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Topical vaginal therapy: Development of a liposomal hydrogel delivery system for epicatechin

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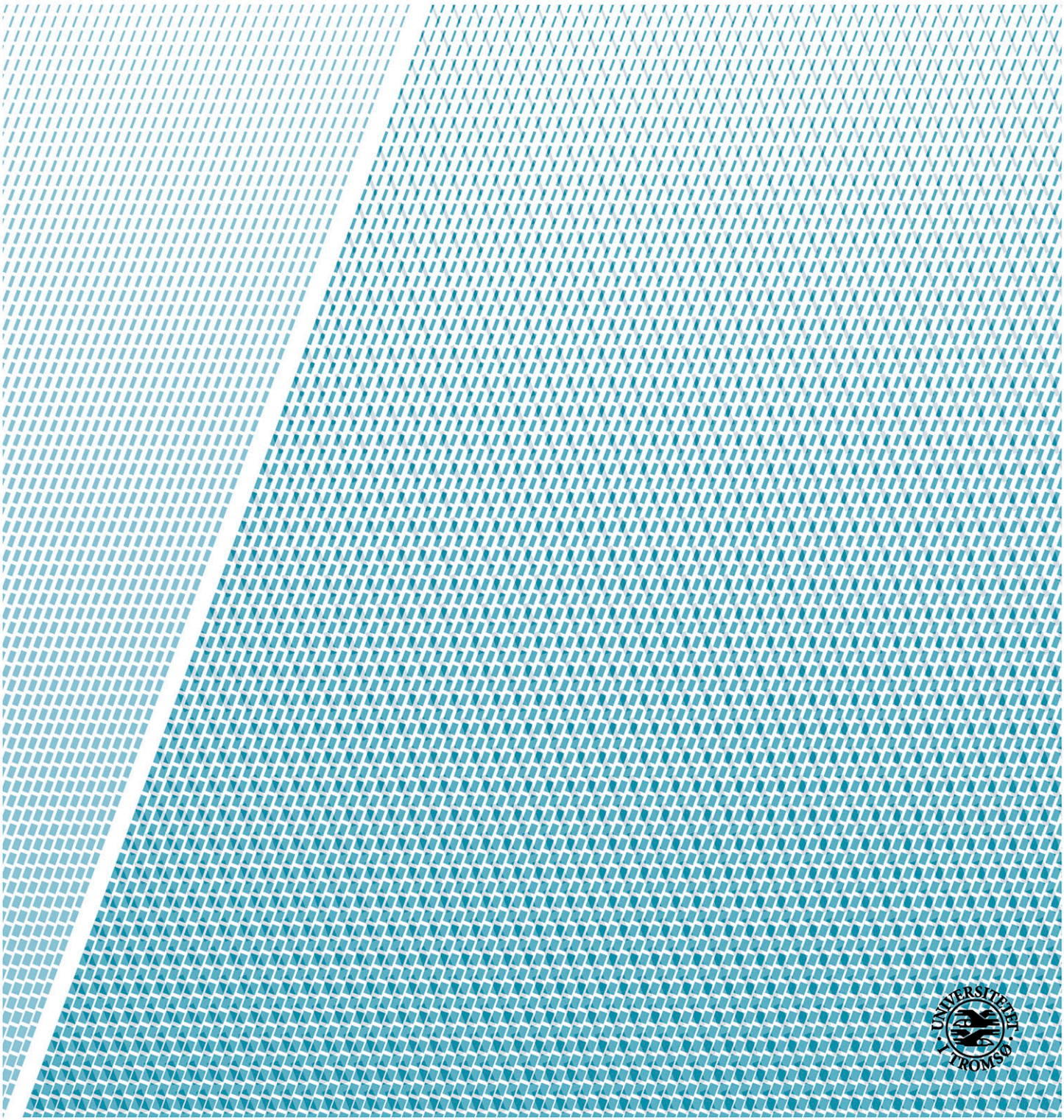

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Abstract

Vaginal infections are common in women of all ages and proper treatment is essential. The vaginal route of administration can be considered as favourable for the local therapy of vaginal infections. There are several vaginal dosage forms currently available; however, all suffer from limitations such as leakage and limited residence time at the site of action resulting in reduced therapeutic effect. Epicatechin (EC) is thought to be a potential substance in the both prevention and treatment of vaginal infections due to its antioxidative and anti-inflammatory effects, potentially also antimicrobial. However, the physiochemical properties of EC limit its use, and EC is a good candidate to exploit the beneficial effects of delivery system on improved therapeutic action. A delivery system with suitable viscosity and good mucoadhesive properties was the focus of this project; liposomal hydrogel comprising liposomes containing EC and chitosan hydrogel as a vehicle was developed.

Phosphatidylcholine liposomes containing EC were made by the thin film method followed by extrusion to desired size of 200 nm. Liposomal EC were characterized for vesicle size, polydispersity and EC entrapment efficiency. We prepared liposomes of desired vesicle size (around 200 nm) with rather high EC load (over 80 % entrapment efficacy). Liposomal EC suspensions were further incorporated in a chitosan hydrogel vehicle, and the texture properties of the hydrogel investigated to optimize the formulation. The hydrogels were found to have satisfactory cohesiveness, adhesiveness and hardness as well as satisfactory mucoadhesive properties, however, further investigation and optimization of the method is needed. Liposomal EC and liposomal EC hydrogel were found to provide prolonged EC release. High accumulation of EC at the *ex vivo* vaginal tissue confirmed that liposomal EC hydrogels could assure localized vaginal delivery.

Key words: Liposomes, chitosan hydrogel, epicatechin, mucoadhesion, vaginal delivery

Abstract (Norwegian)

Vaginale infeksjoner er vanlig hos kvinner i alle aldre, og riktig behandling er viktig. Den vaginale administrasjonsveien kan anses som gunstig for lokal behandling av vaginale infeksjoner. Det finnes per dags dato mange vaginale doseringsformer, men begrensninger som lekkasje og redusert retensjonstid på administrasjonsstedet kan resultere i redusert terapeutisk effekt. Epicatechin (EC) er antatt å være en substans som potensielt kan brukes i forebygging og behandling av vaginale infeksjoner på grunn av dets anti-inflammatoriske, antioksiderende og potensielt antimikrobielle effekt. De fysikalske og kjemiske egenskapene til EC gjør det vanskelig å utnytte disse effektene, og et egnet leveringssystem er nødvendig for å bedre den terapeutiske virkningen. For å utvikle et leveringssystem for EC med passende viskositet og gode slimhinneklebende egenskaper for vaginal applikasjon, var fokuset i dette prosjektet å utvikle en liposomal hydrogel som inneholder liposomer med EC.

Liposomer laget av fosfatidylkolin med EC inkorporert ble fremstilt ved hjelp av tynnfilm metoden etterfulgt av ekstrudering til ønsket vesikkel størrelse på 200 nm. Liposomene ble karakterisert for deres størrelse, størrelsesfordeling og mengde EC inkorporert. Vi fremstilte liposomer med ønsket vesikkelstørrelse (rundt 200 nm) med ganske høyt innhold av inkorporert EC (80 %). Videre ble liposomene inkorporert i en kitosan hydrogel, der hydrogelens teksturegenskaper ble undersøkt for å optimalisere formuleringen. Hydrogelen hadde god kohesivitet, adhesivitet og hardhet. I tillegg hadde hydrogelene gode slimhinneklebende egenskaper, men ytterligere undersøkelser og optimalisering av metoden er nødvendig. Liposomal EC og liposomal EC hydrogel ble funnet å gi en forlenget frisetting av substansen. Høy akkumulering av EC på *ex vivo* vaginalt vev bekreftet at liposomal EC hydrogel kunne sikre en lokalisert vaginal levering.

Nøkkelord: Liposomer, kitosan hydrogel, epicatechin, slimhinneklebing, vaginal levering

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List of Abbreviations

ABTS	2,2- Azino-bis (3-ethylbenzothiazoline-6-sulfonic-acid) diammonium salt
$ABTS^{+\bullet}$	cationic ABTS in free radical form
DPPH	2,2 Diphenyl-1-picrylhydrazyl
$DPPH^{\bullet}$	DPPH in free radical form
EC	epicatechin
EE	entrapment efficiency
EE (%)	percent entrapment efficiency
FDC	franz diffusion cell
GUV	giant unilamellar vesicle
HSV	herpes simplex virus
HPLC	high performance liquid chromatography
HPV	human papilloma virus
LUV	large unilamellar vesicle
MLV	multilamellar vesicle
PC	phosphatidylcholine
PPOS	potassium peroxidsulfate
STI	sexually transmitted infections
SUV	small unilamellar vesicle
VFS	vaginal fluid simulant
WHO	world health organization

1 General introduction

According to the World Health Organization (WHO) and other research centres such as the U.S Centre for Disease Control and Prevention, the incidence of sexually transmitted infections (STIs) has increased and represents a major health problem affecting both female and male population (Blakney *et al*., 2017; Satterwhite *et al*., 2013). Further, the most common viral infections are caused by human papilloma virus (HPV) and *Herpes simplex virus* (HSV) which have specific therapeutic challenges (Viera *et al*., 2010). Today's treatment of HSV and HPV is limited due to challenges in the complete elimination of viruses. The therapy should therefore aim to treat symptoms and prevent recurrence. There is an urgent need for improved treatment options.

Vaginal infections are common in women of all ages and proper treatment is essential in order to avoid serious consequences of failed therapy such as infertility, preterm delivery and induction of malignancy (Fenton and Lowndes, 2004). Vaginitis is an inflammation in the vagina often caused by vulvovaginal candidiasis, bacterial vaginosis and trichomoniasis (Quan, 2010). Topical or oral treatment of those infections are found to be equally efficient, however, local treatment is preferred due to minimal side effects. To obtain an efficient local treatment, the design of effective topical formulation should consider the characteristics of infected vaginal site (Palmeira-de-Oliveira *et al*., 2015). The vaginal self-cleansing mechanism contributes to reduced therapeutic effect of currently available drug dosage forms (Baloglu *et al*., 2009). Thus, the development of an effective drug delivery system that can prolong the residence time and enable sustained release of incorporated drug for improved drug therapy is needed.

Natural polyphenols are considered one of the most promising nutraceuticals in health care and have been recently investigated for the prevention and treatment of STIs (Date and Destache, 2016). Catechins are natural polyphenols found in green tea, of which epicatechin (EC) is one of the primary catechin components. They are known to have strong antioxidant, antibacterial and antiviral activity, and are associated with many health benefits (Cabrera *et al*., 2006). However, EC suffers from poor solubility and low bioavailability, thus making the pharmacological properties hard to be fully utilized. Nanomedicine can improve the therapy outcome due to the potential to address some of the challenges that cause limited therapy (Vanić and Škalko-Basnet, 2013). By incorporating EC in suitable delivery systems, such as liposomes, these challenges can be overcome (des Rieux *et al*., 2006; Hu *et al*., 2017). Liposomes have

been widely used as drug carriers in different applications and are able to incorporate both lipophilic and hydrophilic compounds (Akbarzadeh *et al*., 2013). Liposomes have the ability to perform sustained and controlled release of entrapped compounds and are therefore considered suitable for vaginal applications. However, a major limitation of liposomes for topical application is their low viscosity resulting in reduced retention time at vaginal site. To overcome this challenge, liposomes can be incorporated in well suited vehicles, such as hydrogels, that will prolong the residence time at the vaginal site (Pavelić *et al*., 2001).

Hydrogels are water-swollen polymeric gels made of natural or synthetic materials. Due to their soft and rubbery consistency they are similar to living tissue, making the hydrogels ideal for variety of applications, including those for vaginal administration (Ullah *et al*., 2015). Prolonged residence time at the vaginal site improves the therapy by increasing the contact time between the drug and the vaginal mucosa (Pavelić *et al*., 2001). Some hydrogels contain polymers with good mucoadhesive properties to prolong the residence time, such as chitosan. Chitosan is a natural polysaccharide compound exhibiting good mucoadhesive properties. The increased interest for medical and pharmaceutical applications of this polymer is due to its good biocompatibility, non-toxicity and antibacterial properties (Kast *et al*., 2002). Based on chitosan´s positively charged amino groups and the anionic substructures of the mucus, mucoadhesion can occur enabling an increased retention time at the site of application (Bernkop-Schnürch and Dünnhaupt, 2012). In addition, several factors will influence the mucoadhesion at the vaginal site. The challenging factors affecting the efficacy of this route of the administration are the variation in the amount of mucus, vaginal fluid, pH and the thickness of the epithelium (das Neves *et al*., 2010).

Incorporating liposomes into a hydrogel can assure a high concentration of drug in surrounding tissue over an extended period of time (Hoare and Kohane, 2008). The focus of this project was to develop a delivery system with suitable viscosity and good mucoadhesive properties for vaginal application of EC. This was achieved by incorporating liposomes containing EC into a chitosan-based hydrogel.

2 Introduction

2.1 Common vaginal infections

Genital infections represent a major public health problem in the world, with an increasing incidence due to the lack of effective prevention and treatment strategies (Fenton and Lowndes, 2004). According to World Health Organization (WHO) more than one million sexually transmitted infections (STIs) are acquired everyday worldwide (WHO, 2016). Vaginal infections can be caused by bacteria or viruses and changes in the vaginal microflora can increase the risk for infection (Nardis *et al*., 2013; Noyes *et al*., 2018). However, preventative and therapeutic methods are under investigation, mainly for STIs such as HSV, HPV and some vaginal bacterial infections (Blakney *et al*., 2017).

2.1.1 Vulvovaginal candidiasis

Vulvovaginal candidiasis is a common infection of the female genital tract, however, it is not considered a STI as candida is considered a part of the vaginal flora in reproductive age (Sobel, 1997). *Candida albicans* can be referred to as an opportunistic pathogenic fungus that causes mucosal infections. It is considered that more than 85 % of vulvovaginal candidiasis is caused by *Candida,* and at least 75 % of all women will suffer from a candida infection at least once in their lifetime (De Bernardis *et al*., 2018). The vaginal microflora can be disturbed by different factors such as pregnancy, antibiotics and oral contraceptives, and disturbance of the normal vaginal microflora can result in infection caused by *Candida* (Sobel, 1988). The infection can take place by the adhesion of *Candida* to epithelial cells (De Bernardis *et al*., 2018). *Candida* infections can be treated both local and orally, and the efficacy is considered equal (Sobel, 2014). Azole products as antifungal agents are widely used in the treatment of *Candida* infections. There are several advantages in local drug therapy such as reduced systemic drug exposure and reduced incidence of adverse effects (das Neves *et al*., 2015).

2.1.2 Human papilloma virus

Human papilloma virus (HPV) is a non-enveloped virus in the Papillomaviridae family (zur Hausen, 2002). HPV is an infectious organism and one of the most common STIs which gets transmitted through mucosal surfaces (Bansal *et al*., 2016; Forcier and Musacchio, 2010).

Several HPV subtypes have been identified and are categorized as high- and low risk HPV infections (zur Hausen, 2002). High risk mucosal HPV types 16 and 18 are associated with cervical and vaginal cancers (Boda *et al*., 2018). HPV is also responsible for genital warts, where low-risk HPV type 6 and 11 are responsible for 85 % of the cases (Joura and Pils, 2016). Even if HPV is known to be responsible of cervical and vaginal cancer, the infections are often underestimated, asymptomatic and unrecognized (Boda *et al*., 2018). Currently there are no antiviral therapy for HPV infections, and for genital warts the treatment is often a combination of removal of the warts combined with local treatment. Polyphenon E (sinecatechins 15 % ointment) is a local treatment approved by the Food and Drug Administration (FDA) for external genital warts where the active ingredients are green tea catechins which is thought to have antioxidant, antiviral and antitumor activities (Fathi and Tsoukas, 2014). There are developed vaccines for prevention of HPV infections composed of virus-like particles (Dillner *et al*., 2007). Another great potential in the treatment of HPV infections is the use of interferon alpha (IFN-α) (Kollipara *et al*., 2015).

2.1.3 Herpes simplex virus

HSV is one of the most common STIs that is transmitted by direct contact with infected individuals. There are two main types of HSV, where HSV-1 infections affect the face and HSV-2 is predominantly transmitted by sexual contact and is the major cause of genital herpes (Sauerbrei, 2016). Most genital HSV-2 infections are undiagnosed and unrecognized which contributes to the spreading of the virus (Gupta *et al*., 2007). HSV enters the body through mucus membranes and skin and then replicates within the epithelial cells. When infected, recurrent infections will occur, and studies show that HSV-2 infection presents an increased risk for obtaining HIV infections, which makes HSV a significant health concern (Gupta *et al*., 2007). Another serious complication of genital herpes is neonatal transmission. Treatment of genital herpes with antiviral drugs aims to faster symptom resolution, healing and prevention of new lesions. The treatment does not eradicate infection or prevent new eruption (Gupta *et al*., 2007). Strategies to prevent HSV transmission and development of new antiviral products are under development, and many natural products from plants, such as EC, are under investigation (Docherty *et al*., 2005).

2.2 Vaginal anatomy and physiology

The human vagina is the female genital, where the main functions are sexual intercourse and menstruation discharge. The vagina is an S-shaped tubular fibromuscular organ with a length around 6-9 cm that extends from the cervix of the uterus to the vaginal vestibule (das Neves and Bahia, 2006). The vaginal wall consists of four noticeable layers (Figure 1); stratified squamous epithelium, lamina propria, muscular layer and tunica adventicia (Wong *et al*., 2014).

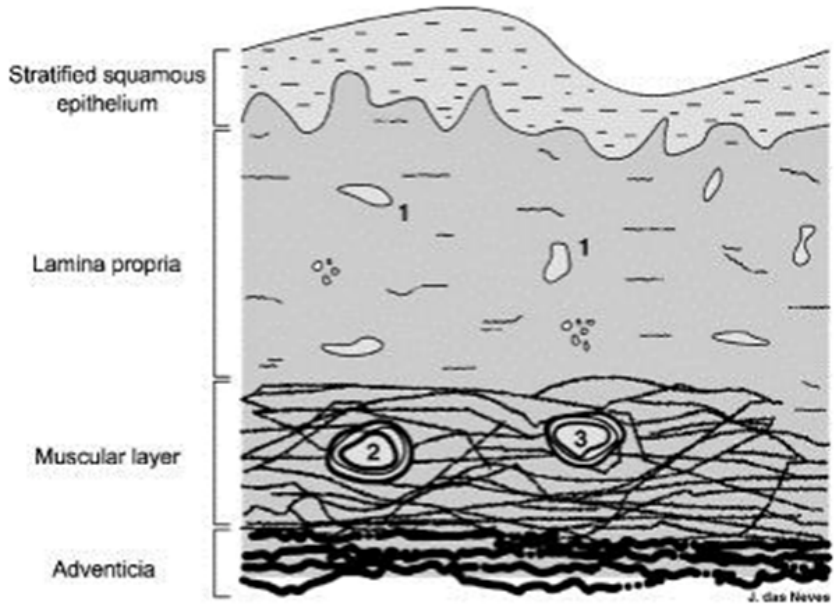


Figure 1: Schematic drawing of the vaginal mucosa. 1: capillary vessels; 2: artery; 3: vein (das Neves and Bahia, 2006). Copyright © with permission from RightsLink ®

Stratified squamous epithelium is highly folded or "rougae" that increases the surface area and makes it suitable for drug absorption. The epithelial mucosa is covering the vaginal wall with a thickness of 200-300 μm (Wong *et al*., 2014). The thickness of the vaginal epithelium is age-dependent and the epithelium changes through the menstrual cycle because of the variations in hormone levels (Poonia *et al*., 2006). The amount of estrogen decreases with increasing age, which leads to lower the thickness, discomfort and dryness.

The vagina is referred to as a mucosal tissue although it does not have any secretory glands, however, a certain amount of fluid is secreted mainly by cervical cells (Vanić and Škalko-Basnet, 2013). Studies are claiming that the daily vaginal fluid production is around 6 g (Palmeira-de-Oliveira *et al*., 2015). Vaginal fluid is a mixture composed of enzymes, proteins,

electrolytes, carbohydrates, amino acids and cervical mucus covering the vaginal wall (Wong *et al*., 2014). The vaginal mucus forms a viscoelastic barrier that protects the tissue from infections. The mucus layer consists of 95 % water, 1-2 % mucin glycoprotein fibers, and lower amounts of lactic acid, salts, proteins and enzymes. Mucin fibers are large molecules with size ranging from 0.5 to 40 million Daltons (Wong *et al*., 2014). The vaginal mucus has a rapid clearance that removes unwanted pathogens and particles before they reach the vaginal tissue. Changes in the vaginal fluid or mucus viscosity by environmental changes can reduce the protecting mucus barrier and increase the risk of infections (Palmeira-de-Oliveira *et al*., 2015). The mucus viscosity also varies depending on the menstrual cycle. When estrogen levels are high, the mucus consistency is thinner and more easily penetrable, and a decreasing estrogen levels increases the mucus viscosity (Caramella *et al*., 2015).

pH in healthy premenopausal women varies between 4.5-5.5 (das Neves and Bahia, 2006). The acidic environment of the vagina is mainly caused by the lactobacilli that is a part of the natural microflora in the vagina. Lactobacilli converts glycogen into lactic acid that gives arise to the low pH (Boris and Barbés, 2000). Presence of menstrual blood, semen (pH 7.0 - 8.0) or disease can temporarily change the pH level (Vanić and Škalko-Basnet, 2017). The pH level may also be disturbed by sanitary products, soap and tampons. Maintenance of a normal vaginal pH is important for the prevention of microbial growth, that can cause vaginal infections (Fashemi *et al*., 2013). Additionally, the application of vaginal dosage forms could disturb the vaginal flora.

2.2.1 Vaginal drug delivery

The vagina has been used as a route for drug administration for several decades (Hussain and Ahsan, 2005). It is an acceptable site for local and systemic drug delivery with many advantages due to the large surface area, avoidance of first pass metabolism, good blood supply and drug penetration. The self-cleansing mechanism of the vagina is both an advantage and disadvantage because it offers the possibility of self-removal of the drug delivery system that is favorable, however the reduced retention time is a disadvantage. The vagina is less sensitive compared to ocular and buccal administrations and allows the presence of drug formulation for a longer period without irritating the administration site (das Neves *et al*., 2010). The vagina as a site of action also presents some limitation where the main is the gender specificity. Other disadvantages such as cultural issues about genital manipulation, insertion of objects in the

vagina, variability in drug absorption, personal hygiene, menopause and pregnancy can limit the vagina as a route for drug administration (das Neves *et al*., 2010).

There are several vaginal dosage forms that are available at the market, such as vaginal rings, tablets, powders, capsules, suppositories, gels and creams. The most traditionally used vaginal products is for the locally active drugs such as antimicrobial, antiviral and contraceptives. The limitations with those dosage forms is that they suffer from leakage and limited residence time at the site of action because of the vaginal self-cleansing mechanism which may result in reduced effect (Baloglu *et al*., 2009).

In the development of new vaginal formulations, vaginal physiology must be studied. The formulation must be suitable for this route of administration and to achieve a successful delivery system it has to overcome several factors. Some physical changing factors that may disturb the efficacy of this route of administration is by the presence of mucus and variation in the vaginal fluid, pH and thickness of the epithelium (das Neves *et al*., 2010). The physiochemical properties of the drug such as ionization, molecular weight and lipophilicity will affect the absorption through the vaginal epithelium. Another important factor to evaluate is, if it is for local or systemic treatment. For local therapy it is undesirable for the drug to be absorbed, especially for pregnant woman (Srikrishna and Cardozo, 2013).

To obtain an optimal local therapeutic effect all the mentioned challenges should be overcome. It is also important that the delivery system is easy to use, non-toxic, non-irritating and painless to the patient. The drug concentration over a period of time at the administration site is important for a successful therapy (Srikrishna and Cardozo, 2013).This can be achieved by delivery systems with mucoadhesive properties.

2.3 Mucoadhesion

In pharmaceutical science, mucoadhesion is defined as when an adhesive material attaches to mucus or mucus membrane for an extended period of time by interfacial forces (das Neves *et al*., 2011a). Mucoadhesion can occur in two steps, the contact stage and consolidation stage (Figure 2). The contact stage is when the dosage forms intimate contact with the mucus membrane, and the consolidation stage is when various physiochemical interactions occurs between the dosage form and the mucus leading to a prolonged adhesion (Smart, 2005; das Neves *et al*., 2011a).

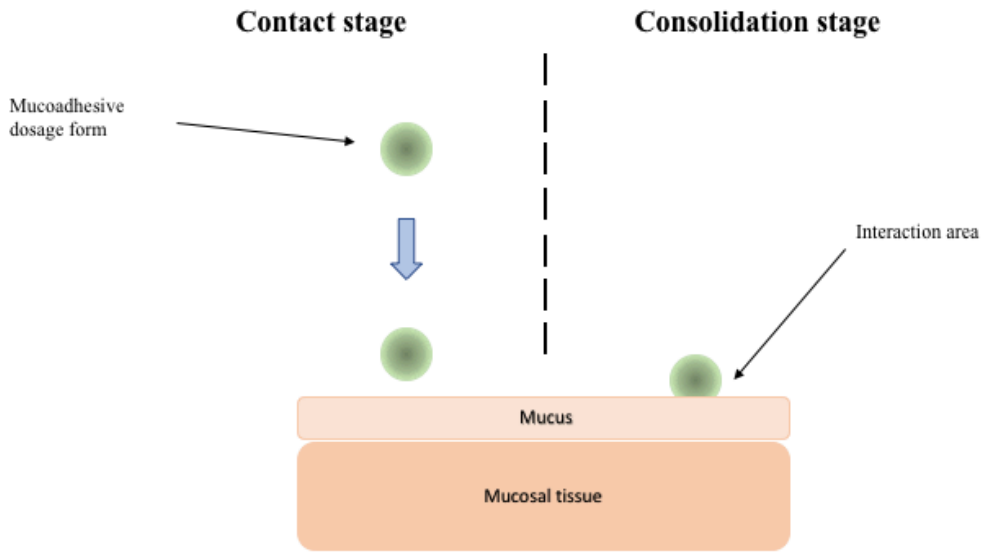


Figure 2: The two steps of mucoadhesion.

The event of mucoadhesion is still not fully understood, and there are six general theories of adhesion. The first theory is the electronic theory that describes the adhesion as electron transfer between the mucoadhesive system and the mucus, forming an electrical double layer at the interface. The second theory is the wetting theory that is applied for liquid systems and describes the ability of a mucoadhesive liquid to spread onto a mucosal surface. The liquid may act as an adhesive depending on the contact angel of the mucoadhesive liquid and the mucosal surface. The third theory is the adsorption theory that describes the adhesion to the mucosa because of hydrogen bonds (van der Waals forces). The fourth theory is the diffusion theory which describes the interpenetration of polymeric chain to decent depth and a semipermanent adhesive bond occurs. The fifth theory is the mechanical theory which speculate that the irregularities on a rough surface makes the adhesive liquid to interlock together. The last theory is the fracture theory that define the force needed for the detachment of two involved surfaces. However, the mucoadhesion process is apparently achieved through a combination of several mentioned theories (Smart, 2005; Boddupalli *et al*., 2010; Vanić and Škalko-Basnet, 2014).

The phenomena of mucoadhesion have been used to develop several dosage forms for oral, nasal, ocular and vaginal delivery. Mucoadhesive vaginal application enables reduced

administration frequency because of the prolonged residence time, better bioavailability and controlled delivery. For vaginal application the mucoadhesion may be affected by the vaginal environment and by the mucoadhesive properties of the polymer (Vanić and Škalko-Basnet, 2014).

2.3.1 Mucoadhesive polymers

Mucoadhesive polymers can be divided into synthetic or natural polymers. Natural polymers such as alginates, chitosan, pectin, gelatine and synthetic polymers such as polyethylene glycol (PEG), carbomers and cellulose derivatives have been widely studied in application for vaginal drug administration (Roy *et al*., 2009). They can also be classified according to their chemical structure or their mechanism of binding (covalent, non-covalent). The surface charge of the polymers will also affect the adhesion mechanism (Hombach and Bernkop-Schnurch, 2010).

Polymers diffuse into the mucosal layer and adhere to it by intermolecular complexes. The higher molecular mass of the polymer chains the greater mucoadhesion. The crosslinking reactions and the hydration of the polymer network effect the flexibility of the polymer chains and the mucoadhesion. Polymers can be manipulated and changed to increase their flexibility and mucoadhesive properties (Roy *et al*., 2009; Hombach and Bernkop-Schnurch, 2010). The existence of functional groups such as hydroxyl, amines and carboxyls on the polymers shows good capacity to stick to mucosal membranes and form adhesive bonds. An example of a polymer with functional groups is chitosan (Smart, 2005).

2.3.2 Chitosan

Chitosan exists in nature as chitin, which is a natural polysaccharide compound. It is the most abundant polysaccharide after cellulose (Hajji *et al*., 2014). The main sources are two marine shellfish; shrimp and crabs (Rinaudo, 2006). Chemical deacetylation of chithin is performed to produce the most common derivative, Chitosan. Chitosan is formed by connecting N-acetyl-2-amino-2deoxy-D-glucoses through β-(1→4) glycoside bonds (Figure 3) (Hajji *et al*., 2014). Parameters that influence the characteristics of chitosan are molecular weight and its degree of deacetylation. These parameters can be modified during the chitosan preparation (Berger *et al*., 2004a).

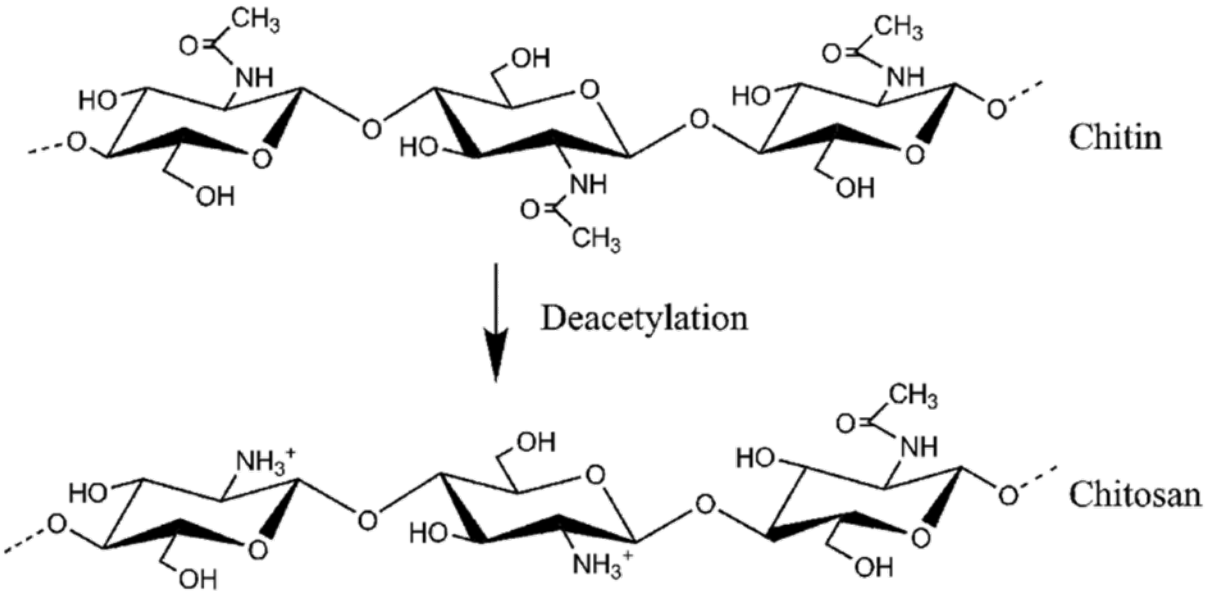


Figure 3: Chemical structure of chitin and chitosan (Zuber *et al*., 2013).

Chitosan has gained increased interest as medical and pharmaceutical applications due to its biocompatibility, non-toxicity, antimicrobial and mucoadhesive properties. Chitosan has been used as a controlled release delivery system for incorporated drugs in different formulations such as tablets, capsules and gels (Kast *et al*., 2002). Based on its positively charged amino groups and the anionic substructures of the mucus, mucoadhesion can occur and an increased retention time at the site of application (Bernkop-Schnürch and Dünnhaupt, 2012). This polymer is hydrophilic in nature and the ability of degradation by human enzymes makes the polymer biodegradable. Another advantage with chitosan is its antibacterial properties which is a benefit when treating vaginal infections (Ahmadi *et al*., 2015). To achieve a prolonged residence time in the vagina, chitosan can be used as a vehicle for the delivery system or as a surface modifying agent (das Neves and Bahia, 2006).

2.4 Hydrogels

Hydrogels are water swollen polymeric gels that are made of natural or synthetic materials, which possess a distinct three-dimensional structure. Because of their soft and rubbery consistency, they are similar to living tissue, making the hydrogels ideal for a variety of applications (Ullah *et al*., 2015). Hydrogels have several characteristics which make them excellent for drug delivery systems. The water content in hydrogels are high and they are

considered highly biocompatible, non-toxic and some are biodegradable (Bhattarai *et al*., 2010). Some hydrogels contain polymers with good mucoadhesive and bioadhesive properties. Hydrogels with good mucoadhesive properties can enhance the residence time and are suitable for vaginal application (Bhattarai *et al*., 2010; Huang *et al*., 2000).

Berger *et al.* classified hydrogels depending on the nature of their network, namely as entangled networks, covalently crosslinked networks and networks formed by secondary interactions (Berger *et al*., 2004a). They can also be classified based on the gelling material, if it is from natural or synthetic source, their degradability and physical structure. Radical polymerization is usually used for the preparation of polymers, then converted into hydrogels by crosslinking of the polymeric chains in moderately concentrated aqueous solutions (Parhi, 2017). The swelling part of hydrogels is a complex process with several steps. The first step is based on hydration of the hydrophilic groups of the hydrogel matrix, also known as primary bond water. The second step is interaction between water and hydrophobic groups and appears in the form of secondary bond water. Both the primary and secondary bound water forms the total bound water in the hydrogel. The third step is driven by osmotic force, and additional water is absorbed and fills the spaces between the chains and the centre of the larger pores (Ullah *et al*., 2015). Due to the crosslinked structure of hydrogels they do not disintegrate during swelling (Kopecek, 2009).

Chemical crosslinked hydrogels are a preparation method to produce permanent hydrogel networks by the presence of a chemical crosslinking agent. Crosslinking can be made by different methods such as crosslinking by radical polymerization, crosslinking by ionic interactions, crosslinking by crystallization and by chemical reaction of complementary groups (Ullah *et al*., 2015).

Physically crosslinked gels have been extensively investigated over the past years as the use of crosslinking agents have been avoided (Ullah *et al*., 2015). Many crosslinking agents are toxic compounds which have to be removed from the hydrogel before used in biomedical/pharmaceutical applications. Physically crosslinked hydrogels are reversible bonds and generally biodegradable (Ullah *et al*., 2015). Under appropriate conditions physically crosslinked polymers are made spontaneously. Chitosan hydrogels are made by physical crosslinking, and the simplest way to prepare a chitosan hydrogel is by solubilisation of chitosan in an acidic aqueous medium. The main interaction between the networks are covalent bonds,

but other interactions such as hydrogen bridges and hydrophobic interactions may also occur (Berger *et al*., 2004b).

2.4.1 Drug release from hydrogels

Hydrogels have a highly porous structure which allows a large amount of substances to be incorporated (Jagur-Grodzinski, 2010). The benefits of local treatment with hydrogels is that they can maintain a high concentration of drug in the surrounding tissue over an extended period of time (Hoare and Kohane, 2008). Several factors influence the drug release from hydrogels and the release is based on the rate-limiting step for controlled release and categorized as: diffusion-controlled, swelling-controlled or chemically-controlled. The most applicable mechanism of drug release is the diffusion-controlled, where the release through the hydrogel mesh is the primary mechanism of release and is dependent of the mesh size within the gel matrix. If the diffusion is faster than the expansion of the hydrogel, then the swelling is considered to control the release. Chemically controlled release depends on the chemical reaction within the gel matrix (Bhattarai *et al*., 2010). The drug release can also be triggered by stimuli such as pH triggered release, enzymatic responsive release and electrical triggered release (Bhattarai *et al*., 2010). However, hydrogels are well suited vehicles for several substances and liposomes.

2.5 Liposomes

During the past years, nanotechnology-based delivery systems for medical purposes have gained an increased attention. Liposomes are attractive and physiological acceptable drug delivery nanosystems. They are widely used in advanced topical delivery of drugs by mucosal routes of administration (Vanić and Škalko-Basnet, 2014). Liposomes are spherical vesicles consisting of one or more phospholipid bilayers (also known as lamellae) surrounding aqueous units where the polar head groups are oriented in the interior and exterior aqueous phase (Figure 4). The liposomes are characterized by their particle size, numbers of lamellae, surface charge and inner/outer aqueous phase (Akbarzadeh *et al*., 2013).

The motive to use liposomes as drug delivery systems was based on the possibility of fusion through the cell membrane, hence the preparation of liposomes had to be from lipids that was

present in the cell membrane. The biological membranes are composed of many different lipids such as glycerolipid, sphingolipids and sterols (Akbarzadeh *et al*., 2013). Liposomes can be prepared by different phospholipids, which will influence the properties of the liposomes, such as the rigidity or fluidity. Adding cholesterol to the liposomal membrane can increase the rigidity of the bilayer membrane and slow the disintegration of the liposomes (Choe *et al*., 1995; Arora *et al*., 2000).

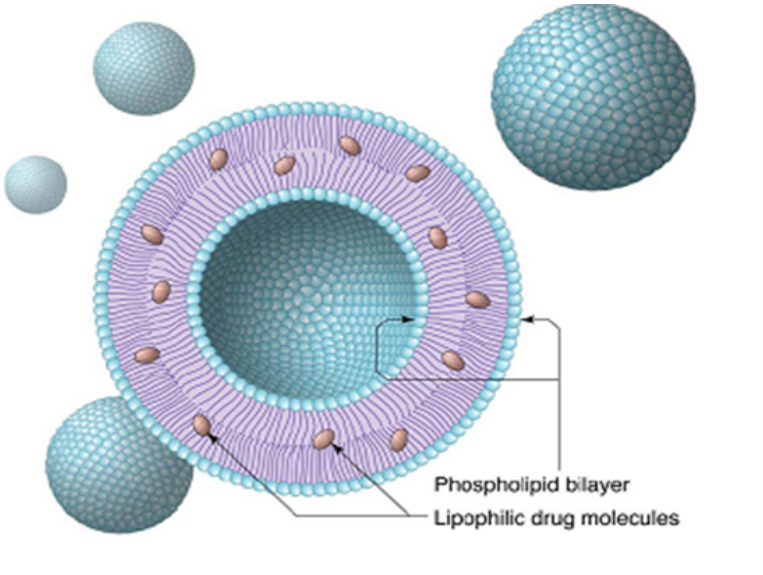


Figure 4: A unilamellar liposome with lipophilic drug incorporated in the phospholipid bilayer (Holsæter, 2004).

One of the most commonly used phospholipids in liposomes is phosphatidylcholine (PC), also known as lecithin. PC and other lipids are amphipathic molecules with a hydrophobic and a hydrophilic component. The polar head group in PC is made of quaternary ammonium moiety choline that is linked to a phosphate group and then to a glycerol backbone by phosphoric ester. The PC tail is made of a fatty acid chain (Brandl, 2001). Phospholipids can be derived from both natural and synthetic sources. The main source is from vegetable oils and animals (i.e. soybean, corn, cotton seed, egg yolk and bovine brain) (Li *et al*., 2015).

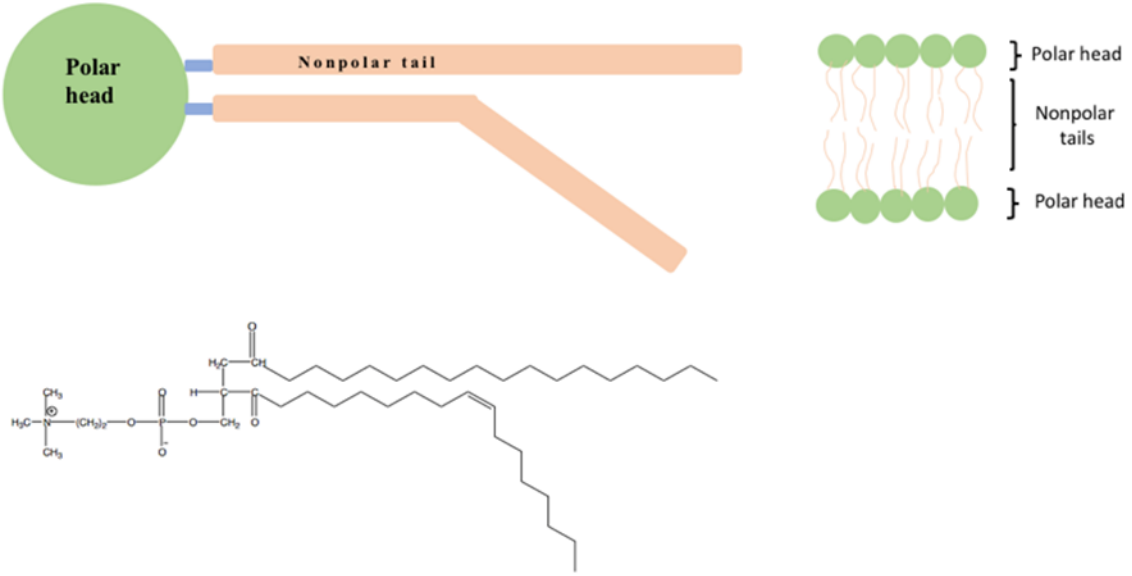


Figure 5: Chemical structure of phosphatidylcholine and schematic illustration of phospholipid.

Liposomes have many advantages as drug carriers for different substances, due to their biodegradability, biocompatibility and low toxicity. Liposomes have the ability to entrap both hydrophilic and lipophilic compounds, ensure controlled release and site-specific delivery of entrapped compounds, which makes them promising as pharmaceutical carriers (Akbarzadeh *et al*., 2013; Vanić and Škalko-Basnet, 2014). Various applications for liposomes are investigated and several formulations are on the market (Fan and Zhang, 2013).

2.5.1 Preparation of liposomes

Liposomes can be prepared by several methods with each process influencing liposome properties including size, lamellarity and entrapment efficiency (EE). The methods can be categorized into conventional or novel (Pattni *et al*., 2015).

The first described method for liposome preparation was the Bangham method, also known as thin lipid film hydration (Bangham *et al*., 1967). The method is based on creating a thin film of lipids in a round bottom flask by evaporating the organic solvent. The lipid film was then re-hydrated and hand shaken, causing the lipids to form liposomes spontaneously (Figure 6). With

the lipid film hydration method, size reduction of the liposomes may be needed. Size reduction can be generated by sonication or by multiple extrusions through a polycarbonate membrane (Pattni *et al*., 2015). The size of the liposomes depends on the number of extrusion cycles and the size of the polycarbonate pores in the extrusion membrane (Hope *et al*., 1985).

Figure 6: Preparation of liposomes by film hydration method.

The conventional methods have limitations such as low aqueous core entrapment and low EE for hydrophilic substances. This technique is for small-scale preparations and not convenient for industrial scale because of disadvantages such as board distribution, inconstant encapsulation and the difficulty with sterilization (Pattni *et al*., 2015). For industrial purpose, novel methods for preparation was developed.

2.5.2 Classification of liposomes

Liposomes are classified by their size and their lamellarity. The desirable size of liposomes in drug delivery applications are between 50 – 200 nm (Patil and Jadhav, 2014). The size of the liposomes can affect the circulation half-life of liposomes. Furthermore, liposomes can have one or many bilayer membranes (Akbarzadeh *et al*., 2013). Multilamellar vesicles (MLV) are liposomes with many membrane layers with a large diameter in size. They are usually over 100 nm in size. Large unilamellar vesicles (LUV) are liposomes with a single layer membrane with a diameter between 100-1000 nm. Small unilamellar vesicles (SUV) are also liposomes with a

single layer membrane, but smaller in size with a diameter between 10-100 nm. Giant unilamellar vesicles (GUV) are single layer membrane liposomes with sizes up to 1 µm. Multivesicular liposomes are many small vesicles inside a bigger vesicle (Figure 7) (Samad *et al*., 2007; van Swaay and deMello, 2013).

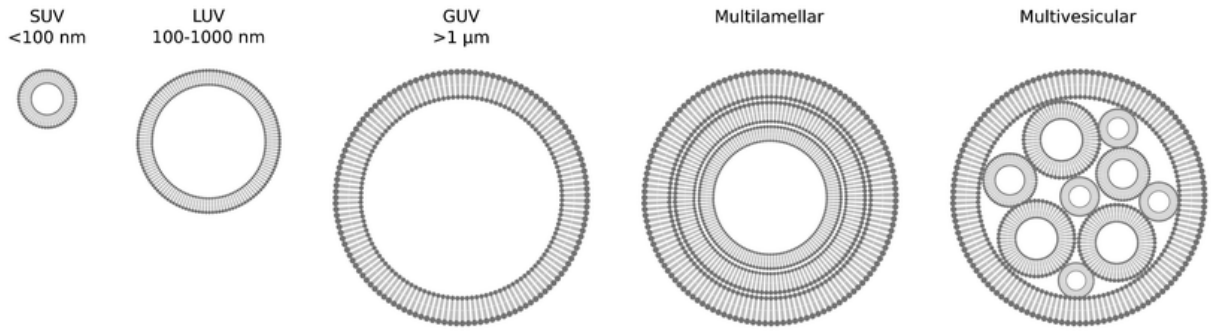


Figure 7: Liposomes classified by size and bilayers. SUV= small unilamellar vesicle, LUV= large unilamellar vesicle, GUV= Giant unilamellar vesicle, multilamellar and multivascular (van Swaay and deMello, 2013). Copyright © with permission from RightsLink ®

Liposomes can also be classified by their lipid composition, which may affect the surface charge of the liposomes. The surface charge of liposomes can be negative, positive or neutral. Another type of liposomes are immunoliposomes. Those are modified liposomes with antibodies on the surface and are designed especially for targeted delivery (Eloy *et al*., 2017). Another class is long circulating liposomes, known as PEGylated liposomes (Samad *et al*., 2007).

Liposomes as drug carrier systems offers a sustained release of substances administrated topically, however the liquid nature of liposomes makes it difficult to achieve a prolonged retention at the administration site. Incorporating liposomes in hydrogels can reduce the frequency of administration and better the treatment of vaginal infections (Hurler *et al*., 2012a). Liposomes are acceptable carriers for different substances that are poorly soluble and undergoes enzymatic degradation such as EC, which is a substance found in green tea. Incorporating EC into liposomes can protect it from degradation and enhance the stability of the substance (Date and Destache, 2016).

2.6 Green tea catechins

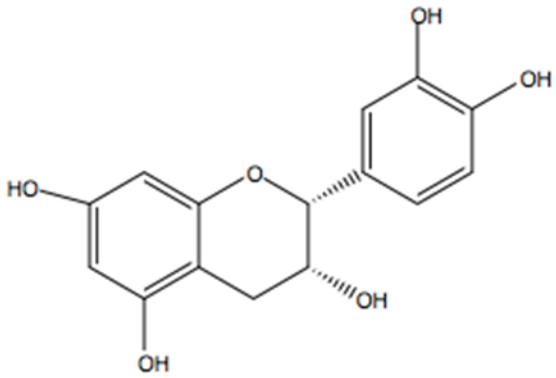
Green tea (*Cammelia sinensis*) is one of the most popular beverages in the world with abundant health benefits (Saeed *et al*., 2017). The green tea plant originated from Southeast Asia, but is cultivated in several continents. (Graham, 1992). The composition of green tea varies with climate, season and manufacturing conditions. Catechins are polyphenols found in the leaves of green tea. There are four major catechin derivatives, such as EC, epigallocatechin, epicatechin gallate and epigallocatechin gallate (EGCG). They are well known for their antioxidative, antibacterial, antiviral and anti-inflammatory effects. Recent studies found that these polyphenols have many benefits in the prevention and treatment of different diseases such as cancer, diabetes and other health concerns (Varilek *et al*., 2001; Frias *et al*., 2016). However, the mechanism of how the catechins in green tea works and affect the body are still not fully understood (Botten *et al*., 2015).

Figure 8: Illustration of the plant *Camellia sinensis* (Urtekilden).

The use of natural polyphenols in prevention and treatment of vaginal infections have been studied for several decades and the main substance used for this project was EC (Date and Destache, 2016).

2.6.1 Epicatechin

EC is one of the active components found in green tea and one of the most important ones. EC is considered to have anticancer, anti-inflammatory and antioxidative properties. The substance can protect the plant from stress by scavenging reactive oxygen species and protect plants from UV radiation from the sun because of its UV absorbing properties. Those effects of EC has been associated to their antioxidant activities and makes it suitable for multiple applications in the pharmaceutical and food industries (Mendoza-Wilson and Glossman-Mitnik, 2006). The mechanism of ECs antioxidant activity has not been proven, although several studies have been performed. Most of the studies are still in the experimental and theoretical level (Mendoza-Wilson and Glossman-Mitnik, 2006; Botten *et al.*, 2015).



(-)-epicatechin

Chemical Formula: $C_{15}H_{14}O_6$
Exact Mass: 290.08
Molecular Weight: 290.27

Figure 9: Chemical structure of EC.

2.6.2 Antioxidative effects of epicatechin

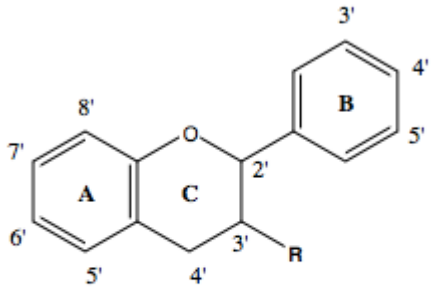
Free radicals are atoms or molecules that contains unpaired electrons. Those electrons alter the reactivity of the atom or molecule comparable to non-radicals (Lobo *et al.*, 2010). Free radicals are formed in the human body by normal metabolic processes caused by physiological stress, and by external sources such as environmental radiation, smoking and industrial chemicals (Bagchi and Puri, 1998; Frias *et al.*, 2016). Normal levels of free radicals are important for the cell signaling processes. High levels of free radicals are harmful and may cause cell damage

and death (Lobo *et al*., 2010). Oxidative stress is a result of imbalance between free radical production and antioxidant defense. Oxidative stress has been postulated in many conditions, including inflammatory conditions and degenerative disorders. Catechins are well known for their antioxidative and anti-inflammatory effects and have the capacity to scavenge free radicals, therefore they have a potential in disease prevention and treatment (Lobo *et al*., 2010; Frias *et al*., 2016)

There are two pathways for oxidation where antioxidants can have a preventative role (Wright *et al*., 2001). The first one is the hydrogen-atom transfer, based on the capacity of a functional phenol group to donate a hydrogen atom to a free radical ($R^{\bullet}$). The other mechanism is based on the single electron transfer from a phenolic antioxidant (ArOH) to a $R^{\bullet}$ with formation of a stable radical cation $ArOH^{\bullet+}$ (Wright *et al*., 2001).

- H-atom transfer: $R^{\bullet} + ArOH \rightarrow RH + ArO^{\bullet}$
- Single- electron transfer $R^{\bullet} + ArOH \rightarrow R^{-} + ArOH^{\bullet+}$

It is proposed that EC exhibit its main antioxidative activity through these mechanisms. In addition to the mentioned mechanisms, EC can also exhibit protective roles by chelating metal ions such as iron and cupper. Structural features for EC that is important for the antioxidative activity include the presence of two hydroxyl groups on the B ring at position 3 and 4, and the hydroxyl group at R on C ring (Higdon and Frei, 2003).



R=OH

Figure 10: General structure and nomenclature of catechins.

However, EC suffer from poor solubility and low bioavailability. To overcome these problems EC should be incorporated into suitable delivery systems such as nanocarriers.

2.6.3 Delivery systems for epicatechin

Green tea catechins, especially EC, have shown many physiochemical limitations that are challenging in the development of new therapeutic agents. Loading EC into nanoparticles can increase the absorption and bioavailability, protect EC from degradation by enzymes, enhance stability and prolong the circulation time (Chuan *et al*., 2015; Wang *et al*., 2014). There are several delivery systems that have been considered suitable for EC.

A study made by Fang *et al.* developed liposomal formulations of catechins, including EC for topical and intratumor applications. They wanted to evaluate the possibility of using liposomes for the local delivery, including skin and tumor deposition. They also tried to establish the *in vivo* effect and relationship between physiochemical properties of catechins encapsulated in liposomes. The results of this study showed no significant increase in skin deposition of catechins after topical administration with liposomes, but a higher amount of catechins in liposomal formulation were delivered into the solid tumor than by the aqueous solution with catechins (Fang *et al*., 2005). Another study conducted by Wisiuitiprot *et al.* investigated the cutaneous absorption of catechins from green tea extract alone and loaded catechins from green tea extract in chitosan microparticles. The results of the study suggested that chitosan microparticles improved the penetration in skin compared to free substances. The results also demonstrated that chitosan microparticles limited the degradation of catechins by enzymatic degradation on the skin (Wisuitiprot *et al*., 2011).

Liposomes have been widely used in different areas such as in drug delivery. Liposomes have been studied as a promising carrier system for catechins and several studies have investigated the effect of catechins in liposomes (Rodrigues *et al*., 2013). EC may prevent or treat different diseases, but until now, there are no studies of EC in liposomes for topical vaginal application.

3 Aim of the study

The main aim of the study was to develop a liposomal hydrogel delivery system for EC intended for local treatment of vaginal infections. Liposomes were expected to enable a controlled and sustained release of entrapped EC and the use of hydrogel as vehicle was expected to enable mucoadhesion and prolonged residence time at vaginal site.

The aim can be divided in:

- Evaluation of the antioxidative effects of EC on DPPH and $ABTS^{+\bullet}$ radicals
- Characterization of liposomal size, size distribution and EC entrapment efficacy
- Characterization and optimization of chitosan hydrogel containing liposomal EC, in regards to texture properties
- *In vitro* EC release testing of liposomal preparations (both suspensions and hydrogels) to confirm controlled and prolonged release
- Determining the *ex vivo* penetration of EC through sheep vaginal tissue in presence of vaginal fluid simulant (VFS) to assure localized EC effect
- Testing the *ex vivo* mucoadhesive properties of the liposomal EC hydrogel on cow vaginal tissue to prove system´s bioadhesive potential
- Preliminary testing of the stability of novel system

4 Materials and methods

4.1 Materials

4.1.1 Materials used

Acetic acid (glacial), Sigma Aldrich Chemie GmBH, Steinheim, Germany

Ammonium acetate ≥ 89 %, Sigma Aldrich Chemie GmBH, Steinheim, Germany

L-Ascorbic acid (Vitamin C), Sigma Aldrich Chemie GmBH, Steinheim, Germany

2,2- Azino-bis (3-ethylbenzothiazoline-6-sulfonic-acid) diammonium salt (ABTS), Sigma Aldrich, Steinheim, Germany

Bovine serum albumin, Sigma Aldrich Chemie GmBH, Steinheim, Germany

Calcium hydroxide, Sigma Aldrich Chemie GmBH, Steiheim, Germany

Chitosan (MMW), Chitinor, Tromsø, Norway

2,2 Diphenyl-1-picrylhydrazyl (DPPH), Sigma Aldrich Chemie GmBH, Steinheim, Germany

Distilled water

Epicatechin, (-)-epicatechin ≥ 90 %, Sigma Aldrich Chemie GmBH, Steinheim, Germany

Ethanol 96 % vol, Sigma Aldrich Chemie GmBH, Steinheim, Germany

Glucose ≥ 99.5 %, Sigma Aldrich Chemie GmBH, Steinheim, Germany

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

Hydrochloric acid, Sigma Aldrich Chemie GmBH, Steinheim, Germany

Lactic acid, Sigma Aldrich Chemie GmBH, Steinheim, Germany

Lipoid S 100, phosphatidylcholine from soybean, Lipoid GmBH, Ludwigshafen, Germany

Methanol, VWR International S.A.S., Fontenay-sous-Bois, France

Methanol, Sigma Aldrich Chemie GmBH, Steinheim, Germany

Milli-Q water

Potassium hydroxide, Norsk medisinaldepot NMD, Oslo, Norway

Potassium peroxidesulfate, Merk KGaA, Darmstadt, Germany

Potassium phosphate monobasic, Sigma Aldrich Chemie GmBH, Steinheim, Germany

Sodium chloride ≥ 99.5 %, Sigma Aldrich Chemie GmBH, Steinheim, Germany

Sodium phosphate dibasic dehydrate, Sigma Aldrich Chemie GmBH, Steinheim, Germany

(±)-α-Tocopherol (Vitamin E), Sigma Aldrich Chemie GmBH, Steinheim, Germany

Trifluoroacetic acid (TFA), Sigma Aldrich Chemie GmBH, Steinheim, Germany

Ureum (carbamide), Apotekproduksjon AS, Oslo, Norway

4.1.2 Instruments

Büchi Waterbath B480, Büchi Vac V-500, Büchi vacuum controller B-721, Büchi rotavapor R-124, Büchi labortechnik, Flawil, Schwitzerland

Branson B-Series 5510 Ultrasonic Cleaners, Danbury, USA

Franz Diffusion Cell 15 mm with 12 ml receptor volume, flat ground joint clear glass, clamp and stirbar, Permgear, Hellertown, USA

High Performance Liquid Chromatography, Waters e2795, Separations Module, Waters 2489, UV/Visible Detector, Milford, USA

Julabo heating circulator, Julabo F12-ED, JULABO GmBH, Seelbach, Germany

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

PermGear V6A Stirrer, PermGear, Hellertown, USA

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

TA.XT.Plus Texture analyzer, Stable Microsystems, Surrey UK. Backward Extrusion Rig A/BE, Stable Microsystems, Surrey, UK

Zetasizer Nano Zen 2600, Malvern Instruments Limited, Engima Business Park, Grovewood Road, Malvern, Worcestershire, UK

4.1.3 Computer programs

High Performance Liquid Chromatography, Empower™ 3 Software, Build 3471, Waters, 2010, Dublin, Ireland

Photon correlation spectroscopy, CW 388 version 1.68, NICOMP Particle Sizing Systems, Santa Barbara, California, USA

Texture analyzer, Exponent, 32 (3.0.5.0) Stable microsystems, Surrey, UK

Zeta potential, Zeta potential report version 2.2, Malvern Instruments Limited, Malvern, UK

4.1.4 Tissues

Vaginal tissue from cow was provided by Nortura Målselv, Tromsø, Norway

Vaginal tissue from sheep was provided by Laboratory Animal Centre, University of Oulu, Finland

4.2 Methodology

4.3 Antioxidative assays for epicatechin

Antioxidative activity is the capacity of EC, vitamin E and vitamin C to scavenge free radicals such as $ABTS^{\bullet+}$ and DPPH. The antioxidative activity of EC was compared with vitamin E and vitamin C under the same experimental conditions as mentioned below.

Stock solutions were prepared of EC, vitamin C and vitamin E in 96 % ethanol to the concentrations 1 mg/ml. Sample solutions were prepared by diluting the stock solutions to the concentrations 5, 10, 25, 50 and 75 μM.

4.3.1 Measurement of DPPH radical scavenging activity

DPPH radical scavenging capacity of EC was determined by the method described by Basnet *et al. (*Basnet *et al.,* 2012*).* DPPH was dissolved in ethanol to the final concentration 134 μM and stored in a dark environment at room temperature for 30 minutes before further use. DPPH solution (134 μM, 0.3 ml) was mixed with 0.3 ml of each sample solution. The mixed sample solutions were shaken and stored in the dark for 30 minutes at room temperature. Determination of the antioxidative activity of EC was measured by UV spectrophotometer at wavelength 519 nm. All experiments were performed in triplicates.

4.3.2 Measurement of $ABTS^{\bullet+}$ radical scavenging activity

Equal volumes (2.5 ml) of $ABTS^{\bullet+}$ solution (7.4 μM) were mixed with potassium peroxidsulfate (PPOS) solution (2.6 μM). The reaction mixture was stored in the dark at room temperature for 3 hours to stabilize. The mixture was then diluted with 96 % ethanol to a final volume of 100 ml. $ABTS^{\bullet+}$ solution (0.3 ml) was mixed with an equal volume of the sample solutions and kept in the dark at room temperature. After 30 minutes the absorbance was detected with UV spectrophotometer at wavelength 757 nm. All experiments were performed in triplicates.

4.4 Liposomal preparation and characterization

4.4.1 Preparation of liposomes with epicatechin

Liposomes were prepared by the conventional film method described by Jøraholmen *et. al.* EC (10 mg) and Lipoid S 100 (200 mg) were dissolved in excess methanol in a round bottom flask. Methanol was evaporated on Büchi rotavapor R-124 with Büchi Vac V-500 Vacuum Pump System for at least 2 hours at 55 mBar and 50°C. The film was then re-suspended in 10 ml distillated water and hand shaken until a homogenous suspension. Liposomal suspensions were stored in the refrigerator (4-8 °C) overnight before further experiments (Jøraholmen *et al.,* 2015).

4.4.2 Size reduction of liposomes

Liposomal size was reduced by extrusion through Nuclepore® Track-Etch Membrane Polycarbonate with different pore sizes. The extrusions were performed 5 times through the membranes with pore sizes 0.8 µm, 0.4 µm and 0.2 µm. Liposomal suspension was placed in the refrigerator for stabilization overnight before further use.

4.4.3 Vesicle size analysis

Particle size distributions of the liposomes were measured by photon correlation spectroscopy. The determinations were performed on NICOMP submicron Particle Size model 370. To avoid any contamination of particles from the environment, the preparations were carried out in a laminar airflow bench using particle free equipment. Test tubes were filled with distilled water and sonicated for 30 minutes, then rinsed with filtrated water (0.2 µm pore size syringe filter) in order to avoid any particles that may interfere during the analysis. The sample was diluted with filtrated water to obtain an intensity of 200 - 350 kHz. All analyses were run in vesicle mode and intensity distribution. Three parallels, each with a run time of 10 min, were run of each sample.

4.4.4 Epicatechin calibration curve

A stock solution was prepared of EC in methanol with the concentration 1 mg/ml. The standard solutions were prepared by diluting the stock solution to the concentrations 2.5, 5, 10, 25, 50, 75 and 100 µg/ml. The UV plate was filled with 200 µl of the solutions in three parallels. The absorbance was measured at wavelength 280 nm on UV spectrophotometer.

4.4.5 Entrapment efficiency determination

Dialysis was used to separate free EC from entrapped liposomal EC, and the dialysis was performed in dialysis tubing. One ml of liposomal EC sample was dialyzed against 250 ml distilled water for 6 hours. Aliquots of the sample and the dialysis medium were diluted in methanol and measured spectrophotometrically at 280 nm to determine the percent entrapment efficiency EE (%).

4.5 Hydrogel preparation and characterization

4.5.1 Preparation of chitosan hydrogel with glycerol

The preparation of hydrogels were based on the method of Hurler and colleges. Glycerol (10 % w/w) was mixed with acetic acid (2.5 % w/w) to a homogenous mixture. Medium molecular weight (MMW) chitosan (3 % w/w) was then dispersed in the mixture of glycerol/acetic acid and stirred manually for 10-15 minutes. The mixture was bath-sonicated for 30 minutes to remove entrapped air. The gel was allowed to swell at room temperature for 48 hours.

Gels with a starting concentration of 2.5 % (w/w) chitosan and gels with the final chitosan concentration of 2.5 % (w/w), after incorporating liposomal EC were also prepared (Hurler *et al*., 2012b).

4.5.2 Incorporation of liposomal epicatechin in chitosan hydrogels

Different amounts of liposomal EC suspensions, free from unentrapped EC, were incorporated into the different chitosan hydrogels. The amount of liposomal EC incorporated was 10 % (w/w;

liposomal suspension/total weight) and 20 % (w/w). Liposomal suspension hand stirred to evenly dispersed within the chitosan hydrogel (Hurler *et al*., 2012b).

4.5.3 Zeta potential measurement

Zeta potential was measured for liposomal EC and the liposomal EC hydrogel. The measurements were performed on a Malvern Zetasizer Nano ZS. Before use, the measurement cell was rinsed with ethanol and filtrated water (0.2 μm pore size syringe filter). Liposomal EC samples (50 μl) was diluted with filtrated water (950 μl). The liposomal EC hydrogel was diluted equally, and samples were loaded into the measurement cells. All measurements were performed at 25°C and the number of runs for each sample were 3 cycles.

4.5.4 Texture analysis

To determine the texture properties (cohesiveness, adhesiveness and hardness) of the hydrogels, a Texture Analyzer TA.XT plus (Stable micro systems Ltd., Surrey, UK) was used. The measurements were conducted by backward extrusion by a probe disk with a diameter of 40 mm. The analysis was performed on the liposomal EC hydrogel, hydrogels with EC in acetate buffer (composition as described below) and plain hydrogels.

Hydrogels were left in room temperature over the night prior to experiment and 40 g of gel formulation were filled in a standard 100 ml beaker. Calibrations were performed, and the experimental conditions used for the test were as followed:

Test mode: compression
Pre test speed: 4 mm / sec
Test speed: 4 mm / sec
Post test speed: 4 mm / sec

Target mode: distance
Distance: 10 mm; return to the start point

Three parameters were measured; maximum compressing force (hardness) (force 1), cohesiveness (area 1) and the adhesiveness (area 2) (Figure 11) (Hurler *et al*., 2012b). Five replicate analysis were performed.

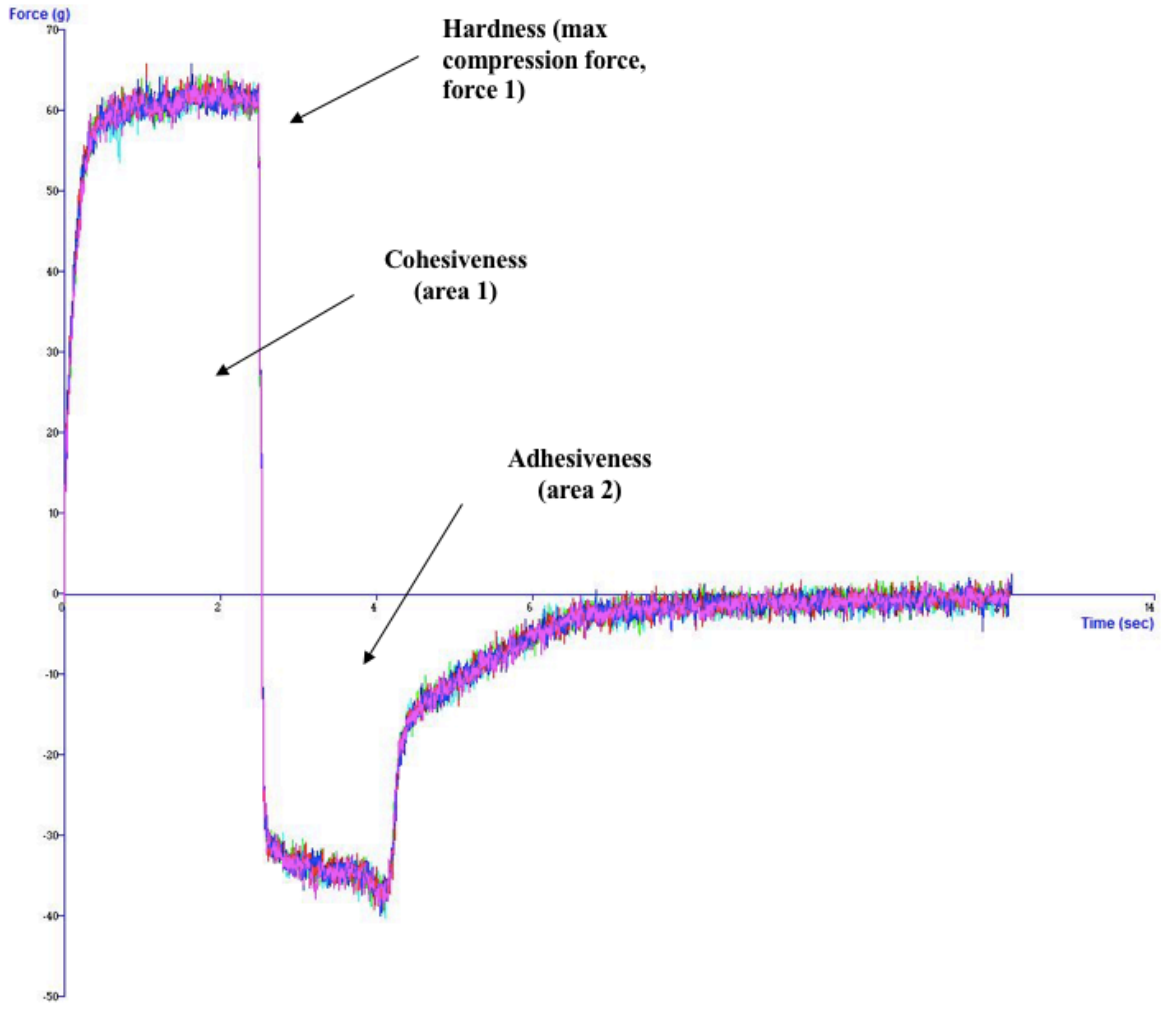


Figure 11: Parameters measured for texture analysis.

4.5.5 Preparation of vaginal tissue from cow and sheep

Cow and sheep vaginal mucosa was prepared by removing access fat and underlying tissue. The tissues were cleaned with phosphate buffer (pH 7.4), packed in clinging film and frozen (- 20 °C). Prior the experiments the tissue was thawed at room temperature.

4.5.6 Mucoadhesive properties

The mucoadhesion test was performed based on the method of Hurler and Skalko-Basnet (Hurler and Škalko-Basnet, 2012). The analysis was performed on a Texture Analyzer TA.XT plus (Stable micro systems Ltd., Surrey, UK) with a mucoadhesion rig. Prior the experiment vaginal tissue from cow was cut to appropriate sized pieces and rinsed with ethanol and

phosphate buffer before the slice was clamped onto the membrane holder. The thickness was measured to be around 1.52 mm. Approximately 150 µl of the gel formulations were applied onto the probe with a one ml syringe. The die was pinched for exactly 10 sec with a pressure of 25 g onto the mucosal tissue. The die was redrawn from the tissue at a speed of 0.1 mm / sec until the gel slipped. The detachment force was recorded.

The mucoadhesive properties were further determined by the amount of the formulation left on the tissue. The probe was weighed before and after the tests, and after each measurement, the tissue was rinsed with ethanol and phosphate buffer (pH 7.4). All tests were performed in triplicates and each formulation was tested five times.

4.5.7 Preparation of acetate buffer

Acetate buffer (pH 4.6) was prepared by dissolving 38.55 g $CH_3COOHNH_4$ in distilled water, 35 ml glacial acetic acid was added to the mixture and the volume was adjusted to 500 ml with distilled water. pH was measured to 4.6.

4.5.8 *In vitro* release of epicatechin

To determine the *in vitro* release of EC from liposome preparations (both suspensions and gels), a Franz diffusion cell (FDC) system was used (Figure 12). Before use, donor chamber and receptor chamber were well cleaned with methanol for 30 minutes and twice with distilled water for 30 minutes. Prior to the experiment the receptor chamber (12 ml) was filled up with acetate buffer (pH 4.6) and the heating circulation was set to 37°C. A magnetic stirrer was inserted to the receptor chamber to assure a reliable stirring during the experiment. Cellophane membrane was evenly cut to fit the top of the receptor chamber and presoaked in acetate buffer for 30 minutes before use. The membrane was placed on top of the receptor chamber and the donor chamber was placed on top with a joint packing in between.

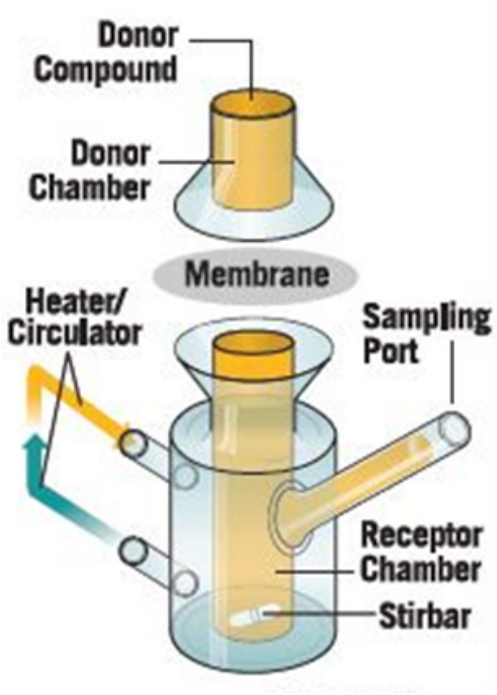


Figure 12: Schematic presentation of Franz Diffusion Cell (Sciences, 2010).

Samples (600 µl) were added to the donor chamber and sealed with a rubber plug. The sampling ports were covered with parafilm to avoid any evaporation of the acceptor medium. Samples of 500 µl were collected after 1, 2, 3, 4, 6 and 8 hours. After each sampling the volume taken out was replaced with fresh buffer. When the last samples were taken, the residue in the donor chamber was collected and the membrane was left in methanol. All samples were diluted in methanol and drug content was determined with UV spectroscopy as described above.

Following preparations were tested (all samples were prepared with the same concentration of EC; Control (EC in acetate buffer), liposomal EC (liposomal EC in distilled water), control in hydrogel (EC in acetate buffer in hydrogel) and liposomal EC hydrogel.

4.5.9 Preparation of vaginal fluid simulant

VFS was prepared from 3.51 g/l NaCl, 1.40 g/l KOH, 0.222 g/l $Ca(OH)_2$, 0.0018 g/l bovine serum albumin, 2 g/l lactic acid, 0.16 g/l glycerol, 0.4 g/l urea, 5 g/l glucose, distilled water and 0.1 M HCl to adjust the pH to 4.5 (Owen and Katz, 1999).

4.5.10 Preparation of phosphate buffer

Phosphate buffer (pH 7.4) was composed of 8 g/l NaCl, 0.19 g/l KH_2PO_4 and 2.38 g/l Na_2HPO_4. pH was adjusted with NaOH.

4.5.11 *Ex vivo* penetration of epicatechin

Sheep vaginal tissue was used for the *ex vivo* penetration study and thawed in phosphate buffer before sectioning small pieces to fit the franz cells. Excess fat was removed, and the thickness of the pieces aimed to be the same. The thickness was measured to be around 1.1 mm. Prior to the penetration experiments the receptor chambers (12 ml) were filled with phosphate buffer (pH 7.4). Samples (550 µl) were added to the donor chamber with VFS (50 µl). Following preparations were tested; Control (EC in acetate buffer), liposomal EC (liposomal EC in distilled water) and liposomal EC hydrogel.

The samples were filtrated through Acrodisk® 13 mm Syringe Filter with 0.2 µm Nylon Membrane. After the filtration, the samples were diluted in methanol and the content of EC was determined by HPLC. The method used in HPLC analysis was based on the method described by Li *et al.* (Li *et al.*, 2012). A reverse phase column (Symmetry® C18 5µ: 3.9x150 mm Column, Waters, Dublin, Ireland) installed in a Waters e2795 separations module coupled to a Waters 2489 UV/Visible detector were used in the measurements. The mobile phase consisted of 0.1 % TFA in Milli-Q water (pH=2.0) mixed with methanol in a ratio 75:25 (v/v). The HPLC measurement settings were as follows: flow rate 0.8 ml / min, column temperature 25 °C, sample temperature 25 °C, injection volume 20 µl, run time 13 min and the detection wavelength 280 nm. The measurements were performed in triplicates.

4.6 Statistical evaluation

When suited, a student *t*-test were performed to determine the level of significance. The significance level was set to $P \leq 0.05$.

5 Results and discussion

5.1 Antioxidative potential of epicatechin, vitamin C and vitamin E

In order to evaluate and compare the *in vitro* antioxidative effect of EC with vitamin C and vitamin E, well established antioxidants, the DPPH and $ABTS^{+\bullet}$ radical scavenging assays were utilized. The assays are widely used to determine the antioxidative activity of different substances, due to the wide availability, simplicity and accuracy (Basnet *et al*., 2012; Kedare and Singh, 2011; Erel, 2004).

The antioxidative activity of EC is expressed as the capacity to scavenge the stable free radicals of DPPH and $ABTS^{+\bullet}$. Both DPPH and $ABTS^{+\bullet}$ are concentration dependent where higher concentrations correlate to high absorbance and lower concentration to low absorbance. This is also reflected in the colour change; the DPPH and $ABTS^{+\bullet}$ are deep violet and deep green colour, respectively; and a decrease in colour intensity indicates a decrease in absorbance, corresponding to the decrease in free radicals concentration (Kedare and Singh, 2011; Erel, 2004).

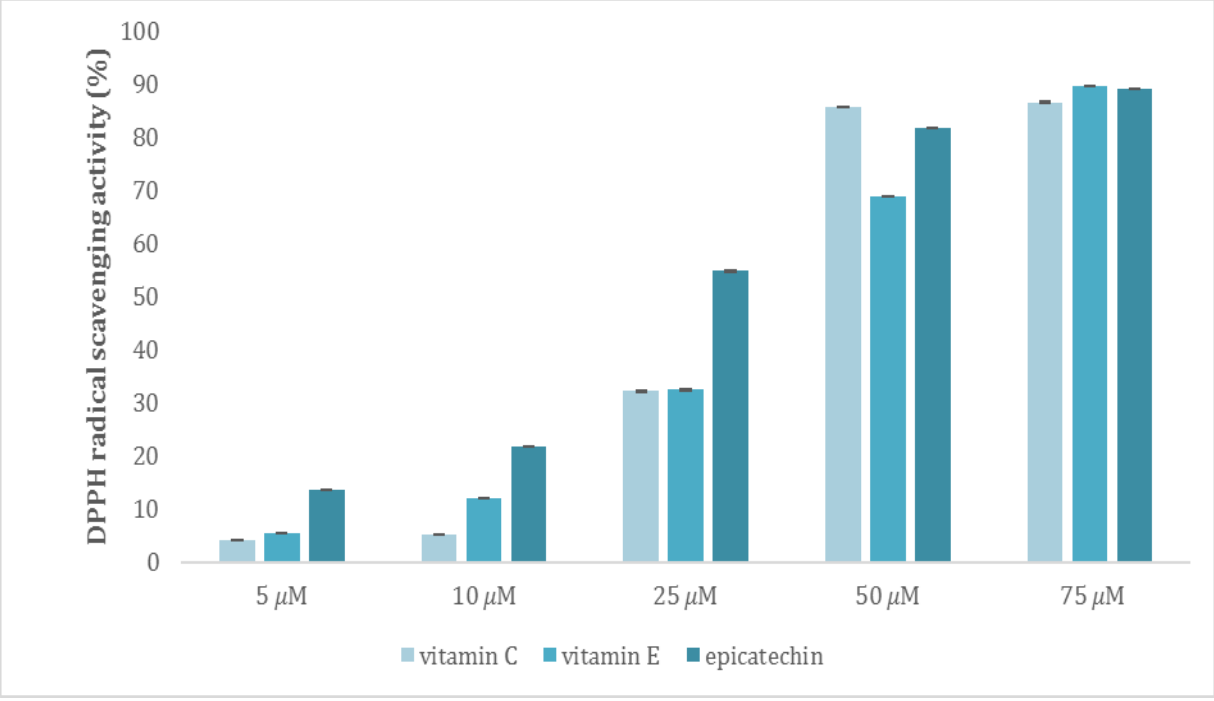


Figure 13: DPPH radical scavenging activities (%) of epicatechin ± SD (n=3).

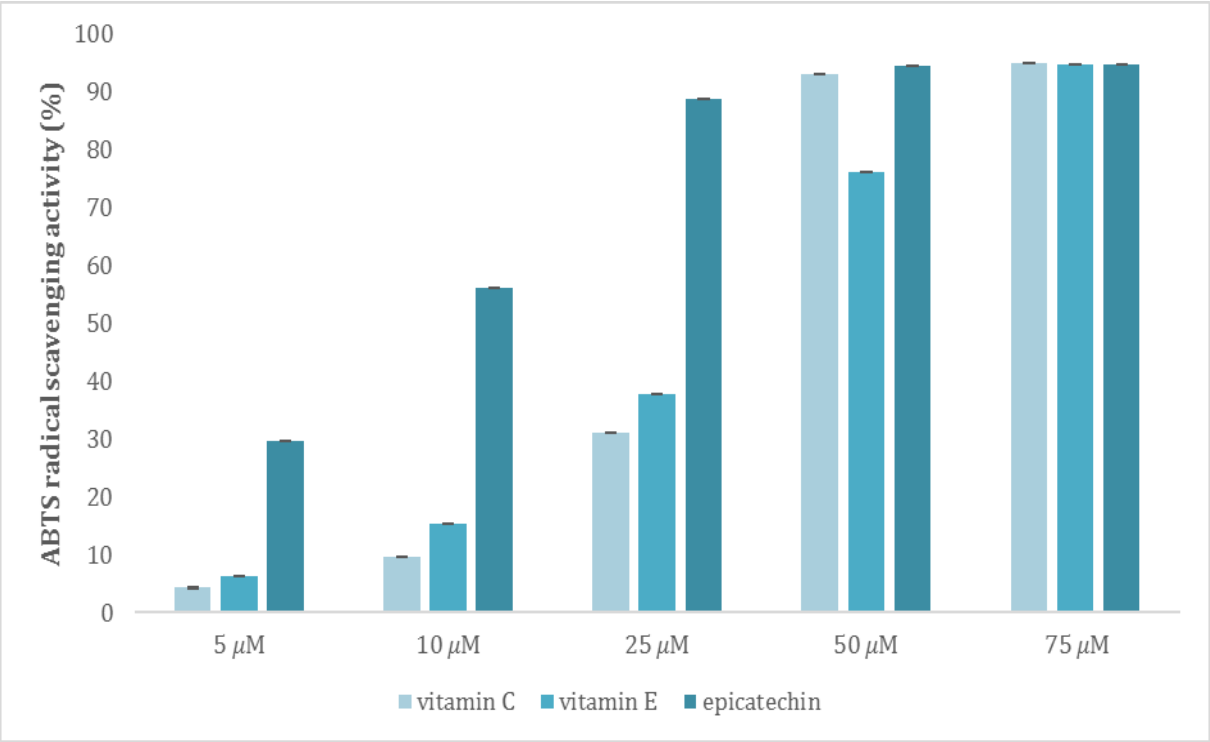


Figure 14: $ABTS^{+\bullet}$ radical scavenging activities (%) of epicatechin ± SD (n=3).

EC is well known for its antioxidative activities, thus, its activities was compared to those of vitamin C and vitamin E. Vitamin C is a water-soluble substance and vitamin E is a lipophilic substance, that are well known for their antioxidative effects (Dreosti, 2000). The antioxidant concentrations chosen were 5, 10, 25, 50 and 75 μM; all the concentrations were measured at the same time. EC, vitamin C and vitamin E expressed a concentration-dependent radical scavenging activity for both $ABTS^{+\bullet}$ and DPPH (Figures 13 and 14), where higher concentrations of the antioxidants corresponded to more of the free radicals being scavenged. The concentration-dependent scavenging activity indirectly indicates that the substances solubility does not affect its antioxidative activity.

EC expressed a slightly superior antioxidative effect towards $ABTS^{+\bullet}$ than DPPH (Figure 13 and 14). The differences in radical scavenging activity may be due to the structural differences between the radicals used and how they react with EC (Cai *et al.*, 2006). The radical scavenging activity of vitamin C and vitamin E were almost identical in both.

In order to consider the differences in antioxidative activity among EC, vitamin C and vitamin E, we used an independent *t*-test where all absorbance values of EC were paired with

the comparable values for vitamin C and vitamin E at the same concentrations. The independent *t*-test was performed for all concentrations and both $ABTS^{+\bullet}$ and DPPH measurements. For DPPH measurements we found that EC was significantly more potent compared to both vitamin C and vitamin E. Our findings are supported by other studies reporting that catechins and EC are more efficient scavengers of DPPH radicals than both vitamin C and vitamin E (Higdon and Frei, 2003; Nanjo *et al*., 1996). Figure 14 indicates that EC is more potent antioxidant in lower concentrations than both vitamin C and vitamin E, which is favourable considering that the amount of EC in the hydrogel formulations is expected to be low (discussed below). Vitamin C was found to have a better antioxidative effect than EC in the concentration of 50 μM. Results of the *t*-test indicated that vitamin C was significantly stronger antioxidant than EC in that concentration.

As mentioned, we found that ECs radical scavenging effects on $ABTS^{+\bullet}$ were much stronger than for DPPH, especially in the lower concentrations (Figure 14). The results from the independent *t*-test confirmed that EC was significantly more potent antioxidant than both vitamin C and vitamin E ($P \leq 0.05$ for all substances), in the lower concentration. This correspond to the findings of Higdon and Frei who reported that the radical scavenging activity of EC was greater than both vitamin C and vitamin E (Higdon and Frei, 2003).

The results confirmed that EC exhibits strong radical scavenging effects. EC has many health beneficial properties; however, it suffers from several physiochemical limitations such as low solubility and limited bioavailability. To overcome these limitations and increase the biological activity especially for vaginal infection targeting, liposomal EC incorporated in chitosan hydrogel was proposed. Therefore, current research was mainly focused on the development of a liposomal hydrogel delivery system for EC.

5.2 Liposomal characterization

The physiochemical characteristics of the delivery system are important for the effectiveness of local drug delivery to the vaginal tissue. The size of vesicles can affect system´s ability to pass through mucin pores, thus, it is an important parameter to measure (Vanić and Škalko-Basnet, 2013). According to Samad *et al.* the thin film method used to prepare liposomes provide large vesicles often larger than 1 μm in diameter (Samad *et al*., 2007). Smaller

liposomes with uniform size distribution are preferred for improved drug delivery properties, hence, a reduction of liposomal size is needed.

One of the most common methods for size reduction of liposomes is by extrusion (Berger *et al*., 2001). This method is based on size reduction by input of mechanical energy to push an aqueous lipid suspension through the polycarbonate membranes with defined pore sizes. Based on the vaginal mucus physiology the mesh pore sizes are estimated to be around 340 ± 70 nm (Lai *et al*., 2010). Considering the optimal size of particles intended for vaginal drug delivery, there are very few studies reporting the effect of vesicle size. However, a study conducted by Takeuchi *et al.* indicated that a reduction in liposomal size to approximately 100 nm increased the penetration through intestinal mucus. This finding cannot be directly correlated to the penetration through vaginal mucosa. On the other hand, das Neves *et al.* suggested that a size range between 200-500 nm is preferable for vaginal mucosal delivery, since both larger and smaller nanosystems failed to achieve mucus penetration (das Neves *et al*., 2011b; Takeuchi *et al*., 1996).

In this project the membrane pore sizes chosen for the extrusion were 800, 400 and 200 nm to achieve a desired vesicle size around 200 nm, and the obtained liposomes were found to be in the desired size range (Table 1).

Table 1: Characterization of liposomes containing epicatechin.

	Particle size (nm)	**Polydispersity index (PI)**	**Entrapment efficiency (%)**	**Zeta potential (mV)**
Liposomal epicatechin	197.89 ± 26.83	0.074	81.46 ± 1.61	-1.52 ± 2.03

The values are presented as mean ± SD (n=3).

Advantages of using extrusion as a method to reduce the liposomal size is the easiness of achieving the desirable size and a more uniform size distribution, which is favourable. A PI below 0.7 is acceptable, however, it indicates a broad distribution, thus a PI below 0.3 is

preferable and considered a more homogenous size distribution. Many studies use sonication as a size reduction method which is not optimal due to the difficulties to achieve the desirable size and increased stress to the liposomes that may cause deformation or loss of entrapped substance (Patil and Jadhav, 2014; Akbarzadeh *et al*., 2013). Table 1 indicates that PI for our liposomes were lower than 0.1 that indicates a very homogeneous size distribution. The low PI also confirmed that extrusion is a suitable method to achieve desired vesicle size with limited polydispersity.

It is suggested that EC is incorporated in the lipid bilayers of liposomes (Hashimoto *et al*., 1999). The physiochemical properties of EC will affect the incorporation efficiency and the solubility of the substance is crucial. Due to the lipophilic nature of the drug (logP of 1.8 (Drugbank), EC was dissolved with the lipid in methanol prior to liposomes preparation and was expected to be incorporated within the lipid bilayer. To assure a sufficient amount of active substance able to achieve an optimal therapeutic effect, a high entrapment efficiency of the active substance in the liposomes is desirable (Pavelić *et al*., 2001). According to Basnet *et al.* there is a balance between the vesicle size and entrapment efficacy where a reduction in vesicle size leads to reduced entrapment efficacy, due to reduced number of lipid bilayers available to accommodate lipophilic substances (Basnet *et al*., 2012). As described earlier, free EC was separated from liposomal EC by dialysis. Dialysis principle is based on separating entrapped substance from the non-entrapped substance using dialysis tubes and aqueous medium. Time used in dialysis was set to 6 hours considering that this is enough time to remove free EC. It is known that dialysis can be used as a release method, a longer dialysis of EC could lead to release of incorporated EC. The dialysis was performed in the sink conditions, to assure that free EC, with limited solubility in water, can be separated from liposomal EC.

The entrapment efficiency was calculated on the basis of a standard curve for EC, and a representative standard curve used in the calculation is shown in Figure 15. The correlation coefficient was determined to be 0.999. The entrapment efficiency (EE) for EC was found to be 81.46 ± 1.61 % (Table 1). The entrapment was slightly higher than previously reported data; Fang *et al.* reported EE from 20 – 67 % depending on type of liposomes; Jain *et al.* reported EE of 64.7 % for extruded liposomes with EC (Fang *et al*., 2006; Jain *et al*., 2013). However, Huang *et al.* have shown similar entrapment values for EC (Huang *et al*., 2011). The mentioned studies refer to different lipid composition and preparation methods used for liposome preparation. When determining the EE, the method of separation of free drug from liposomally-associated drug can affect the EE to certain degree. Lipophilic compounds can also be separated

from liposomes by centrifugation, depending on their size. The use of this method can cause a higher strain to the liposomes and an increased loss of liposomally associated substance, in addition, larger liposomes may settle at the bottom and be removed, which may be an explanation for lower entrapment reported in the above mentioned studies.

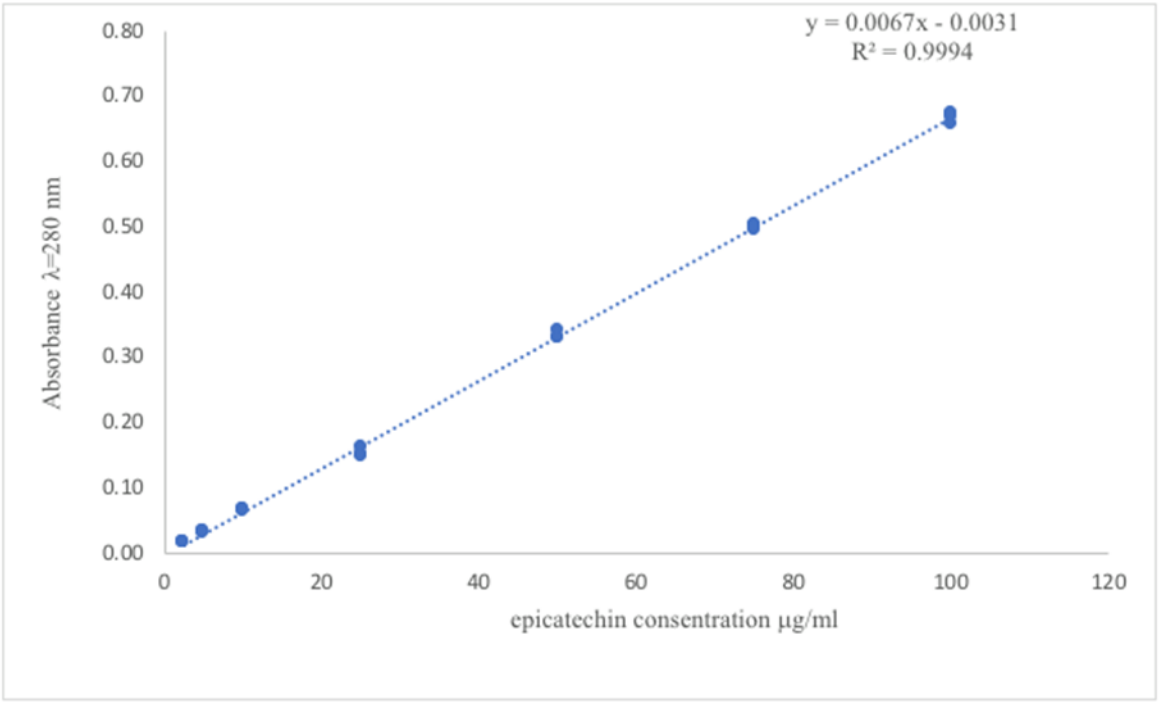


Figure 15: Calibration curve of epicatechin in methanol.

Zeta potential is used to characterize the surface charge of nanoparticles, such as liposomes (Singh and Lillard, 2009). Zeta potential is defined as the electric potential in the interfacial double layer of colloidal systems (Honary and Zahir, 2013). The surface charge of liposomes can be influenced by the liposomal compositions and the medium dispersed in. Positively charged vesicles are expected to interact with the negatively charged mucus layer by electrostatic interactions (Singh and Lillard, 2009). Vesicles with a net charge near neutral may achieve better mucus penetrating properties by avoiding interactions with mucus (Cu and Saltzman, 2008). Our liposomes were composed of PC and EC, and the zeta potential was expected to be neutral as a result of the neutral lipids used in liposome preparation (Pasenkiewicz-Gierula *et al*., 1999). It is expected that neutral liposomes will interact less with chitosan hydrogel (Hurler *et al*., 2013). Our liposomes were slightly negative with a zeta potential of -1.52 ± 2.03 (Table 1); the results were supported by literature (Fang *et al*., 2006).

The slightly negative zeta potential may be caused by a minor lipid oxidation due to exposure to air (Reis and Spickett, 2012).

5.3 Hydrogel characterization

In order to make the liposomal suspension applicable for vaginal administration and prolong their residence time at the site of action, the liposomes was incorporated in a chitosan hydrogel which functions as a vehicle for the liposomes. Prolonged residence time at the site of action improves the therapy by increasing the contact time between the active substance and the administration site (Pavelić *et al.*, 2001). It can also reduce the frequency of administration and contribute to improved patient compliance. Considering that the mucus is negatively charged due to the anionic substructures in the mucus, ionic interaction between the cationic amino groups of chitosan and vaginal mucus can occur (Bernkop-Schnürch and Dünnhaupt, 2012). In this project we used chitosan hydrogel and the overall positive surface charge was confirmed by the measured positive zeta potential (Table 2).

Table 2: Zeta potential of liposomal EC hydrogel.

Type of hydrogel	Zeta potential (mV)
Liposomal EC hydrogel (2.5 % chitosan, 20 % liposomes (w/w))	75.62 ± 1.98

The value is presented as mean ± SD (n=3).

The texture properties of the hydrogels are important parameters for topical formulations, as they will affect the applicability of the formulation and therapeutic efficacy at the administration site (Hurler *et al*., 2012b). To assure that the properties of our hydrogels correlate to the desired properties, we investigated the cohesiveness (Area 1), adhesiveness (Area 2) and the hardness (Force 1) (Figure 11) for different hydrogel formulations containing liposomal EC. The texture properties are presented in Figure 16.

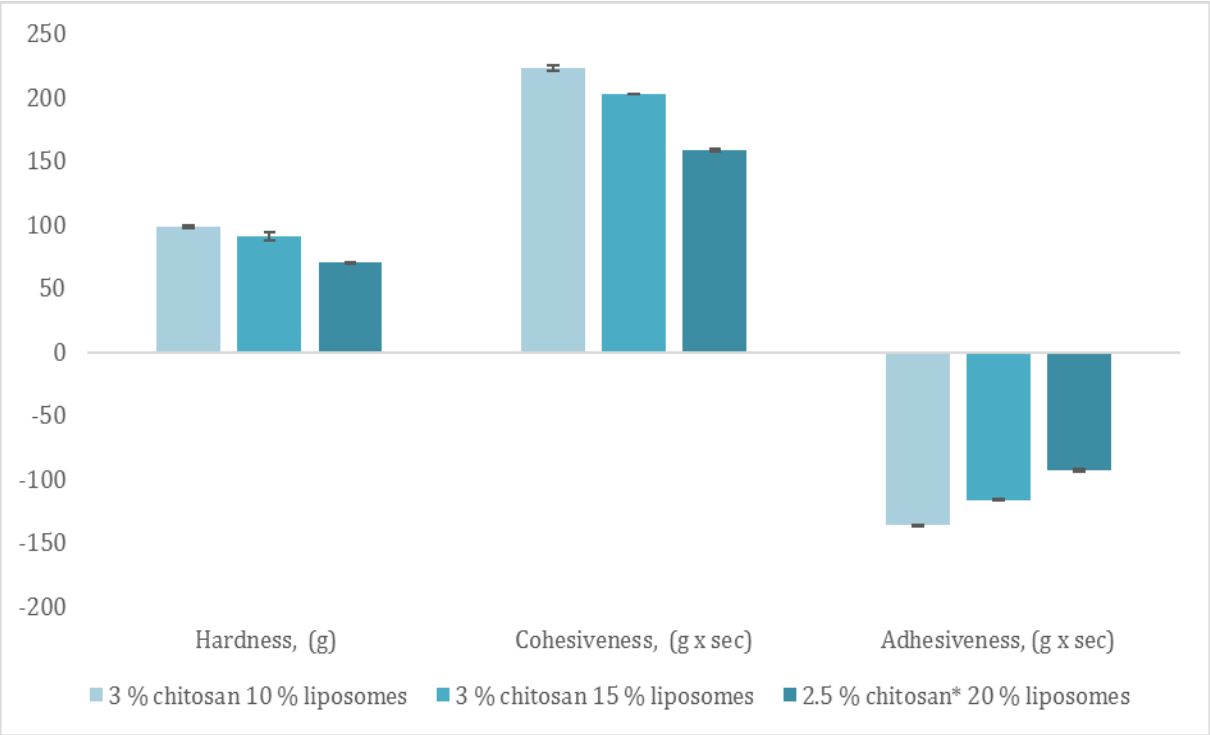


Figure 16: Texture properties of different liposomal EC hydrogels, the values are presented as mean ± SD (n=2). * Final concentration of chitosan (w/w).

Important features of gels are gel cohesiveness, adhesiveness and hardness, considering optimization of the gel properties (Hurler *et al*., 2012b). The adhesiveness together with the cohesiveness give an indication of the retention time at the site of action while the hardness of the formulation indicates the applicability of the gel onto tissue and those are directly correlated to the polymer concentration (Hurler *et al*., 2012b). As presented in Figure 16, the hardness, cohesiveness and adhesiveness increase with increased chitosan concentration. Sezer *et al.* reported similar observations, where the gel adhesiveness, cohesiveness and hardness increased with the increase in chitosan concentration (Sezer *et al*., 2008). However, the experimental setup as well as the compositions were different to those in this project. The hydrogels used for further experiments consisted of 2.5 % (w/w) chitosan and 20 % (w/w) incorporation of liposomal EC although this hydrogel exhibited the lowest values of the tested parameters, the formulation was still considered to be acceptable for vaginal application and further use. Table 3 represent the texture properties of the hydrogels used in further experiments.

Table 3: Texture properties of liposomal EC hydrogel.

Type of hydrogel	Force 1 (g)	Area 1 (g x sec)	Area 2 (g x sec)
Liposomal EC hydrogel (2.5 % chitosan, 20 % liposomes (w/w))	70.15 ± 0.62	158.53 ± 0.89	-93.42 ± 1.33

The values are presented as mean ± SD (n=3).

The preparation method used for the hydrogels was simple and the texture properties indicate that the method produced reproducible hydrogels, as indicated by the small standard deviations.

Hurler *et al.* investigated the influence of glycerol and liposomes when incorporated into a chitosan hydrogel and reported stabilizing effect of glycerol on the chitosan gel structure (Hurler *et al.*, 2012b). To confirm the proposed glycerol effect, we tested hydrogels with different concentrations of glycerol (Table 4).

Table 4: Texture properties of plain hydrogel, liposomal EC hydrogel with different amount of glycerol and control in hydrogel.

Type of hydrogel	Force 1 (g)	Area 1 (g x sec)	Area 2 (g x sec)
Plain hydrogel*	65.09	146.87	- 80.05
Control in hydrogel*** (2.5 % chitosan, 20 % liposomes (w/w))	59.79	134.28	- 69.52
Liposomal hydrogel* (2.5 % chitosan, 20 % liposomes (w/w))	71.01	159.79	- 94.31
Liposomal hydrogel*** (2.5 % chitosan, 20 % liposomes (w/w))	66.29	148.09	- 86.27

The values denote the mean of 5 runs (n=1), * Amount of glycerol in the formulations is 10 % (w/w,) ** EC in acetate buffer. *** Amount of glycerol in the formulation is 5 % (w/w).

Table 4 reports slight differences in the gel properties of the different formulations. Liposomal EC hydrogels exhibited better cohesiveness, adhesiveness and hardness compared to the empty hydrogel and the control in hydrogel. Hurler *et al.* reported similar findings where the addition of liposomal dispersion to a glycerol/chitosan hydrogel increased the cohesiveness, hardness and adhesiveness of the hydrogel (Hurler *et al*., 2012b). Ruel-Gariepy *et al.* reported that incorporation of liposomes into a chitosan-β-glycerophosphate gel expanded the gel strength, however this was concentration-dependent and inclusion of higher amounts of liposomal suspension led to decrease in gel strength (Ruel-Gariepy *et al*., 2002). In order to confirm the effect of the incorporation of liposomes into the hydrogel, more testing is needed. In addition, Hurler *et al.* claimed that glycerol stabilizes the chitosan network (Hurler *et al*., 2012b). Table 4 indicates better cohesiveness, adhesiveness and hardness for the formulation with higher glycerol content. However, more research is needed, including testing over a longer period of time to investigate if the concentration of glycerol will affect the stability of the hydrogel. In addition, several parallels would assist in drawing a conclusion. Our results can therefore be considered as an indication of a trend.

To investigate the stability of chitosan hydrogel a stability test was performed where the texture properties were measured after the storage in the refrigerator (4-8 °C) for one and two months (Table 5).

Table 5: Texture properties of liposomal EC hydrogels (2.5 % chitosan and 20 % liposomes (w/w)) after storage.

Measurements after (n) months	**Force 1 (g)**	**Area 1 (g x sec)**	**Area 2 (g x sec)**
0	70.45 ± 0.52	158.79 ± 1.62	-92.93 ± 2.20
1	66.03 ± 0.30	147.78 ± 1.73	-87.62 ± 2.63
2	63.57 ± 0.95	144.04 ± 2.11	-78.29 ± 3.01

The values denote the mean of 5 runs (n=2).

As shown in Table 5 we could observe a slight decrease in the values from the measurements after one and two months of storage. The decline in the values was not considered critical; however, further investigation is needed especially for gels stored at room temperature. It would be beneficial to include more parallels in the testing.

To obtain an optimal therapeutic effect, the formulation applied to the vaginal mucosa should enable a prolonged residence time. Mucoadhesion is an important parameter for optimization of drug delivery systems, as it can influence substance retention and subsequently, its bioavailability. To mimic the *in vivo* conditions, the barrier used in the experiment was vaginal mucosal tissue from cow, since the cow vaginal mucosa is a good simulation of human vaginal mucosal properties (das Neves *et al.*, 2008). The results are presented as the detachment force in Figure 17; the detachment force is defined as a force needed to overcome the adhesive bonds between the formulation and the mucus. This force can also be correlated to the cohesiveness of the formulation (Hurler and Škalko-Basnet, 2012).

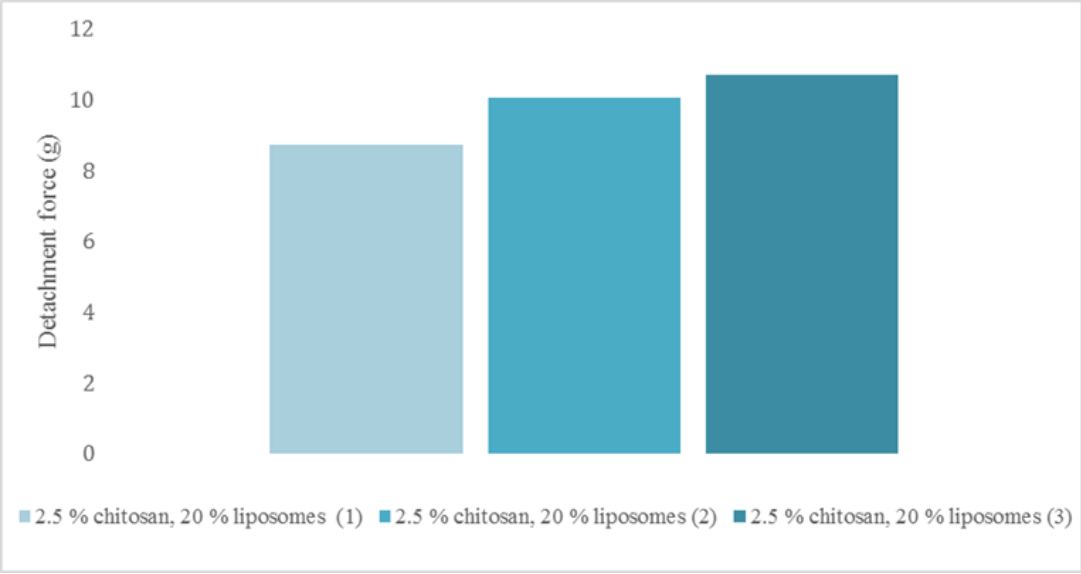


Figure 17: Mucoadhesiveness of three liposomal EC hydrogels determined as detachment force. The figure represent the mean of five runs for each formulation.

The formulations represented in Figure 17 and 18 are liposomal hydrogels prepared in triplicate comprising the same concentration of chitosan and incorporated liposomal EC. Liposomal hydrogel with 3 % chitosan and 20 % liposomes (w/w) was tested only one time and is not included. Figure 17 indicates that the force needed to detach the formulation from the tissue is

relatively similar. Given the similarity of the gel formulations it is expected good reproducibility, however, there are some variation and may be due to the soft vaginal tissue (discussed below). To provide a deeper insight on the mucoadhesion of the formulations, determination of the exact amount retained on the tissue was performed.

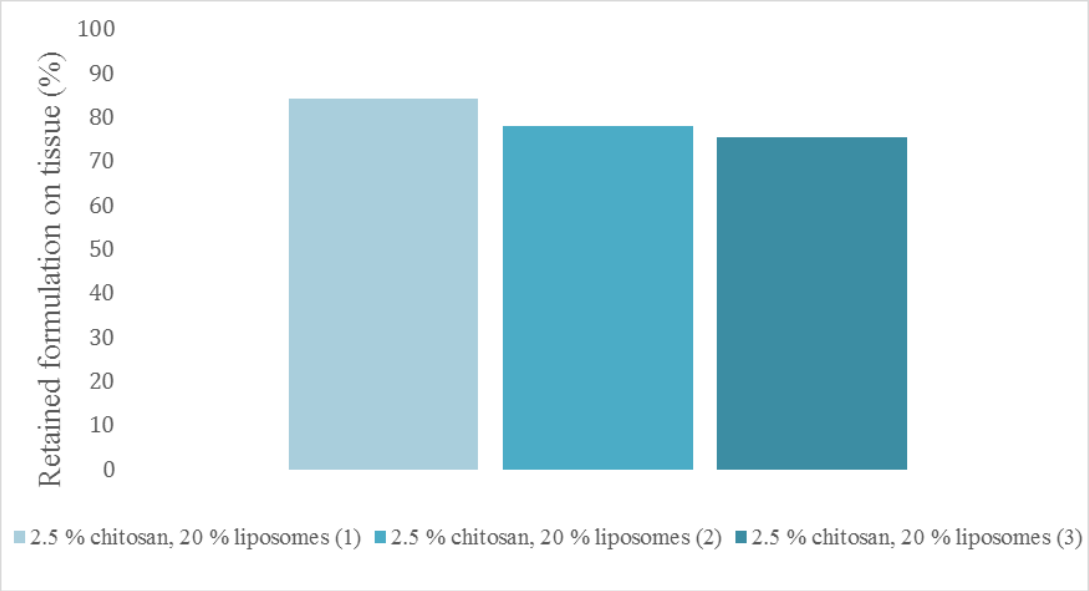


Figure 18: Mucoadhesiveness of three liposomal EC hydrogel formulations determined as the amount of retaining formulation on vaginal tissue. The figure represents the mean of five runs of each formulation.

The amount of formulation retained on the tissue after removing the die/probe is presented in Figure 18. The results indicate that high amounts were retained on the tissue and the results indicate good reproducibility due to only slight variation in the measurements. For the chitosan hydrogels, the detachment forces reported by Hurler and Skalko-Basnet (2012) were similar as our results, however the amounts of formulation retained on the tissue were higher in our study (75 - 85 %) compared to Hurler and Skalko-Basnet (2012) who reported about 50 % of formulation retained on the skin. An explanation of the differences can be attributed to the different tissues used. Skin as a tissue is much more rigid and robust, whereas vaginal tissue is very soft and rather slippery. After each measurement the tissue was rinsed with ethanol and phosphate buffer, which may affect the integrity of the tissue, however, the method is reproducible and the same cow mucosa sample where used up to five times. The results are not entirely close to *in vivo* conditions considering that the experiments were performed in the

absence of VFS. The reason VFS was not applied were due to difficulties in adjusting for the additional liquid. According to das Neves *et al.* the use of VFS is important as it may influence the performance of the test (das Neves *et al*., 2008). However, the time did not permit us to optimize the bioadhesion study and allow for the use of VFS.

Another method tested was with vaginal tissue attached to the probe where the mucosal surface covered the lower end of the probe. The probe with tissue was then lowered until it touched the gel surface on a beaker and then retracted to the starting position. However, the results were not included due to high variations in the detachment force. The instrumental setups for this measurement was developed for studies on skin and might not be optimal for vaginal mucosal tissue (Hurler and Skalko-Basnet, 2012).

5.4 *In vitro* release of epicatechin

In the optimization of a drug delivery system, a controlled release of the substance is an essential parameter. To evaluate the release of EC from liposomal preparations (both as suspensions and suspension-in gels), an *in vitro* study was performed. FDC systems is considered as one of the most appropriate *in vitro* method for evaluating the drug release from different formulations such as those destined for vaginal use (das Neves and Bahia, 2006). The FDC was used in our release studies. As the main focus of the project was localized vaginal drug delivery, the experimental setups were closely mimicking the human conditions; the acceptor medium (pH 4.6) simulated the pH of the healthy vagina (4.5-5.5), and the temperature was set to 37 °C (das Neves and Bahia, 2006). The release profile of EC was evaluated for 8 hours considering that the vaginal discharge, presence of semen or menstruation can contribute to the clearance of formulation from the vagina (das Neves *et al*., 2011a). The release profile of EC through cellophane membrane is presented in Figure 19.

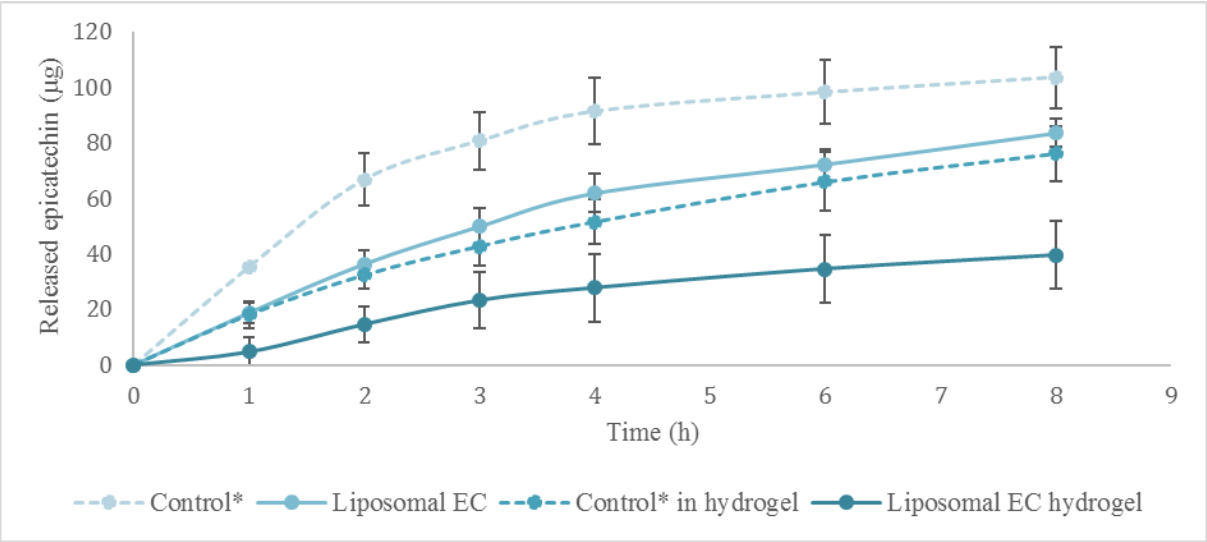


Figure 19: *In vitro* epicatechin (EC) release from different formulations through cellophane membrane (n=3). EC concentration in all formulations was ~120 µg. *EC in acetate buffer.

A more prolonged release would be favourable to reduce frequency of administrations, which is considered favourable for the patient. In order to confirms that the prolonged release is indeed due to the liposomes as carrier system and their incorporation in hydrogel, EC in acetate buffer as well as hydrogel containing EC in buffer were used as the controls. Figure 19 indicates that the release rate of EC from the liposomal hydrogel is slower than from the other formulations, as expected. After 8 hours, the release of EC from our liposomal hydrogel was approximately 40 µg. Additionally, it is essential that the active substances are released in a quantity able to induce a desired therapeutic effect. However, this cannot be evaluated in an *in vitro* study, and would require at least artificial vaginal tissue model. Another interesting finding observed was the release of EC from the control in hydrogel, which was slower than for liposomal EC. This indicates that the hydrogel plays an important role in defining the release profile of entrapped substances, especially in the dual system such as liposomes-in-hydrogel. Liposomes are known to prolong the release of different substances in synergy to hydrogels, however, our findings indicate that the hydrogel is the actual rate limiting step. Considering the ECs structure and logP of 1.8, it is expected that the substance would be preferably retained in the liposomal bilayer due to its lipophilicity and release would be slower than from a hydrogel.

A study conducted by Vanic *et al.* investigated the *in vitro* drug release from liposomal hydrogels under the conditions simulating vaginal environment. The results of the study showed

great potential of liposomal hydrogel as an efficient delivery system for the controlled and sustained release of antimicrobial drugs in the vagina (Vanić *et al*., 2014). Mulik *et al.* investigated the release properties of cytatabine-loaded liposomes incorporated in a gel in comparison with free catatabine in gel. The study confirmed sustained release from the liposomal gel (Mulik *et al*., 2009). Another study by Pavelic *et al.* optimized liposomal drug carrier system, able to provide sustained and controlled release of drug for local vaginal therapy. Liposomes containing model molecule calcein were incorporated in Carbopol gels and calcein *in vitro* release was evaluated. The study confirmed that incorporation of liposomes in Carbopol gels resulted in a novel drug carrier system for vaginal delivery (Pavelić *et al*., 2001).

Many studies indicated that incorporation of liposomes into a hydrogel led to prolonged release of active ingredient, the studies cannot be directly compared due to the different substances used in different delivery systems in addition to the differences in experimental setups. It is rather misleading to directly compare the release of hydrophilic molecules to the release of lipophilic molecules from liposomes. The lower release of EC from liposomal hydrogel may also be contributed to the amount of active substance incorporated into the hydrogel as well as the chitosan concentration. Dragicevic *et al.* reported that increase in polymer concentration can lead to a decrease in the release of liposomally entrapped drug for liposomal carbomer (Carbopol) hydrogels (Dragicevic-Curic *et al*., 2009).

5.5 *Ex vivo* penetration of epicatechin

After assuring that the liposomal formulation is able to release EC for potential penetration, the next focus was on the penetration profile of EC, in respect to the time. Thus, the next step was to determine *ex vivo* penetration of EC through a vaginal mucosal tissue to get a closer insight on the possible *in vivo* situation. To determine the *ex vivo* tissue penetration of EC, FDC was used as this method is well known to be suitable for this purpose (das Neves *et al*., 2013; Jøraholmen *et al*., 2015). When aiming for local treatment at the vaginal site a limited absorption of the substance is desirable; assuring that a higher drug concentration at the vaginal site is achieved. To closer mimic the *in vivo* conditions, the barrier used for the experiment was vaginal tissue from sheep, as the sheep vagina is rather similar to human vagina (Holt *et al*., 2015). Sheep vaginal epithelium is stratified squamous tissue comparable to the human (Moss *et al*., 2012). The experimental setup was the same as for *in vitro* EC release, except for

the acceptor chamber where the medium was phosphate buffer simulating the pH in blood (pH 7.4). To further mimic *in vivo* conditions, the VFS was included in the donor chamber.

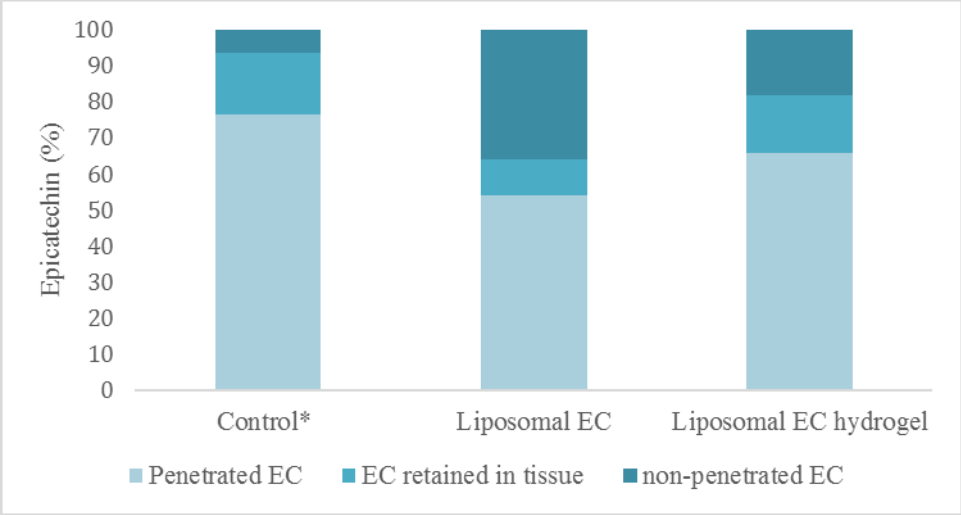


Figure 20: Epicatechin (EC) distribution (%) in *ex vivo* studies (8 hours) on sheep vaginal tissue (n=2). * EC in acetate buffer.

In the *ex vivo* penetration studies, three formulations were included; liposomal EC hydrogel, liposomal EC suspension and control EC solution (EC in acetate buffer). Figure 20 indicates that EC from the control had the highest amount of penetration through the tissue relative to the other formulations, possibly due to the lack of a release controlling vehicle. The amount penetrated from liposomal EC were approximately around 50 % and lower than expected considering that from the *in vitro* release study, where a higher amount of EC was released relative to the other formulations. Liposomal EC had the lowest amount of EC penetrated compared to the other formulations. In addition, non-penetrated EC was the highest for the liposomal EC.

Figure 20 indicates a higher amount of EC penetrated from the liposomal hydrogel formulation as compared to liposomal EC. An explanation for this can be attributed to chitosan as it is known that chitosan has permeation enhancing properties and may act on the sheep tissue through decomposition of the tight junctions (Bernkop-Schnürch and Dünnhaupt, 2012), allowing EC penetration. In addition, the amount of EC retained in the tissue and non-penetrated EC were higher. A higher amount retained in the tissue for the liposomal hydrogel is considered favourable regarding the non-invasive treatment of HPV. Considering that local

HPV treatment should reach deeper into the vaginal mucosal tissue and obtain a prolonged release within the affected tissue, while avoiding high systemic levels (Foldvari, 2012), our findings are encouraging.

In order to evaluate the differences in the EC penetration from different formulations an independent *t*-test was performed for all formulations and the determined amount of penetrated EC (%). The *t*-test indicated that liposomal EC and liposomal EC hydrogel showed significantly reduced penetration of EC compared to the control.

A control experiment with pure phosphate buffer (pH 7.4) in the donor chamber was performed under the same experimental setup to eliminate interference of tissue in the measurements. The results from this experiment indicated that even where there was no EC present (buffer alone as control), and those readings were taken into account when determining the EC concentration.

In order to come closer to the evaluation in *in vivo* conditions, further investigation is needed, including the anti-inflammatory potentials, safety and toxicity studies.

6 Conclusions

The present research was focused on the development of a liposomal EC hydrogel for local vaginal therapy. EC exhibited strong antioxidative effects, as confirmed by its $ABTS^{+\bullet}$ and DPPH scavenging activity and was found to be superior to both vitamin C and vitamin E at lower concentrations. Liposomes prepared by the thin film method were shown to incorporate high amount of EC; by applying extrusion as a size reduction method we were able to prepare rather homogenous liposomes in the desired size range. Liposomal EC hydrogel exhibited acceptable textural properties along with mucoadhesive properties enabling prolonged retention time at vaginal site. In addition, the formulations were shown to provide prolonged release both for liposomal EC and liposomal EC hydrogel, which is favourable for vaginal application. EC may be effective in prevention and treatment of vaginal infections, however further investigation is needed. The *ex vivo* penetration study indicated that EC penetrated to some extent into and through vaginal mucosal tissue, however, more studies and optimization of the method are required.

7 Prospective

Further optimization of *ex vivo* mucoadhesion measurements for semisolid formulations applied to mucosal tissues are required, as there are no validated methods available so far. Further, several testing conditions that closely mimic the vaginal environment, such as temperature, pH and the presence of vaginal fluid simulant or semen simulant should be investigated as it will affect the mucoadhesiveness of the formulation destined for vaginal site. In addition, stability testing at room temperature conditions for liposomal EC and liposomal EC hydrogel should be carried out, as well as the stability in vaginal environment. The method used to determine amount of EC in *ex vivo* penetration studies should be optimized due to the interference from the tissue. However, our data can serve as a very good starting point in further optimization.

Evaluation of anti-inflammatory and antimicrobial properties of EC is necessary and further investigation is needed. In addition, evaluation of cellular toxicity of liposomal EC and liposomal EC hydrogel should be performed. Moreover, the possible interference of novel delivery system on the vaginal flora should be performed to prove its safety. Finally, *in vivo* animal studies in the model of vaginal infection should be performed.

8 References

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