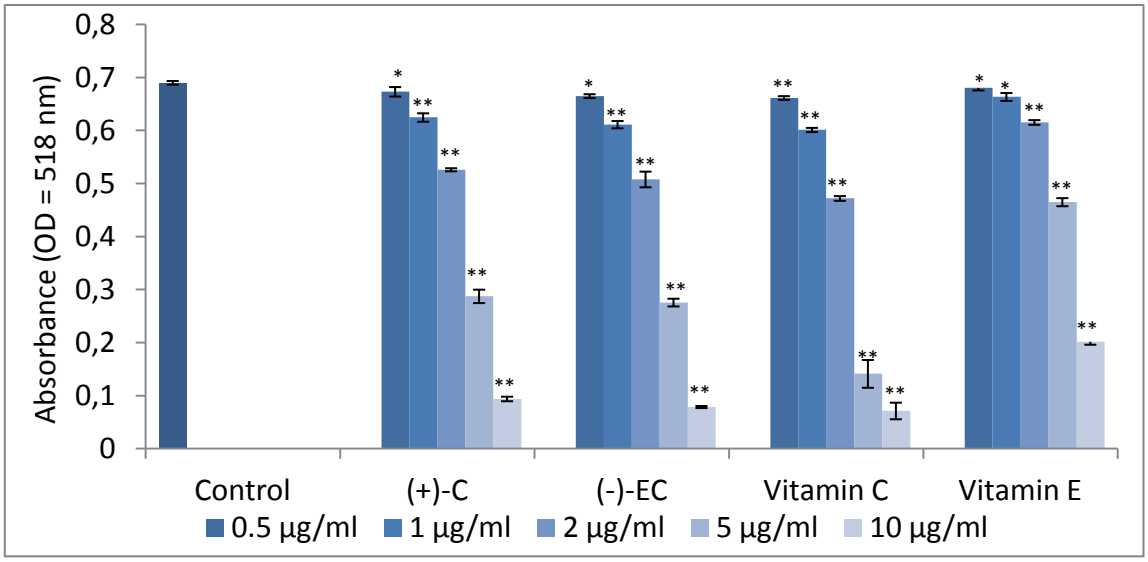

Figure 8: Antioxidative activities of catechin, epicatechin, vitamin C and vitamin E. The values are expressed as mean ± SD (n = 3). *p < 0.05 **p < 0.001 compared to control.

We examined the antioxidative activities of catechin and epicatechin, each at 0.5, 1, 2, 5 and 10 µg/ml concentrations and they both showed concentration-dependent radical scavenging activity (Figure 8).

In order to investigate if there was a difference in antioxidative activity among catechin and epicatechin, we applied a paired t-test. In the paired t-test, all absorbance values of catechin were paired with the corresponding values for epicatechin at the same concentrations. The paired t-test was then applied on all pairs for all concentrations. The results showed that epicatechin was a significantly stronger antioxidant than catechin ($p<0.01$).

This is of particular interest, since both substances have the same structure except at position 3 in the C-ring (see Figures 2 and 3) where the configuration is opposite. Although these are very preliminary results, it seems that the stereochemistry plays an important role in their antioxidative activities and it is not discussed enough to elaborate their biological activity. There are numerous examples showing that stereochemistry plays an important part in related biological activity. For instance, the analgesic drug ibuprofen exists in a form of two enantiomers and only one of the enantiomers is active (Johnson, 1999). However, a study by Nanjo et al. (1996), found no significant difference between tea catechins and their epimers when assessing their antioxidative activity on DPPH radical by electron spin resonance

spectrometry. More research is therefore needed in order to draw a conclusion regarding whether stereochemistry and antioxidative activity of green tea catechins are indeed linked.

In addition, we compared the antioxidative activities of catechin and epicatechin to those of vitamin C and vitamin E, under the same experimental conditions (Figure 8). Vitamin C and E are well known antioxidants and were used as reference antioxidants. Vitamin C is a water-soluble (hydrophilic) antioxidant, whereas vitamin E is a lipophilic substance (Dreosti, 2000). Both Vitamin C and vitamin E showed concentration-dependent antioxidative activity at concentrations of 0.5, 1, 2, 5 and 10 μg/ml, which indicates that the substance's solubility is not the pre-determinant of antioxidative activity.

We found that both catechin and epicatechin were significantly more potent antioxidants than vitamin E ($p<0.01$ for both substances) (Figure 8). Vitamin C was found to be more potent than catechin ($p<0.05$) and epicatechin ($p<0.05$) and also more than vitamin E ($p<0.01$). However, in some studies (Higdon and Frei, 2003; Zaveri, 2006) catechins were reported to be stronger antioxidants than vitamin C. This is in a way conflicting to our own findings, but may be due to different experimental setup or that the other groups have used mixtures of catechins, instead of single purified components as we did.

Nevertheless, our results confirmed that both catechin and epicatechin exhibit strong antioxidative effects. Like other polyphenols, they are known to have low bioavailability and poor solubility (Fang and Bhandari, 2010). As a mean to overcome these physiochemical limitations and enhance their biological activity, liposomal delivery of catechin and epicatechin was proposed. Liposomes are known to be able to solubilize poorly soluble substances of natural origin and enable their preparation in aqueous formulation (Basnet et al., 2012). Therefore, current research was mainly focused on liposomal formulations of catechin and epicatechin.

5.2 Liposome characterization

Liposomes prepared by the film-hydration method are often assumed to be MLVs, with a mean diameter in a range of 1 μm (Brandl, 2001). For topical administration, a mean diameter of vesicles around 200 nm is preferable (Cevc, 2004). As our liposomes were intended for topical vaginal therapy, SUVs or oligolamellar vesicles with the approximate average size of 200 nm were more relevant. There are various methods available to reduce original particle

size of MLVs, among which sonication is a well-known process that can reduce the size of liposomes in a straight forward manner. The time and power used in sonication can be correlated to the final vesicle size (Woodbury et al., 2006), and was thus applied for that purpose. A high entrapment efficiency of active substance in liposomes is desirable, in order to assure that a sufficient amount of substance can be delivered to achieve optimum therapeutic effect (Pavelic et al., 2001). However, optimization of liposomal characteristics requires a balance between the vesicle size and entrapment efficiency, as vesicle size reduction leads to lower entrapment efficiency (Basnet et al., 2012). Therefore, finding an optimum drug-lipid ratio for required vesicle size is very important. In order to optimize liposomal formulation for catechin, we prepared liposomes with different drug-lipid ratios by varying the starting amount of catechin and keeping the amount of lipid fixed (200 mg). The effect of sonication time (2 and 4 minutes) on liposome size and entrapment efficiency was examined.

Figure 9 shows the size characteristics of liposomes prepared from 0, 15, 20 and 25 mg of catechin as starting amounts of liposomal suspensions (10 ml). The vesicle size is presented as mean diameter, but we need to state that with polydisperse systems mean diameter is actually an estimate, and was calculated from intensity of particles with similar particle sizes. That is the reason for rather large standard deviations (SD) (Figure 9).

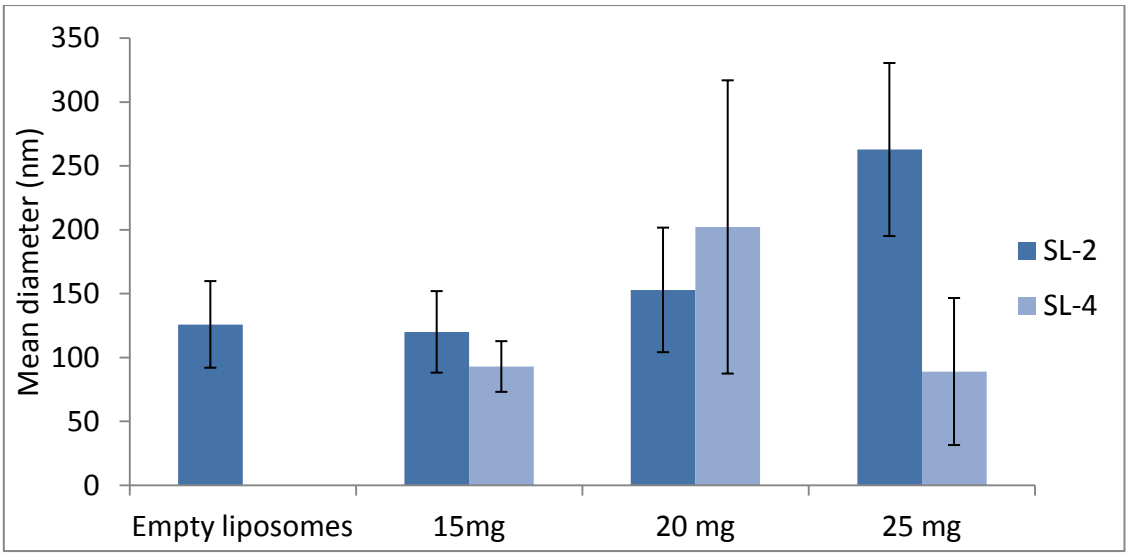

Figure 9: Influence of drug-lipid ratio on the liposomal size. The drug-lipid weight ratios were 0 (empty liposomes), 3:40 (15 mg of catechin in 200 mg of lipid), 1:10 (20 mg of catechin in 200 mg of lipid) and 1:8 (25 mg of catechin in 200 mg of lipid). The values are expressed as mean ± SD (n=2).

We could observe that empty liposomes sonicated for 2 minutes (SL-2 in text) had a similar size as the SL-2 prepared with 15 mg catechin. Results in Figure 9 indicate that the vesicle size increases as the amount of catechin used in preparation of liposomes increases. This would indicate that catechin is accommodated in liposomal bilayers and exhibits similar behavior as cholesterol, namely increasing the rigidity of bilayer. Similar findings were seen for liposomes containing curcumin (Basnet et al., 2012) and those containing mupirocin (Berg, 2011).

The size of liposomes sonicated for 4 min (SL-4 in text) was smaller than the corresponding SL-2 sizes, as expected. At this point we need to comment on the formulation for which 20 mg of catechin was used as staring amount. Although it appears that SL-4 for this liposomal composition is bigger than SL-2, the large SD indicates that this is not the case. We have performed additional experiments with this formulation, but the size polydispersity remained the same. It would be very interesting to perform additional experiments which would provide insight on where exactly catechin is placed within liposomal bilayer, but it would require more sophisticated methods such as X-ray diffractions and differential scanning microscopy (New, 1990). In addition, sonication is a step in liposome preparation, which has potential source of an error. It is very important to place the probe of the sonicater in a similar manner during every sonication. Improper sonication can also contribute to the large standard deviations observed.

Another point worth commenting is the polydispersity index (PI) of the liposomal preparations. PI describes the size distribution of vesicles, or rather the degree of monodispersity of dispersion. A low PI value is desirable, as it indicates a more homogeneous liposomal sample. The obtained PI values are presented in Table 3.

Table 3: Polydispersity (PI) of liposomal preparations prepared and sonicated in 2 or 4 minutes.

Amount of (+)-C in preparation (mg)	SL-2	SL-4
0	0.33 ± 0.03	-
15	0.42 ± 0.03	0.39 ± 0.02
20	0.33 ± 0.08	0.41 ± 0.08
25	0.68 ± 0.02	0.51 ± 0.09

The values represent the mean ± SD (n=2).

In our case, only liposomal dispersion for which 25 mg of catechin was used (SL-2) had a PI too high to be acceptable. It also indicates that so high drug-lipid ratio results in precipitation of unentrapped catechin, which causes high polydispersity.

In an attempt to evaluate the effect of sonication time on vesicle size characteristics, it become evident that 25 mg (per 200 mg of lipid) as starting amount is too high amount, as we were able to visually observe precipitates of catechin. Therefore, maximum amount we could prepare liposomes applying this lipid composition and method of preparation would be 20 mg. In the case of this liposomal composition, we observed large standard deviations for vesicles sonicated for 4 min (Figure 9), therefore the choice was made to focus on vesicles with this liposomal composition but sonicated for shorter period of time (2 min).

However, the second important parameter was the entrapment efficiency of which the results are presented in Figure 10.

Figure 10 shows the entrapment efficiency of liposomal preparations prepared from 15, 20 and 25 mg catechin (non-sonicated N-SL, SL-2 and SL-4).

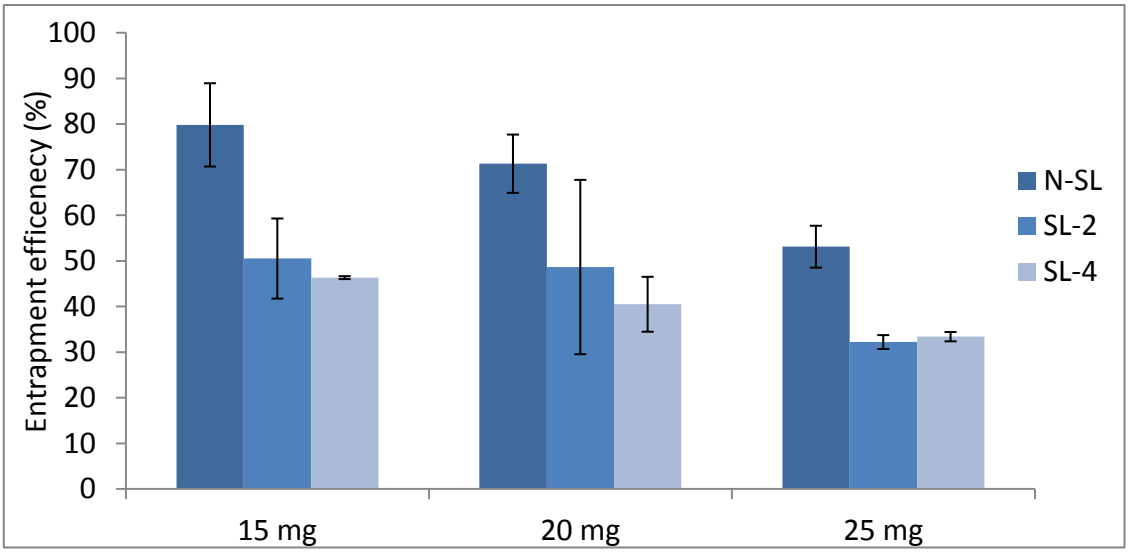

Figure 10: The effect of drug-lipid ratio and sonication time on entrapment efficiency of catechin.
The values represent mean ± SD (n=2).

Entrapment efficiency was determined as described in section 4.3.6 and calculated on the basis of a standard curve obtained under section 4.3.7. The standard curve was prepared in ethanol, while entrapment efficiency determination of liposomal suspension (free of unentrapped catechin), was performed with methanol. Methanol is often used in entrapment efficiency determination (New, 1990). To limit the health risks, we tried to replace methanol, with ethanol whenever applicable, therefore the standard curve was determined in ethanol. In order to assure that dissolved lipid in methanol is not interfering with spectrophotometrical readings, we measured absorbance of empty liposomes and found negligible absorbance. The dialysate was used directly in spectrophotometrical analysis, without any further dilution. The volume of dialysate (distilled water) was adjusted to be below solubility limit for catechin. Catechin is reported to be slightly soluble in water, according to Fang and Bhandari (2010).

The highest entrapment efficiency was found for the non-sonicated liposomes (N-SL), for all liposomal preparations, regardless of the starting amount of drug (Figure 10). We observed that the entrapment efficiency decreased with time of sonication applied to the liposomal suspensions, as expected. Although it may appear that better entrapment efficiency was achieved for liposomes with lower starting amount of catechin, the real indicator, namely the drug-lipid ratio (Table 4) confirms that vesicles for which preparation 20 mg of catechin was used and sonicated for 2 min are the optimal for this method of vesicle preparations. This preparation had a drug-lipid ratio of 5.0 μg/mg and was the best among the sonicated

preparations. Although the drug-lipid ratio is higher for the N-SL, we were not aiming at using them because their average mean diameter was expected to be too large for our purpose.

Table 4: Drug-lipid ratio.

Starting amount of (+)-C	Drug-lipid ratio (μg/mg)		
	N-SL	SL-2	SL-4
15 mg	6.0	3.8	3.5
20 mg	7.1	5.0	4.0
25 mg	6.6	4.0	4.1

After selecting the conditions for liposomal preparation of catechin, we aimed at preparing liposomes with epicatechin and compare their characteristic. Table 5 shows the characteristics of liposomes prepared from 20 mg epicatechin and sonicated for 2 minutes.

Table 5: Characteristics of liposomes (SL-2) containing epicatechin.

Amount of (-)-EC (mg)	Mean diameter (nm)	PI	Entrapment efficiency (%)
20	89.63 ± 5.24	0.40 ± 0.04	49.85 ± 1.13

The preparation contained 200 mg lipid (n=3).

Liposomes containing epicatechin were found to be smaller than corresponding liposomes containing catechin (Table 5 and Figure 9), however, the entrapment efficiencies were very similar (Table 5 and Figure 10).

The third important parameter in optimization of liposomal delivery systems is liposomal stability. Therefore, the next step was to evaluate the stability of selected liposomal formulations of catechin and epicatechin.

5.3 Stability testing

When assessing the stability of liposomal formulations, particularly interesting parameters are the changes in original vesicle size and leakage of originally entrapped drug (Basnet et al., 2012). To evaluate the stability we performed an accelerated stability testing (30 days storage at 40 ºC).

Figure 11 shows the change in original vesicle size during the accelerated stability testing.

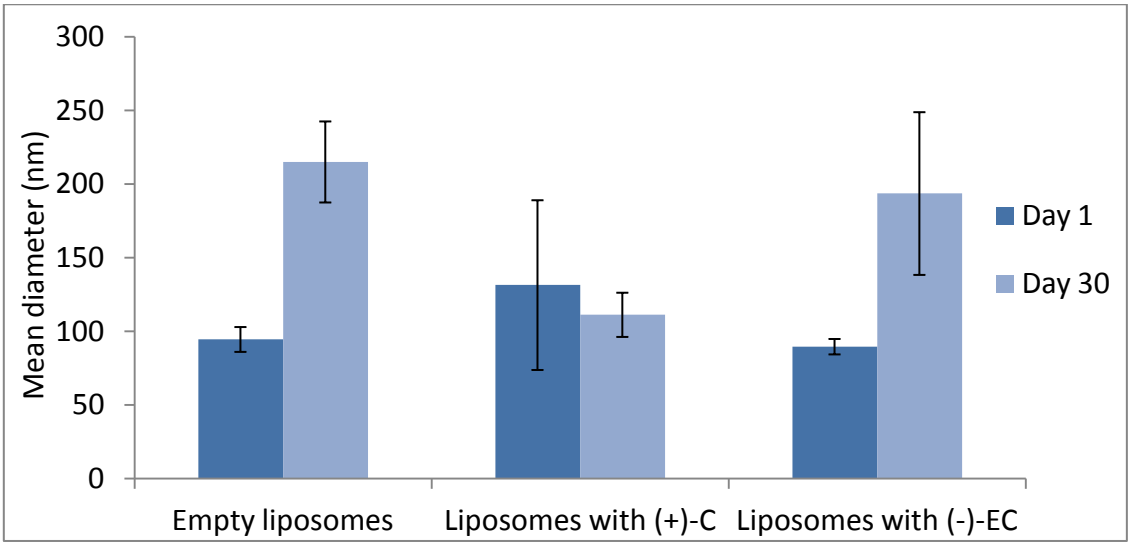

Figure 11: Changes in vesicle size during storage at 40 ºC for 30 days (n=3).

We observed an increase in mean particle size for both empty liposomes and liposomes containing epicatechin. This is likely due to fusion and/or aggregation of particles and has earlier been reported as a phenomenon of physical instability of liposomal suspensions (Grit and Crommelin, 1993). Changes in vesicle size and size distribution due to aggregation are affected by phospholipid composition of the lipid bilayer. Liposomes with a net electrical charge on their surface will repel each other by electrostatic forces and are thus less susceptible to aggregation. All liposomes were made of PC, which has no net charge at physiological pH, and may thus be more prone to aggregation. Considering that the final formulation would require the use of vehicle, such as hydrogels, as liposomes are liquid in nature (Pavelic et al., 2001), this finding is not considered to be of serious consequences. The hydrogel vehicle is expected to preserve the original liposomal size (Pavelic et al., 2001). Although the vesicle size of liposomes with catechin seems to decrease, phenomena earlier observed by Basnet et al. (2012), due to the large standard deviations the difference was not

significant and cannot be considered relevant. Further experiments, including measurements of zeta potential and possibly electron microscopy, are required.

Regarding the leakage of originally entrapped drug during the accelerated stability testing, the results showed a loss of liposomally-associated drug for liposomes containing both catechin and epicatechin respectively (Figure 12). As we were dealing with rather small vesicles, most probably unilamellar and it is known that SUVs have a tendency to lose or exchange the entrapped material more easily than MLVs (New, 1990), the findings are as expected. The phospholipid composition may also affect the leakage of drug, as it affects the properties of the lipid bilayers. A study by Hussain (2010) showed that addition of cholesterol to a PC membrane increased the curcumin retention in liposomes.

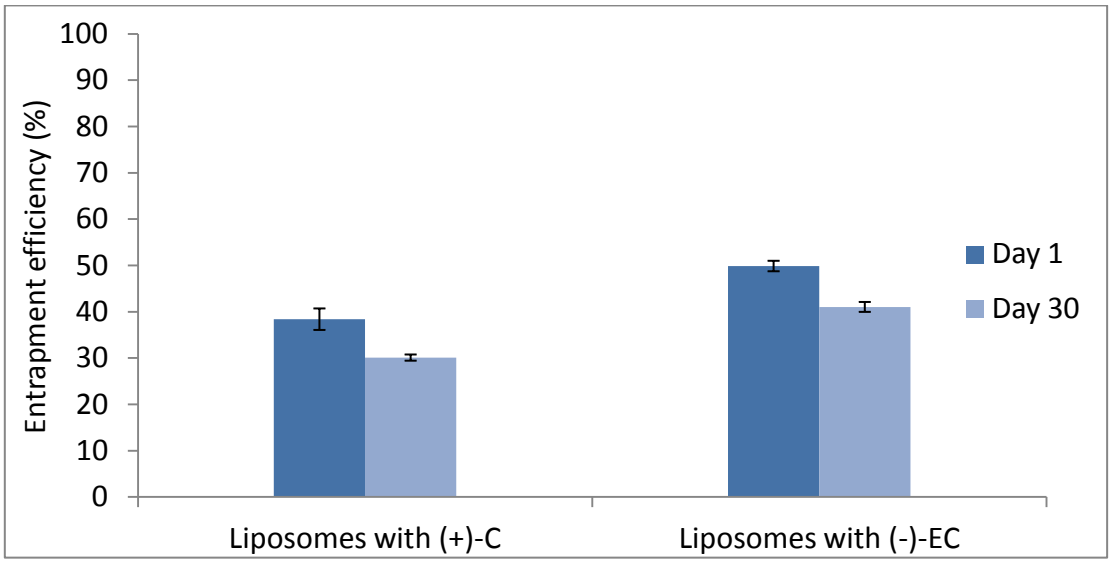

Figure 12: Loss of entrapped drug during the accelerated stability test (n=3).

Again, as our liposomes will be incorporated in a hydrogel and not stored for a long period of time before incorporation in a hydrogel, the findings of accelerated stability testing are satisfactory. We have proceeded with optimizing combined delivery system comprising of liposomes with entrapped catechin and hydrogels which served as the vehicle.

5.4 Hydrogel characterization

5.4.1 Textural properties of empty and chitosan-based liposomal hydrogel

The hydrogel is supposed to function as a vehicle for liposomes in order to make the viscosity of the liposomal suspension more applicable for vaginal administration and to prolong residence time at the administration site (Pavelic et al., 2001). Prolonged residence time increases the contact time between the drug and the destined area and thus improves therapy. It also reduces a frequency of dosing and thus contributes to better patient compliance. In order to assure that the properties of the hydrogel corresponded to the desired properties, it was important to investigate the cohesiveness (Area 1) and adhesiveness (Area 2) of the hydrogel before and after incorporation of liposomes (as 10 %; w/w). Table 6 shows the textural properties of empty and liposomal hydrogel.

Table 6: Texture properties of empty and liposomal hydrogel.

Type of gel	Force 1 (g)	Area 1 (g x sec)	Force 2 (g)	Area 2 (g x sec)
Empty hydrogel	92.00 ± 0.00	217.36 ± 0.77	-0.85 ± 0.00	-174.63 ± 1.27
Liposomal hydrogel	68.00 ± 0.00	159.61 ± 0.32	-0.63 ± 0.00	-129.79 ± 0.61

The values denote the mean of 5 runs ± SD.

Gel cohesiveness and adhesiveness are important features of gels, including hydrogels, in respect to gel optimization (Hurler et al., 2012). These values provide an indication of hydrogel potential to reside at the site of administration over a longer period of time. The respective values for the empty hydrogel were in range suitable for topical application (Engesland, 2010). We observed that the values declined to some extent as a result of incorporation of liposomal suspension. However, according to previous research in our laboratory, the values were found to be acceptable for topical application.

A similar trend was observed in a study by Berg (2011). He evaluated the textural properties of an empty chitosan hydrogel and the textural properties of the gel after incorporation of liposomes containing mupirocin and the values declined to some extent. It is very important to evaluate and follow up the changes in hydrogel properties upon inclusion of liposomal suspensions in original polymer network (Hurler et al., 2012).

After assuring that liposomal hydrogel has the desired textural properties, we have focused on determination of the release profile of catechin. Therefore, the next step was to determine the *in vitro* release of catechin from liposomal hydrogel.

5.5 *In vitro* release studies

5.5.1 *In vitro* release of catechin

The in *vitro release* of catechin from liposomal preparations (both suspensions and gels) under different conditions (se Methods part) was determined by the use of a FDC. This system is considered the most appropriate *in vitro* method for evaluating the drug release from topical formulations for vaginal use (das Neves and Bhaia, 2006).

As the project focused on vaginal drug delivery, the initial *in vitro* release of catechin from a liposomal hydrogel, was tested under conditions simulating the vaginal environment (Figure 13). The vaginal pH of healthy pre-menopausal women is usually between 4.0 and 5.0 (Caillouette et al., 1997) therefore, the experiments were performed in VFS with pH 4.0 (Owen and Katz, 1999).

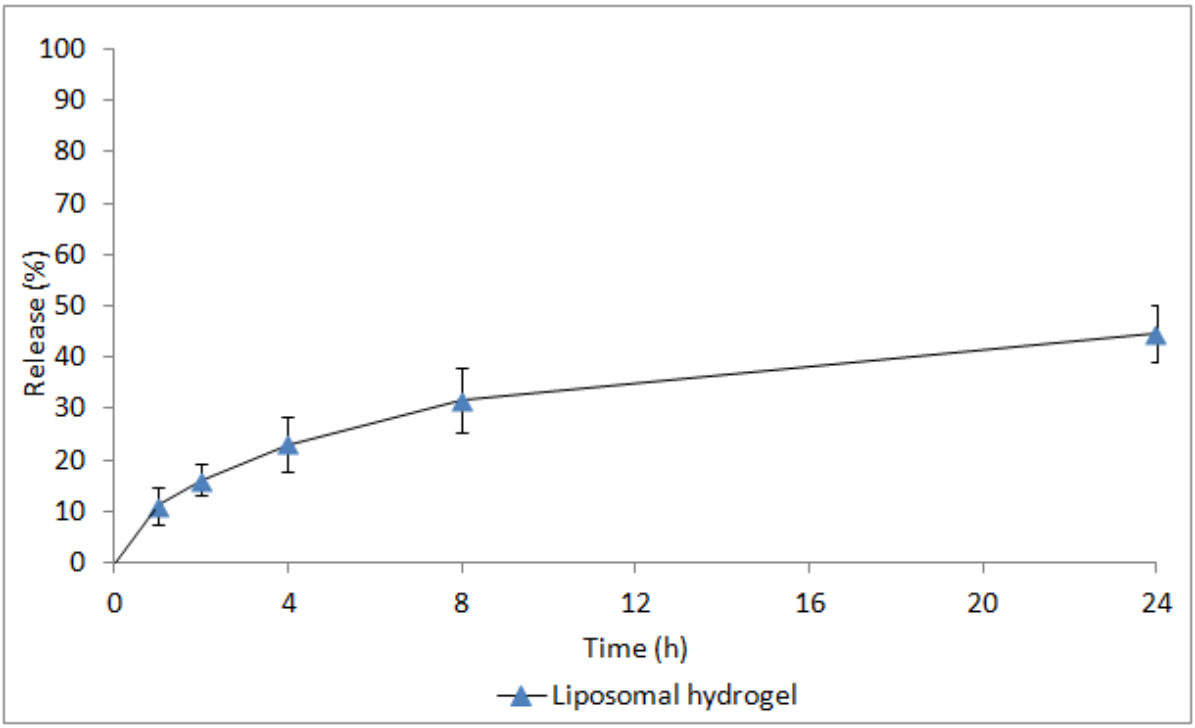

Figure 13: *In vitro* release of catechin entrapped in liposomes incorporated in hydrogel (VFS, pH 4.0).
The values denote the mean ± SD (n=3).

We could confirm that the release rate of catechin was slow, indicating sustained release. After 24 hours, only 45 % of catechin was released. This also means that over 50 % of catechin was still retained within the delivery system. It would be advantageous if the liposomes-in-hydrogel delivery system could provide sustained release, as that would implicate a reduced frequency of administration for the patient. In addition, vaginal site is known to be challenging due to vaginal discharge, menstruation and several limiting factors (Hussain and Ahsan, 2005). On the other hand, the drug has to be released in a sufficient quantity to induce desired therapeutic effect. This needs to be taken into account when optimizing the formulation destined for vaginal administration. Figure 13 confirms sustained release potential of newly developed system. Its therapeutic potential can only be evaluated in suitable *in vivo* animal model. We did not perform the *in vitro* release experiments with liposomes (not incorporated in hydrogels) as it was shown earlier that the release from liposomal hydrogels is sustained as compared to the release from liposomes (Berg, 2011). In addition, it is not feasible to expect that non-modified liposomes will be applied directly to vagina, on the contrary some suitable delivery system will be used as a vehicle.

We were intrigued to evaluate the effect of pH on the *in vitro* release of catechin and performed the additional experiments at physiological pH (7.4). There were two additional reasons for doing so, one is that post-menopausal women are expected to have vaginal pH in neutral range (Caillouette et al., 1997), and second was related to potential of applying liposomal catechins to various other site of the body, such as for example rectum, skin, and similar.

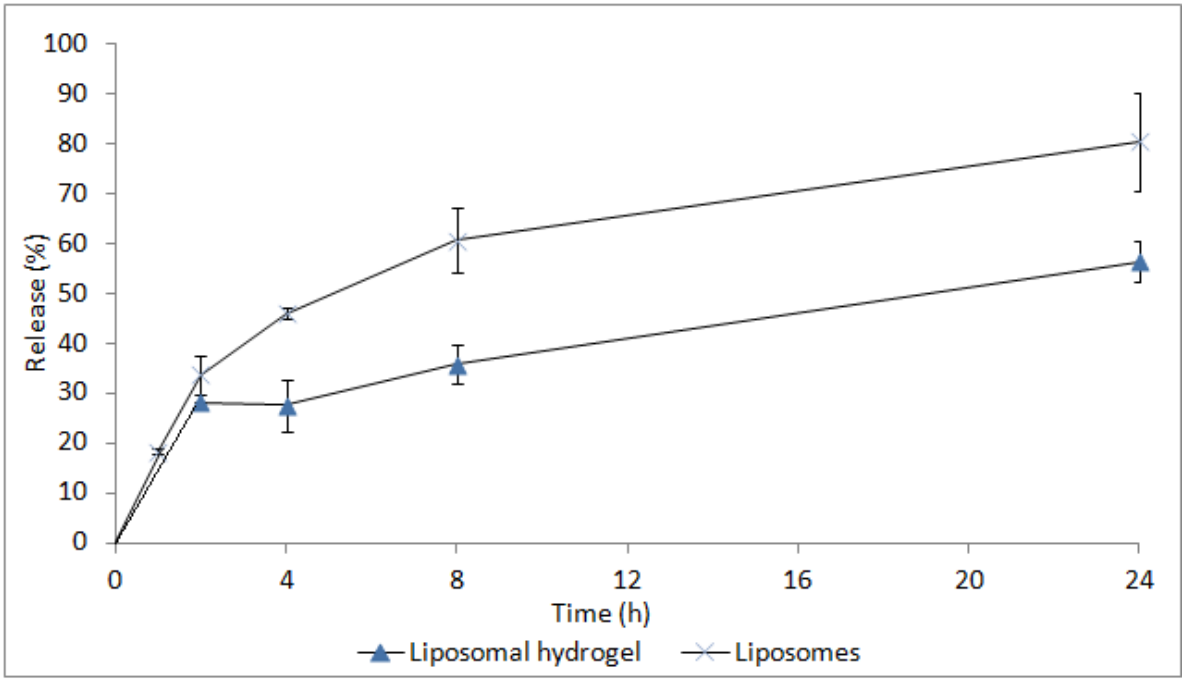

Figure 14: *In vitro* release of catechin entrapped in liposomes and catechin entrapped in liposomes incorporated in hydrogel (PBS, pH 7.4).
The values denote the mean ± SD (n=3).
(Missing marker in graph after 1 hour for liposomal hydrogel, due to lost sample).

The release of catechin from liposomes was much faster than from liposomal hydrogel (Figure 14). After 24 hours, 80 % of catechin was released from liposomes, whereas 56 % was released from the liposomal hydrogel ($p<0.05$). This confirmed that liposomal hydrogels provide sustained release features to liposomally associated drugs. It was interesting to note the effect the pH and buffer composition had on release profile. Less total amount of catechin was released at pH 4.0 then at neutral pH. However, one should also consider the difference in ionic strength and osmolarity between two mediums used for testing at two different pH.

Based on the findings presented in this thesis, we can confirm that encapsulation of catechins in liposomes and there subsequent incorporation in hydrogels provides potentials for development of novel drug delivery systems for vaginal therapy with catechin. There are several additional steps which need to be fulfilled in order to come closer to *in vivo* evaluation in animal models, such as evaluation of safety and toxicity of newly developed systems and confirmation of anti-inflammatory potentials of catechins and catechins in delivery systems. These experiments are currently in progress in our laboratory.

6 Conclusions

We confirmed a strong antioxidative activity of two green tea catechin epimers, namely catechin and epicatechin, through DPPH assay. Epicatechin was found to be a significantly stronger antioxidant than catechin. Although this is a very preliminary result, it seems that stereochemistry plays an important role in their antioxidative activities and it remains to be further discussed in order to elaborate their biological activity.

We were able to develop liposomal formulations for both catechin and epicatechin. The optimized formulations had an appropriate vesicle size for topical administration and carried a sufficient amount of drug. The stability profile of liposomal formulations was found to be satisfactory.

The hydrogel with incorporated catechin-containing liposomes were found to have acceptable cohesiveness and adhesiveness for topical application. *In vitro* release experiments showed sustained release of catechin from liposomal hydrogels, both in a media simulating vaginal environment (pH 4.0) and in physiological pH (7.4).

Based on the findings presented in this thesis, we can confirm that encapsulation of catechins in liposomes and their subsequent incorporation in hydrogels provides potentials for development of novel drug delivery systems for vaginal therapy. Experiments for the evaluation of anti-inflammatory potentials of developed delivery systems for catechin and epicatechin are currently in progress in our laboratory.

7 Perspectives

Short-term perspectives

- Evaluation of anti-inflammatory properties of catechin and epicatechin in delivery system (experiments currently in progress in our laboratory).
- Deeper insight on location of catechin and epicatechin within liposomes by more advanced characterization methods such as X-ray diffractions and differential scanning microscopy.
- Investigation of zeta potential of liposomes containing catechin or epicatechin, respectively, in order to further evaluate stability.
- *In vitro* release studies of developed formulation by using *ex vivo* vaginal tissue.
- Bioadhesion studies of developed formulation on *ex vivo* vaginal tissue.

Long-term perspectives

- Evaluation of safety and toxicity of developed formulation in animal model.
- Preliminary tests on healthy human vaginal mucosa regarding potential irritancy.
- Evaluation of antioxidative activities of other green tea catechins in relation to stereochemistry.
- Development of liposomal formulation of other green tea catechins for their biological evaluation.

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