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Liposomes-in-hydrogel delivery system containing resveratrol for the local treatment of vaginal infections

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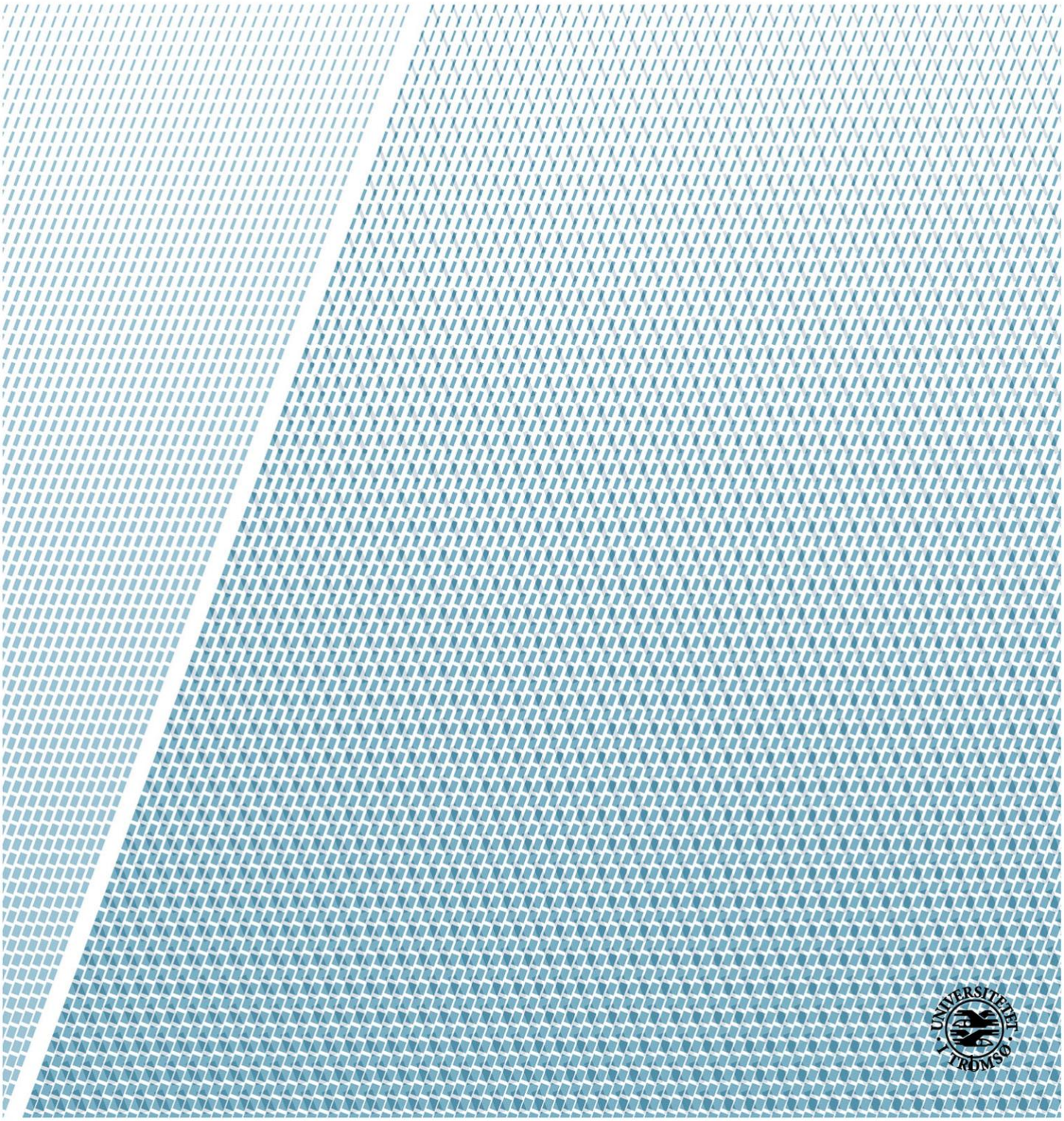

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Sammendrag

Vaginal administrasjon av legemiddelformuleringer er utfordrende på grunn av den selvrensende prosessen i vagina, som gjør at fremmede partikler blir fanget og hurtig fjernet. Dette medfører ofte at oppholdstiden for legemidler er for kort til at en klinisk effekt kan oppnås. Hensikten med dette prosjektet var å utvikle og optimalisere et liposomer-i-hydrogel system som inneholder resveratrol ment for lokalbehandling av vaginale infeksjoner. Resveratrol er en naturlig forekommende forbindelse, som finnes i blant annet druer, bær og peanøtter. Det er knyttet en rekke gunstige effekter til resveratrol, og spesielt interessant er det at forbindelsen har vist seg å ha en potensiell terapeutisk effekt mot *Chlamydia trachomatis*. Det er dessverre vanskelig å utnytte disse gunstige kliniske effektene av resveratrol i vaginal administrasjon. Resveratrol har dårlig vannløselighet og er svært lyssensitiv. For å overvinne disse utfordringene ble resveratrol innkapslet i liposomer. Og for å kunne forlenge virketiden til resveratrol i vagina, ble resveratrol-liposomene inkorporert (10 %, w/w) i kitosan hydrogeler (2.5–3 %, w/w). Kitosan er en polymer som stammer fra skjell av krepsdyr, insekter og sopp. Det er en svært attraktiv polymer på bakgrunn av dens kjente slimhinneklebende egenskapene.

Liposomene ble fremstilt i henhold til «conventional film» metoden. For å oppnå liposomer med ønskelig størrelse (~200 nm), ble de ekstrudert gjennom polykarbonatmembraner. Egenskapene til liposomene ble karakterisert i henhold til mengde resveratrol i liposomene, zeta-potensiale, størrelse og størrelsesfordeling. For å kunne optimalisere kitosan hydrogelene ble teksturegenskapene, zeta-potensiale og slimhinneklebende egenskaper karakterisert. For å bekrefte den forlengede frisettingen av resveratrol fra formuleringen ble det utført en *in vitro* resveratrol frisettingsstudie og en *ex vivo* penetreringsstudie. Resveratrol er også kjent for å ha antioksiderende egenskaper, og for å bekrefte dette ble «radical scavenging assay» utført. Effekten av resveratrol ble sammenlignet med de kjente antioksidantene vitamin C og vitamin E.

Nøkkelord: Vaginale infeksjoner, Vaginal administrasjon, Resveratrol, *Chlamydia trachomatis*, Liposomer, Kitosan hydrogel

Abstract

Administering drug formulations to the vagina is a challenge since the vagina possesses "self-cleaning" features which allow trapping and rapid removing of foreign particles. Therefore, the residence time of drugs is often too short to offer a sufficient clinical effect. The aim of this project was to develop and optimize a liposomes-in-hydrogel system containing resveratrol for the local treatment of vaginal infections. Resveratrol is a natural occurring compound which could be found in grapes, berries and peanuts. It possesses a number of beneficial effects, and of special relevance is that it has shown to have a potential therapeutic effect against *Chlamydia trachomatis*. However, there are challenges related to resveratrol which cause difficulties to exploit the favorable clinical effects of it in vaginal delivery. Resveratrol is poorly water soluble and highly sensitive to the exposure of light. To overcome those limitations, resveratrol was encapsulated in liposomes. Moreover, to prolong the resveratrol residence time in the vagina, the resveratrol-liposomes were incorporated (10 %, w/w) in chitosan hydrogel (2.5–3 %, w/w). Chitosan is a polymer which originates from exoskeleton of crustaceans, insects and fungi. The polymer is very attractive considering its known mucoadhesive properties.

Liposomes were prepared by the conventional film method. To obtain liposomes in the desired size (~200 nm), the liposomes were extruded through polycarbonate membranes. The properties of the liposomes were characterized in respect to entrapment efficiency, zeta potential, size and polydispersity. To optimize the chitosan hydrogels, hydrogels were characterized in regard to texture properties, zeta potential and mucoadhesive properties. The formulation was also tested to confirm the prolonged release profile by performing an *in vitro* resveratrol release study and an *ex vivo* penetration study. Resveratrol is also known to have antioxidant properties, and this was confirmed by radical scavenging assay. The radical scavenging activity of resveratrol was compared to the known antioxidants vitamin C and vitamin E.

Keywords: Vaginal infections, Vaginal delivery, Resveratrol, *Chlamydia trachomatis*, Liposomes, Chitosan hydrogel

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List of abbreviations

ABTS	2,2´-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt
C. trachomatis	*Chlamydia trachomatis*
d-water	Distilled water
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EtOH	Ethanol
GUV	Giant unilamellar vesicles
HPLC	High performance liquid chromatography
Lipoid s100	Approximately 100 % phosphatidylcholine from soybean
LUV	Large unilamellar vesicles
MeOH	Methanol
MLV	Multilamellar vesicles
NPs	Nanoparticles
PCS	Photon correlation spectroscopy (Particle size distribution)
PI	Polydispersity index
PID	Pelvic inflammatory disease
RES	Resveratrol
STDs	Sexually transmitted diseases
SUV	Small unilamellar vesicles
UV	Ultra violet (Spectroscopy)

1 General introduction

Vaginal infections are relatively common, and it is reported that more than 80 % of women will experience some kind of vaginal infection during their lifetime (Wong *et al.*, 2014). These infections include bacterial infections, viral infection, fungal infection and sexually transmitted infections (Spence and Melville, 2007). It is common to distinguish between sexually transmitted infections and non-sexually transmitted infections. It is reported that approximately 18.9 million peoples will acquire a new sexually transmitted infection each year and the most common bacterial sexually transmitted infection is *Chlamydia trachomatis* (Malhotra *et al.*, 2013; Nardis *et al.*, 2013).

Today most of the bacterial vaginal infections are treated with different types of antibiotics which are chosen based on the type of pathogen (Palmeira-de-Oliveira *et al.*, 2015). Antibiotics has over decades saved a lot of human lives, however the pathogens are again starting to become a threat for the humanity with the development of antibiotic resistance (Ventola, 2015). Therefore, it is of huge interest to find new agents with antimicrobial effect. Resveratrol is a natural substance that shows great potential since it is found to have a phytoalexin properties and a potential effect against *Chlamydia trachomatis* development (Summerlin *et al.*, 2015; Petyaev *et al.*, 2017).

Currently, the oral route of administration is most common because it is well-known, convenient, and cost-effectiveness. However, for an orally administrated dose there are challenges linked to first-pass metabolism, gastrointestinal-related side effects, and interaction between co-administrated drugs/active ingredients. Thus, there is an interest to develop formulations that could be administered locally, especially for substances that are poorly absorbed when administered orally (Sosnik and Augustine, 2016).

The vaginal physiology is well known, however, it still remain challenges linked to the delivery of drug/active substance by the vaginal route (Hussain and Ahsan, 2005). The vagina has been used for the delivery of local acting agents such as steroids, antibacterial agents, antifungal agents, antiviral agents, contraception and labor-inducers. The vagina also shows great potential as site of administration for systemically acting agents. This is due to the rich blood supply and the large surface area (Baloglu *et al.*, 2009; Knuth *et al.*, 1993). Other favorable properties of vaginal administration of drugs are the capability of lowering drug doses, low systemic side effects, a non-invasive approach and avoidance of first pass metabolism in the liver (Wong *et*

al., 2014). However, our aim was to develop the formulation for localized delivery of resveratrol to vagina.

Today there are different conventional pharmaceutical formulations approved on the marked, these includes capsules, tablets, pessary, solutions, creams, tampons, emulsions and vaginal rings (Wong *et al.*, 2014; das Neves and Bahia, 2006). However, these formulations have several limitations due to challenges associated to vaginal administration (Valenta, 2005). The available formulations suffer from leakage, low residence time and tissue irritation (Valenta, 2005; Wong *et al.*, 2014). The administration route is also highly gender specific and there are difference between women (Vermani and Garg, 2000). The physiological environment of the vagina is non-constant and is affected by the age and the menstrual cycle. Further, the presence of disease or semen might also affect the formulations applied locally into vagina.

One approach to cope with these challenges is the development of novel drug delivery systems based on nanomedicine and drug carriers. It will provide the opportunity to design more potent therapeutics with less toxicity (Vanić and Škalko-Basnet, 2013). The novel systems must be safe and non-irritating to the vaginal mucosa (Woodrow *et al.*, 2009).

Liposomes are vesicles that consist of one or more phospholipid bilayer, with an aqueous inner core (Sercombe *et al.*, 2015) and they have the potential as pharmaceutical carriers for vaginal administration. Liposomes allow the entrapment of molecules based on physiochemical properties. There are stability issues linked to resveratrol, therefore, liposomes could be a suitable drug delivery system to increase the stability of resveratrol and make the compound more suitable for therapeutic application. However, from the vaginal administration point of view, it could be a challenge to use liposomes in its pure form because of the liquid nature of the formulation. It is also a challenge that the vagina is capable to "self-clean" and thereby remove the pharmaceutical molecules before an therapeutically effect is achieved (Pavelić *et al.*, 2001). To maintain a long enough retention time at the site of action and thereby improve the efficacy of the formulation the solution could be to use a vehicle that has mucoadhesive properties (Pavelić *et al.*, 2001). Hydrogels made of polymers that possess mucoadhesive properties is one approach to implement mucoadhesive properties to a system intended for vaginal application. Commonly used polymers are polyacrylic acid derivates such as carbomer, and cellulose derivates such as chitosan (Caramella *et al.*, 2015).

2 Introduction

2.1 The vagina

The vagina is a part of the female reproductive system and serves an important role in reproduction, outlet for menstrual blood and child birth. The vagina is located from cervix at the uterus, between the urinary bladder, the urethra and rectum (Figure 1) (Baloglu *et al.*, 2009). The anatomy and physiology of the vagina differ in women before puberty, at reproductive age and postmenopausal women.

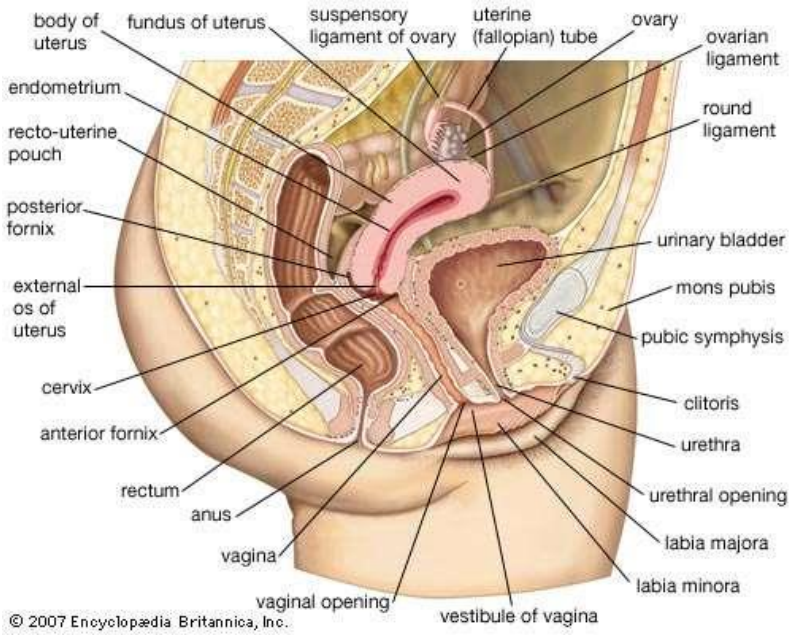

Figure 1: Illustration of location of the vagina, Enclopædia Britannica, 2007

For a healthy woman, the pH of the vagina normally ranges from 4–7. The length is between 4.5–10 cm, and it is 1.5–2.5 cm in diameter (Sahoo *et al.*, 2013). The pH changes with age, sexual activity, menstrual cycle and can be influenced by disease. Microbes are naturally occurring in the vagina, and functions as a first line defense against unwanted microbes. The vaginal flora is dominated by lactic-acid producing *Lactobacillus* spp. which contributes to the acidic pH in the vaginal environment and protects against infection (Smith and Ravel, 2017).

The wall of the vagina is composed by four layers: Stratified squamous phylum, lamina propria, muscular layer, and adventicia (Figure 2) (das Neves and Bahia, 2006).

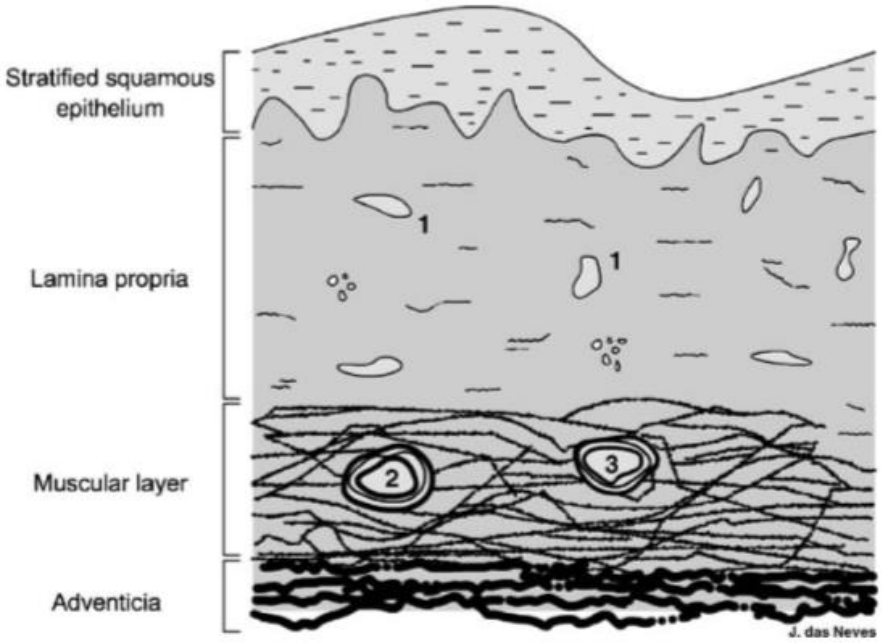

Figure 2: The composition of the different layers of the vaginal wall; (1) capillary vessels, (2) artery and (3) vein. Rreprinted from das Neves and Bahia, 2006 copyright © with permission from RightsLink®

The mucosal layer is composed of folds called rugae which provides distensibility and increase the surface area of the vagina (Alexander *et al.*, 2004).

2.1.1 Vaginal mucus

Even though there are no secretory glands localized in the vagina it is still referred to as mucosal tissue. The fluids present in the vagina originates from different sources, primarily transudate from vaginal and cervical cells, and composes a moist surface film (Vanić and Škalko-Basnet, 2013; Palmeira-de-Oliveira *et al.*, 2015). The vaginal mucus consists of a network of 1–2 % mucin fibers and about 95 % water and function as a barrier towards foreign particles (Wong *et al.*, 2014). The mucus is semipermeable and the stickiness and the viscosity of the mucus make it suitable for trapping and rapidly clearing particles away, however, still allowing nutrients and gasses to enter the underlying epithelium.

2.2 Vaginal drug delivery

There are different groups of drugs that can be administrated through the vaginal route like antibiotics, antifungal, labor inducers, hormones, steroids and antiviral agents (das Neves and Bahia, 2006). This is mainly for the local treatment, however, because of the rich blood supply and a large surface area of the vagina, it also shows good potential for systemic delivery of drugs (Baloglu *et al.*, 2009). It is known that small molecules between 0.2–0.5 μm are able to penetrate mucus and reach the underlying epithelium (Figure 3) (Wong *et al.*, 2014; das Neves *et al.*, 2011). Smaller particles (~100 nm) are able to diffuse through smaller pores in the mucus network, however, this will often trap the particles, thereby preventing the particles from reaching the underlying epithelium. When comparing microparticles with adhesive and non-adhesive properties it has been shown that the adhesive particles would be able to prolong the residence time but the diffusion properties through the mucus is reduced because of bonds that forms between the adhesive and the mucus (das Neves *et al.*, 2011).

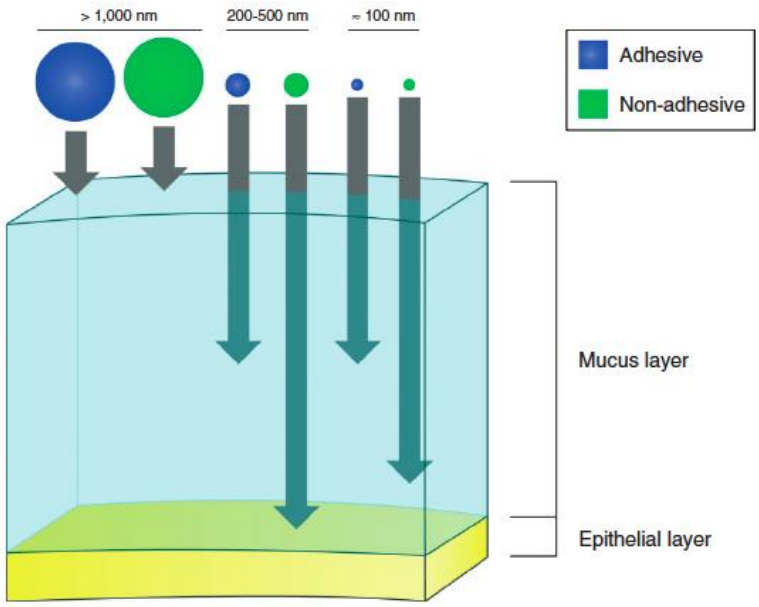

Figure 3: Particles ability to penetrate vaginal mucus based on size and adhesive properties. Reprinted from das Neves et al., 2011 copyright © with permission from RightsLink®

Most substances use diffusion as permeation mechanism. Hydrophilic substances are often absorbed extracellular, through pores in the vaginal mucosa, while hydrophobic substances are absorbed through an intracellular mechanism (das Neves and Bahia, 2006). The vaginal route of administration is of interest due to the avoidance of hepatic first pass metabolism, avoidance of gastrointestinal-related side effects, possibility of reduced administration frequency, easy to

use, discreet and low interaction potential with other medications (Wong *et al.*, 2014). There is also a possibility for enhanced patient compliance as vaginal administration allows longer intervals between doses compared to an oral given dose (Srikrishna and Cardozo, 2013). However, there are challenges linked to this route of administration. The hormone levels are not constant and will differ during the menstrual cycle. This will affect the thickness of the epithelial cell layer, secretions and pH, which will influence the effect of administered drug. The therapeutic effect is also affected by inadequate spreading of drug, low residence time, leakage from the dosage form, local irritation, and low water solubility of drug (Wong *et al.*, 2014). Further, the amount of vaginal fluid present may affect the absorption of poorly soluble drugs and it will also effect the removal of drug from the vaginal cavity (Sahoo *et al.*, 2013).

2.2.1 Formulations for vaginal application

Conventional vaginal delivery systems have been formulated in form of tablets, suppository, cream, capsules, ointments, gels, film, solutions, pessaries and foam (Wong *et al.*, 2014). Gels are often preferred because they show good properties to spread over the vaginal surface and have the potential to relieve dryness and discomfort due to the high water content which will lubricate (Palmeira-de-Oliveira *et al.*, 2015). However, the efficacy of these formulations is often limited by the short residence time at the site of action due to the "self-cleansing" properties of the vagina (Pavelić *et al.*, 2001; Pereira and Bruschi, 2012) Therefore, there is a need for novel formulations for vaginal drug delivery. There have been used drug carriers to improve the efficacy of vaginal administered drugs, these either uses mucoadhesion or mucopenetration as strategies to enhance the efficacy (Schattling *et al.*, 2017). In mucopenetrating systems there are used delivery systems that are able to penetrate deeper into the vaginal epithelium compared to mucoadhesion systems (Netsomboon and Bernkop-Schnürch, 2016). In mucoadhesion systems there are used polymers which have a bioadhesive effect, and these are made to overcome the drawbacks from conventional formulations among other low residence time and leakage (further discussed in the mucoadhesion chapter) (Srikrishna and Cardozo, 2013).

2.3 Vaginal infections

The vaginal mucosa serves as a possible portal of entry for different microorganism, that could lead to infections both locally and systemically (Nardis *et al.*, 2013). Vaginal infections are relatively common, and about 80 % of women will experience some type of infection during they lifetime (Wong *et al.*, 2014). It is also the number one reason for women to seek medical treatment. There are different kind of conditions that could affect the vaginal tract, such as fungal infections, bacterial infections, viral infections and sexually transmitting diseases (STDs) (Ensign *et al.*, 2014). It is common to distinguish between non-sexually transmitted infections (bacterial vaginosis, *Candida* infections) and sexually transmitted infections (*Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, *immunodeficiency virus, human papillomavirus and herpes simplex virus* (Spence and Melville, 2007; Nardis *et al.*, 2013).

High mortality rates are not observed for vaginal infections, but they are associated with reduction in quality of life (Palmeira-de-Oliveira *et al.*, 2015). Inadequate or lack of treatment may lead to persistent symptoms and infection (Palmeira-de-Oliveira *et al.*, 2015). Asymptomatic non-sexually transmitted infections do not always acquire treatment, however sexually transmitted infections need treatment because untreated conditions could result in severe, long-term complications especially for women (Spence and Melville, 2007; Nardis *et al.*, 2013).

It is reported that approximately 18.9 million people will acquire a new STD each year and 48 % of these are young people in the age group of 15–24 (Nardis *et al.*, 2013). There are more than 30 different types of microbes that could be sexually transmitted, and the most common bacterial STD is *Chlamydia trachomatis* (*C. trachomatis*) (Malhotra *et al.*, 2013; Nardis *et al.*, 2013). *C. trachomatis* is an intracellular, non-motile, gram-negative bacterium (Black, 1997; Malhotra *et al.*, 2013). Infected individuals are often asymptomatic or experience minimal symptoms of infection (Malhotra *et al.*, 2013; Black, 1997). When symptoms are reported it is most often vaginal discharge and dysuria (Black, 1997). Untreated infection can cause pelvic inflammatory disease (PID), ectopic pregnancy and even infertility. Also, a concern is an increased risk of transmission of HIV (Malhotra *et al.*, 2013; Spence and Melville, 2007). *C. trachomatis* could also affect the eyelids (trachoma) and causing pulmonary infections (Petyaev *et al.*, 2017). Today *C. trachomatis* is treated orally by doxycycline or azithromycin (Spence and Melville, 2007). Due to the possible threat of antimicrobial resistance, it is of interest to

find new compounds that shows effect against different infections (Petyaev *et al.*, 2017). Resveratrol (RES) is one such compound which has gained a lot of attention in recent years because of the numerous beneficial clinical effects that the compound possesses (Arora and Jaglan, 2017).

2.4 Resveratrol

RES with the chemical name 3,5,4´-trihydroxy-trans-stilbene, is a natural occurring polyphenol in the stilbene family (Figure 4). It was first isolated from the roots of *Veratrum grandiflorum* in 1940´s (Arora and Jaglan, 2017). RES is produced by many plants as a response to stress and it is also known to be a phytoalexin, which means that antibiotic compounds are synthesized by plant as a response to infection (Chauhan, 2015; Summerlin *et al.*, 2015; Veech, 1982). In the human diet, RES can be found in among other grapes, berries and peanuts (Karthikeyan *et al.*, 2013).

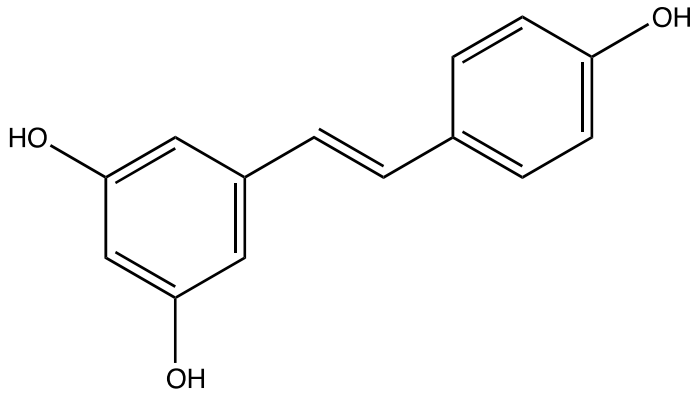

Figure 4: Chemical structure of resveratrol

RES is sensitive to light and unstable at high temperatures (Abba *et al.*, 2015; Chauhan, 2015). When the *trans*-isomer is exposed to light about 80-90 % of the *trans*-isomer gets easily transformed to the more unstable *cis*-isomer (Amri *et al.*, 2012; Arora and Jaglan, 2017). This is unwanted since it is the *trans*-isomer that has shown to have biological activity (Amri *et al.*, 2012). RES is a lipophilic compound, with a log P value of 3.1 (Amri *et al.*, 2012), and can be classified as a class-II compound, according to the Biopharmaceutical Classification system (Amri *et al.*, 2012). Hence, it shows high permeability properties, but low solubility. The water

solubility is <0.001 mol/L, and this will affect the solubility in biological fluids (Bonechi *et al.*, 2012).

2.4.1 Resveratrol in drug delivery

RES has been found to have a numerous beneficial clinical effect (Figure 5). It is reported that RES could exhibit positive effects against cancer, heart diseases, diabetics, pathological inflammation and viral infections (Baur and Sinclair, 2006). RES also has shown to have antimicrobial activity against various bacteria- and fungi-species that could cause vaginal infection and of specially importance it has shown to have effects against the growth of *C. trachomatis* (Pangeni *et al.*, 2014; Jøraholmen *et al.*, 2015; Petyaev *et al.*, 2017).

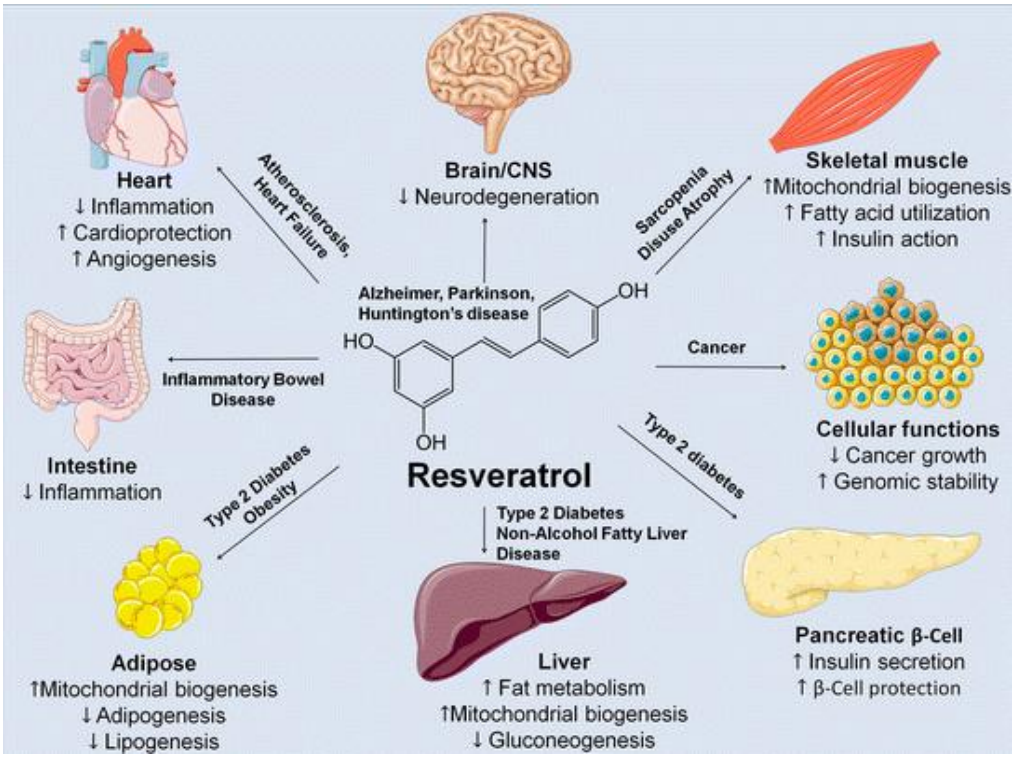

Figure 5: Clinical application of resveratrol. Reprinted from Arora and Jaglan, 2017 copyright © with permission from RightsLink®

Today, RES is marketed as a nutritional supplement (Summerlin *et al.*, 2015). Toxicity studies concluded that it is mostly is well-tolerated, and no severe adverse effects are reported. However, *in vivo* testing is needed to see the long-term effect of use (Cottart *et al.*, 2010). Even though RES shows great pharmacological properties, the limitations linked to the physiochemical properties remains a challenge. Therefor there is a need of a suitable drug

delivery system, such as liposomes, to increase the stability of RES and make it more suitable for therapeutic application (Jøraholmen *et al.*, 2015).

2.5 Nanoparticles as drug carriers

Nanoparticles (NPs) are regarded as particles with size ≤100 nm (De Jong and Borm, 2008). However, larger particles may be needed to load an effective amount of drug for therapeutic application. Therefore, from a pharmaceutical perspective NPs can be viewed as systems in the size range of 10-1000 nm (De Jong and Borm, 2008). NPs and nanotechnology are of interest in the pharmaceutical field due to their large functional surface area and their ability to carry drug molecules. NPs can be used to deliver drug molecules through different administration routes such as oral, vaginal, pulmonary, intraocular, transdermal and parenteral (De Jong and Borm, 2008; Yildirimer *et al.*, 2011). When drug molecules are incorporated in NPs, based on the chemical properties, they can be protected from degradation, undesirable interaction with the environment, and used in targeted drug delivery. Properties that are of interest for NPs is size, shape and surface properties. These properties can be modified to make NPs that can overcome, or "hide" from the natural degradation that take place *in vivo*. By modifying the surface properties of the NPs, they can obtain properties that would prolong the circulation time, enable drug targeting or controlled drug release. Another favorable property of NPs is that a lower amount of the active pharmaceutical ingredient can be used to obtain a therapeutic effect, thereby reducing side effects. There are different types of NPs-systems, such as liposomes, dendrimers, micelles, solid lipid nanoparticles and quantum dots (Mudshinge *et al.*, 2011; De Jong and Borm, 2008).

In the next section, liposomes that are of relevance of this master thesis, will be displayed in further details.

2.5.1 Liposomes

Liposomes can be defined as spherical shaped vesicles, consisting of one or more phospholipid double layers, with an aqueous inner core (Figure 6). The size of liposomes can range for 30 nm to serval micrometers (Akbarzadeh *et al.*, 2013).

Based on size and lamallarity it is common to distinguish between multilamellar vesicles (MLV, 0.1–10 μm) and unilamellar vesicles. Unilamellar vesicles can be further divided into small (SUV, <100 nm), large (LUV, 100–500 nm) and giant (GUV, ≥1 μm) (Ulrich, 2002).

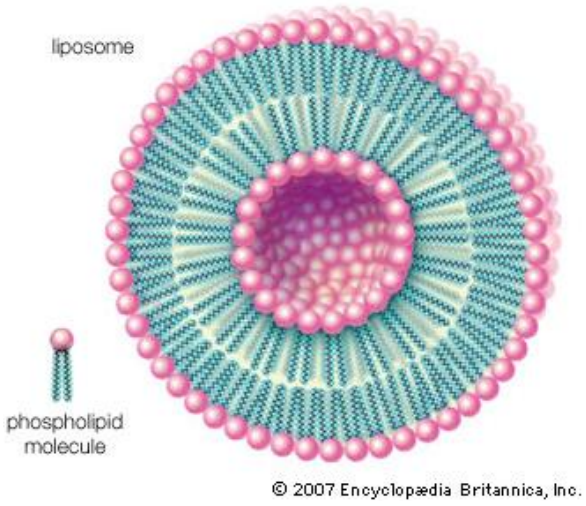

Figure 6: Illustration of the structure of liposomes, Enclopædia Britannica, 2007

The phospholipid molecule has a hydrophilic head group consisting of esterified phosphorylated alcohol, that can have both neutral, positive and negative net charge at pH 7 (Figure 7). The phospholipids do also have a glycerol backbone and two hydrophobic tails, consisting of fatty acids. The tail groups do not contribute to the charge but can consist of different lengths and saturation. Usually the length is between 14 and 18 carbon atoms. Commonly used phospholipids are phosphatidylcholine, phosphatidylserine and phosphatidylglycerol (Kraft *et al.*, 2014).

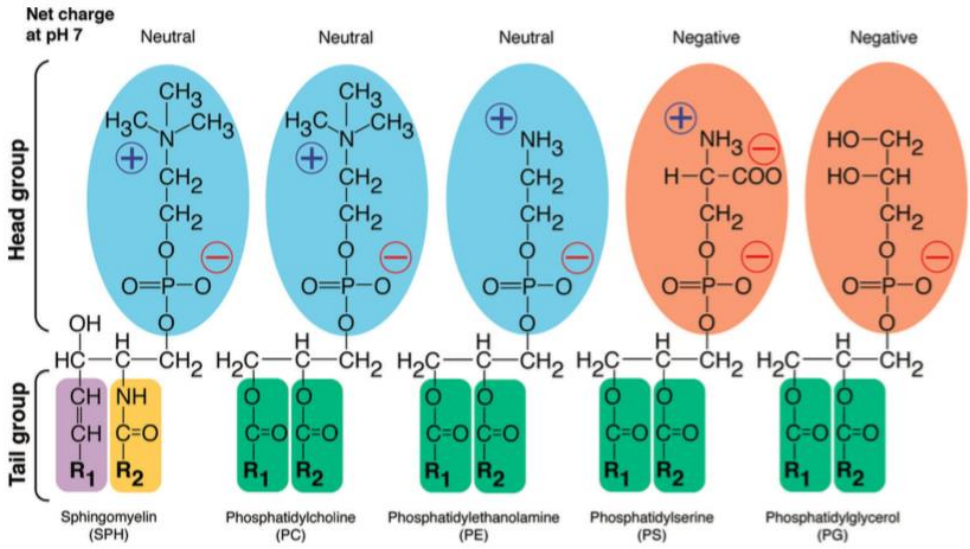

Figure 7: Illustration of composition of phospholipids. Reprinted from Kraft et al., 2014 copyright © with permission from RightsLink®

2.5.2 Liposomes as drug delivery system

Due to the amphiphilic properties of the phospholipids, the liposomes can function as drug carriers for both hydrophilic and hydrophobic drug molecules, where lipophilic molecules are incorporated in the phospholipid bilayer and hydrophilic molecules encapsulated in the core. Liposomes protect the drug from degradation and early inactivation and can thereby prolong the circulation time and bioavailability of the drug molecules. Liposomes are characterized by size, charge, lipid composition, number of lamellae and surface properties (Akbarzadeh *et al.*, 2013).

Liposomes for targeted drug delivery can be classified into four different types: Conventional liposomes, sterically-stabilized liposomes, ligand-targeted liposomes and a combination (Figure 8) (Sercombe *et al.*, 2015).

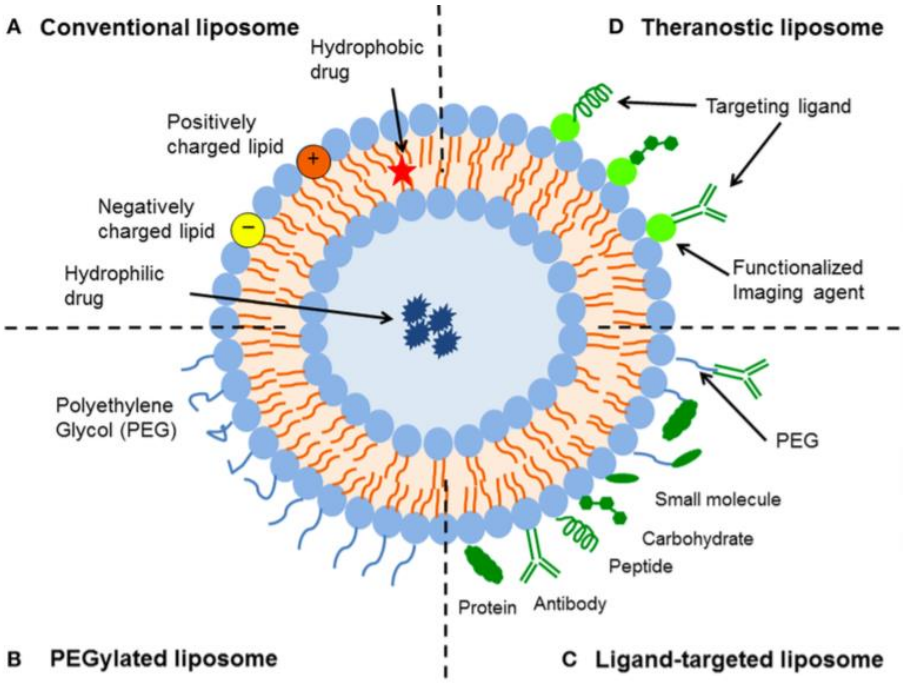

Figure 8: Illustration of different types of surface-modification of liposomes for targeted drug delivery. Reprinted from Sercombe et al., 2014.

In this thesis, conventional liposomes with RES incorporated were made. Due to the self-cleansing action of the vagina, conventional liposomes might be rapidly removed, resulting in reduced therapeutic effect (Pavelić *et al.*, 2001). Therefore, it is necessary to make a system for the RES-liposomes, to prolong the retention time in the vagina. One approach is to incorporate the liposomes in a mucoadhesive hydrogel.

2.6 Mucoadhesive drug delivery system

Mucoadhesion means that bonds are formed between particles and mucosal tissue and contact is maintained over time (Boddupalli *et al.*, 2010). The mechanism of mucoadhesion is often divided into two steps; The contact stage and the consolidation stage (Figure 9). In the first stage the polymer comes in contact with the mucus and start to swell and forming non-covalent bonds. Further, the polymer penetrates into the mucus network and forming covalent bonds to the mucus network (Schattling *et al.*, 2017; das Neves and Bahia, 2006).

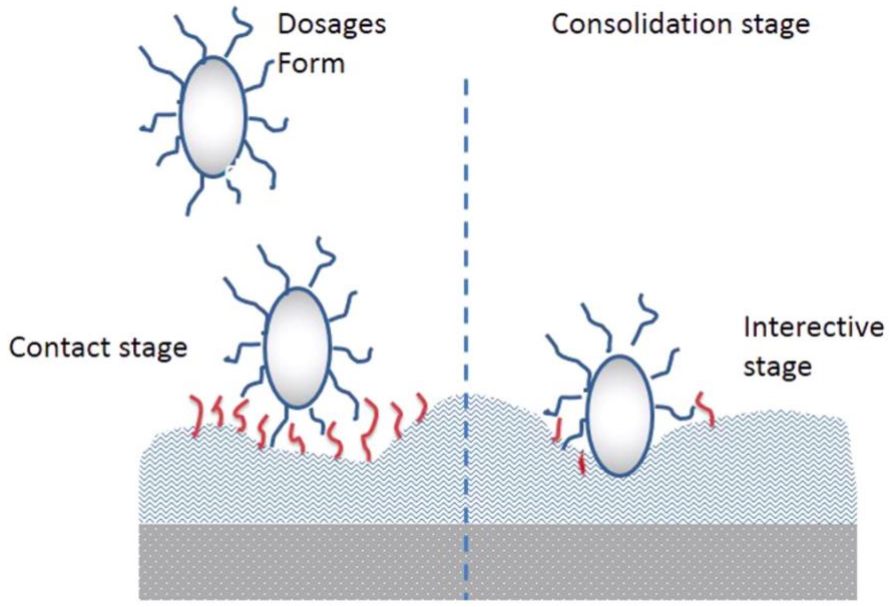

Figure 9: Illustration of the mechanism of mucoadheion showing the contact stage followed by the consolidation stage. Reprinted from Kumar et al., 2014 copyright ® with permission from RightsLink®.

There are different strategies involving covalent bonds that can be formed between the mucus and the polymer:

1. Physical entanglement between the polymer and the mucus
2. Electrostatic interactions. Positively charged polymers can make interactions with negatively charged mucus.
3. Hydrogen bonds between mucus and anionic polymer
4. Formation of disulphide bridges (Schattling *et al.*, 2017).

There are different theories that try to explain the mechanism of mucoadhesion and the mucoadhesion process is probably achieved through a combination of several mechanisms (Kumar *et al.*, 2014; Carvalho *et al.*, 2010; Hombach and Bernkop-Schnurch, 2010; Shaikh *et al.*, 2011; Smart, 2005).

Electronic theory: Attractive forces between a double layer consisting of electrical charges which occurs due to electron transfer between the mucoadhesive system and the mucus.

Adsorption theory: Mucoadhesiveness due to covalent bonds and/or hydrogens bonds and Van der Waals forces

Wetting theory: The affinity for a liquid formulation to the surface and the ability to spread. The contact angle is an important parameter that determines the mucoadhesive properties.

Diffusion theory: The diffusion and interpenetration of the polymeric chains of the formulation into the glycoprotein mucin chains allowing formation of semipermanent bonds.

Fracture theory: Taking in consideration the force needed to brake the interaction (detachment force) between two surfaces (mucus and the polymer) after adhesion is achieved.

Mechanical theory: The mucoadhesiveness is obtained by the interlocking of a liquid formulation due to irregularities in a rough surface.

2.6.1 Factors affecting mucoadhesion

Polymer factors: In general, the higher the concentration of polymer, the stronger mucoadhesion is possible. Low-molecular mass polymers are able to interpenetrate the mucus easier, whereas high-molecular mass polymer allows a higher degree of entanglements (Hombach and Bernkop-Schnurch, 2010). The degree of cross-linking is also important which affects the flexibility properties and hydration/swelling properties. A low cross-linking density is favorable which gives a higher degree of flexibility and hydration rate, allowing more water to penetrate the mucus network. This will in turn lead to an increase in polymer surface area and stronger mucoadhesive properties are obtained. It´s also important that the polymer possesses a good hydrogen bonding capacity (Boddupalli *et al.*, 2010; Shaikh *et al.*, 2011).

Environmental factors: pH will affect the ionization of the polymer. For anionic polymers the degree of swelling is pH independent. This means that at higher pH values the polymer could swell too much, and loose some of the adhesive properties. For cationic polymers the swelling would be highest at a low pH. Presence of water and pressure applied will also affects the mucoadhesion (Hombach and Bernkop-Schnurch, 2010).

There are different types of mucoadhesive dosage forms that are suitable for different routes of administration. Mucoadhesive hydrogels which is of relevance for this thesis will therefore be discussed in further detail.

2.6.2 Hydrogels

Hydrogels are made of polymeric materials that will form a three-dimensional structure due to either chemical- or physical crosslinking between the polymer chains (Lin and Metters, 2006). Hydrogels possesses the capacity to absorb from 10 % up to 1000 times their dry weight (Rosiak and Yoshii, 1999).

Hydrogels are of big interest for the pharmaceutical field, due to the properties similar to human tissue (Lee *et al.*, 2010). Hydrogels are also known to be biocompatible due to the high water content of the formulation (Kopeček, 2009). They have properties that make them capable to hold a large amount of water and swell due to hydrophilic functional groups attached to the monomeric sub units of the polymer. The hydrogels do not dissolve in contact with water due to cross-links in between the network of the polymer (Ahmed, 2015).

Hydrogels can be classified in regard to:

- The source of the material of the hydrogel: Whether the hydrogel consist of naturally molecules, synthetic molecules or a hybrid kind of hydrogel
- How the polymeric network is built: Hydrogels made of homopolymers, copolymer, interpenetrating, or double networks.
- The physical structure: Homogeneous, microporous, or macroporous
- The fate of the hydrogel in an organism: Degradable or non-degradable (Kopeček, 2009).

Different types of hydrogel can be modified and have properties that make them sensitive to changes in external environment conditions. The hydrogels can be sensitive to physical stimuli (temperature, electric field, magnetic field, light, pressure and sound), or chemical stimuli (pH, ionic strength, solvent composition and molecular spices) (Ahmed, 2015).

The release mechanism from hydrogel can be categorized into: (I) Diffusion-controlled, (II) Swelling-controlled and (III) Chemically-controlled (Lin and Metters, 2006).

To avoid the rapid clearance from the vagina, it is of interest to make a delivery system with mucoadhesive properties. Different polymers can be used to prolong the residence time, and thereby the bioavailability (Andrews *et al.*, 2009). Commonly used polymers are polyacrylic acid derivates such as carbomer and cellulose derivates such as chitosan (Caramella *et al.*, 2015). Chitosan is of interest for this thesis and will therefore be discussed in further detail below.

2.6.3 Chitosan

Chitosan (Figure 10) is of high interest in pharmaceutical technology due to the characteristics of being biocompatible, mucoadhesive and biodegradable. It is a polycationic polymer originating from chitin (Szymańska and Winnicka, 2015).

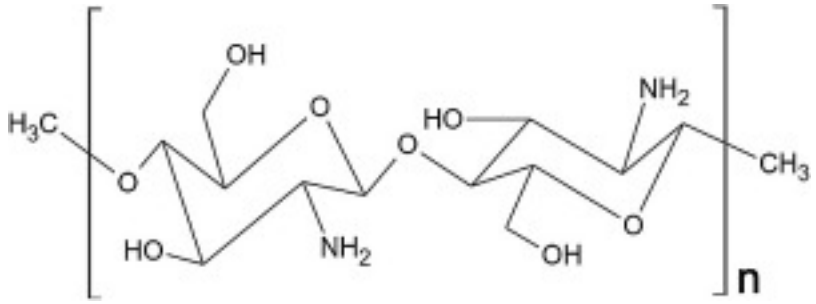

Figure 10: Chemical structure of chitosan

In nature, chitin will be found as a complex with proteins and function as a hardener for shells, and are derived from exoskeleton of crustaceans, insects and fungi (Kumar *et al.*, 2016). Chitin is a macromolecular compound made of N-acetyl-2-amino-2-deoxy-D-glucoses units connected through β-(1,4) glycoside bonds (Teng, 2012). For industrial preparation, it is mainly chitin from the shells of shrimps and crab that is used.

Chitosan will be produced by deacetylation in an alkaline environment form the parent compound chitin through the following steps: shrimp and crab shells → Acid treatment → Alkali treatment → Crude chitin → Decolor → Chitin → Deacetylation → Chitosan (Teng, 2012).

It is common to characterize chitosan by its molecular weight. The molecular weight of commercial chitosan is usually in the range of 10–10,000 kDa (Szymańska and Winnicka, 2015).

Chitosan can be used as a mucoadhesive polymer to prolong the residence time of the drug in vaginal delivery. Often the terminal end of the mucus fibers consists of a sialic acid residue, providing a negative charge (Schattling *et al.*, 2017; Wong *et al.*, 2014). The positively charge of chitosan will be able to interact with the negative charge of mucin fibers. The mucoadhesive properties of chitosan will be affected by pH, showing a higher degree of mucoadhesion with

lower pH making it a good candidate for vaginal application. It is also favorable that the mucoadhesive properties of chitosan does not weaken over time, neither get inactivated in the contact with mucin fibers (Andersen *et al.*, 2015).

The use of chitosan in pharmaceutical preparations is also of interest as chitosan itself has shown antibacterial properties to some pathogens responsible for vaginal infections such as *Candida* spp. (Palmeira-de-Oliveira *et al.*, 2015). It is suggested that chitosan could interfere with the bacterial biofilm. The bacterial biofilm has the potential to protect the bacteria from the antibiotic treatment making it difficult to treat some infections (Kandimalla *et al.*, 2013). It is suggested that the mechanism is that the cationic properties of chitosan will interact with the anionic cell surface of the bacteria and thereby lower the strength of the membrane of the bacteria (Teng, 2012).

3 Aims of the study

The main aim of this project was the development of a liposomes-in-hydrogel system containing resveratrol for the local treatment of vaginal infections. The specific aims were:

- Characterization of RES-liposomes in respect to size distribution and RES entrapment efficiency.
- Characterization and optimization of RES-liposomes-in-hydrogel formulation
- Assuring a sustained *in vitro* RES release from the novel delivery system
- Evaluation of *ex vivo* mucoadhesion properties of hydrogels
- Evaluation of the antioxidative effects of RES

4 Materials and methods

4.1.1 Materials

(±)-α-Tocopherol (Vitamin E), Sigma-Aldrich® Produktions GmbH, Steinheim, Germany

2,2-Diphenyl-1-picrylhydrazyl (DPPH), Sigma-Aldrich® Productions GmbH, Steinheim, Germany

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

Acetic acid 99.8 %, Sigma-Aldrich® Productions GmbH, Steinheim, Germany

Acetonitrile, VWR Chemicals, Fontay-sous-Bois, France

Ammonium acetate, VWR Chemicals, Leuven, Belgium

Bovine serum albumin ≥ 96 %, Sigma-Aldrich® Productions GmbH, Steinheim, Germany

Calcium hydroxide, Sigma-Aldrich® Productions GmbH, Steinheim, Germany

Chitosan (MMW), Chitinor, Tromsø, Norway

D–(+)–Glucose, Sigma-Aldrich® Productions GmbH, Steinheim, Germany

Disodium phosphate, Sigma-Aldrich® Productions GmbH, Steinheim, Germany

Distilled water

Ethanol, Sigma-Aldrich, Sigma-Aldrich® Productions GmbH, Steinheim, Germany

Glyserol 86-88 %, Sigma-Aldrich® Productions GmbH, Steinheim, Germany

Hydrochloric acid, Sigma-Aldrich® Productions GmbH, Steinheim, Germany

L-Ascorbic acid (Vitamin C), Sigma-Aldrich® Productions GmbH, Steinheim, Germany

Lactic acid, Fluka® Honeywell Research Chemicals, Bucharest, Romania

Lipoid S100, ~100 % phosphatidylcholine from soybean, Lipoid GmbH, Ludwigshafen, Germany

Methanol, Sigma-Aldrich® Productions GmbH, Steinheim, Germany

Methanol, VWR Chemicals, Fontay-sous-Bois, France

Mili-Q water

Potassium dihydrogen phosphate, Sigma-Aldrich® Productions GmbH, Steinheim, Germany

Potassium hydroxide, Norsk Medisinaldepot, Oslo, Norway

Potassium peroxodisulfate, Merk KGaA, Darmstadt, Germany

Propylene glycol $\geq$ 99.5 %, Sigma-Aldrich® Productions GmbH, Steinheim, Germany

Resveratrol, Sigma-Aldrich® Productions GmbH, Steinheim, Germany

Sodium Chloride $\geq$ 99.5 %, Sigma-Aldrich® Productions GmbH, Steinheim, Germany

Ureum, Apotekproduksjon AS, Oslo, Norway

4.1.2 Instruments

Branson B-series 5510 Ultrasonic Cleaners, Danbury, USA

BÜCHI waterbath B-480, BÜCHI Vacuum Pump V-700 BÜCHI rotavapor R-124, BÜCHI Labortechnik AG, Flawil, Switzerland

Franz diffusion cell 15 mm, 12 ml acceptor chamber volume, PermeGear, Hellertown, USA

HPLC Separation Module e2795, Waters, Dublin, Ireland

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

Microplate reader, Speactra MAX 190, Molecular Devices, Sunnyvale, USA

PermeGear V6A Stirrer, PermeGear, Hellertown, USA

Submicron Particle Sizer Model 370, NICOMP Particle sizing systems, Santa Barbara, USA

TA XT.plus – texture analyzer, Stable Micro systems, Surrey, United Kingdom

UV/Visible detector 2489, Waters, Dublin, Ireland

Zetasizer, Malvern Instruments Limited, Malvern, United Kingdom

4.1.3 Computer programs

HPLC: EmpowerTH 3 Chromatography Data Software, Build 3471, 2010, Waters, Dublin, Ireland

PCS: PSS CW388 Version 1.68, NICOMP Particle Sizing Systems, Santa Barbara, USA

Texture analysis: Exponent, Version 6,1,5,0, Stable micro systems, Surrey, United Kingdom

UV-microplate reader: SoftMax Pro v5 Software, Molecular Devices, San Jose, USA

Zeta potential: Zetasizer Software, Version 7.11, Malvern Instruments Limited, Malvern, BY, United Kindom

4.1.4 Equipment

0.8 μm, 0.4 μm, 0.2 μm Nuclepore Track-Etch Polycarbonate Membrane, Filtration products, Whatman®, Sigma-Aldrich®, Steinheim, Germany

Acrodisc® CR 4 mm syringe filter, with 0.45 μm PTTFE Membrane, Life Sciences, Sigma-Aldrich®, Steinheim, Germany

Backward Extrusion Rig. Part code. A/BE 40 mm disc, Stable Micro Systems, Surrey, United Kingdom

Cellophane membrane: Zellglas Einmach Fix, Folia Bringmann, Germany

Dialysis Tube, Molecular weight cut-off 12-14 kDa, Medicell Membranes Limited, London, United Kingdom

Disposable glass tubes, 6x50 mm, ASTM Type 1, Borosilicate glass, Kimble®, USA

GHP Acrodisc® 13 mm syringe filter, with 0.2 μm GHP Membrane, Life Sciences

SMS Mucoahesive Rig, A-MUC, Stable Micro Systems, Surrey, United Kingdom

Sterile Syringe Filter, 0.2 μm PES, VWR, Sigma-Aldrich®, Steinheim, Germany

Symmetry® C18 5 μm 3.9x150 mm column, Waters, Ireland

Whatman Swinnex-25 millipore filter holder, Whatman®, Sigma-Aldrich®, Steinheim, Germany

UV Plate, 96 well, With UV transparent flat bottom, Acrylic, CORNING, Sigma-Aldrich®, Steinheim, Germany

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

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

4.2 Liposomal preparation and characterization

4.2.1 Preparation of RES-liposomes

Liposomes were prepared by the conventional film method (Jøraholmen *et al.*, 2015). RES (10 mg) was dissolved in an adequate amount of ethanol (EtOH) in a round bottomed flask and mixed with Lipoid S100 (200 mg) dissolved in an adequate amount of methanol (MeOH). The solvents were removed completely by evaporation (50 °C, 55 mbar) for approximately 3 hours, forming a lipid film at the inner walls of the flask. The lipid film was re-suspended in distilled water (d-water) (10 mL) and shaken by hand until the film was completely dislodged from the flask. Liposomal suspension was stored in the fridge (4–8 °C) overnight prior further use. During the preparation, the formulation was protected from light by aluminium foil.

4.2.2 Vesicle size reduction

Liposomal size was reduced by extrusion through polycarbonate membrane to approximately 200 nm. Membranes with pore a size of 0.8 μm, 0.4 μm and 0.2 μm were used. The liposomes were extruded 5 times through each membrane (Nuclepore Track-Etch polycarbonate membrane), with decreasing pore size of the membrane. The liposomes were stored in refrigerator overnight, prior further use.

4.2.3 Particle size distribution determination

The particle size distribution of liposomes was determined by photon correlation spectroscopy. Glass tubes were filled with d-water and sonicated in ultrasonic bath for approximately 30 minutes. The tubes were emptied and rinsed with filtered d-water (using 0.2 μm filter), in order to avoid interference from dust particles. A small amount of liposomal sample was diluted in d-water to obtain an intensity of approximately 250-350 kHz. All preparations were conducted in a laminar airflow bench and all analyses were run in vesicle mode and intensity distribution. Three parallels were run of each sample, with running time of 10 minutes.

4.2.4 Zeta potential determination

The Zeta-cell was rinsed with EtOH, and then twice with filtered tap water (using 0.2 µm filter). Liposomal sample was diluted to adequate concentrations (typically 1:20) and loaded to the zeta-cell. Zetasizer from Malvern was used to detect the zeta potential and three parallels were measured for all samples. The same preparations were done when measuring the zeta potential of the hydrogels.

4.2.5 RES entrapment efficiency determination

A stock solution containing RES in concentration of 1000 µg/mL was prepared in MeOH and further diluted to standard solutions with concentrations of 0.5, 1, 2, 4, 6, 10 and 12 µg/mL. Liposomally entrapped RES was separated from free RES by dialysis. The dialysis membrane (molecular weight cut-off value 12-14 kDa) was soaked in d-water for 30 minutes. An aliquot of 5 mL of liposomal sample was dialyzed in 1250 mL of d-water for 4 hours, covered by Parafilm® and aluminium foil. The sink conditions were assured. Aliquots of liposomal sample and dialysis medium were diluted in MeOH. Standard solutions and samples (200 µL) were added to a flat bottom transparent UV-plate and measured spectroscopically at 306 nm. A standard curve was prepared based on the measurements and RES content was determined.

4.3 Hydrogel preparation

4.3.1 Preparation of chitosan hydrogel

Hydrogels were prepared as described by Hurler et al., in three different concentrations (Hurler *et al.*, 2012). Briefly, hydrogel was made by mixing chitosan (MMW; 3 %, w/w) in a solution of 10 % (w/w) glycerol in acetic acid (2.5 %, w/w). Hydrogels with 2.5 % (w/w) chitosan and hydrogels with the end concentration of 2.5 % chitosan after incorporation of liposomes were also prepared. The hydrogel was stirred by hand, then bath sonicated for approximately 30 minutes to remove all entrapped air bubbles. The hydrogel was left to swell at room temperature for 48 hours prior further use.

4.3.2 Incorporation of liposomes in hydrogel

RES-liposomes-in-hydrogel (10 % w/w liposomal sample) was prepared. Into a chitosan hydrogel (45 g) 5 g of RES-liposomes were added and gently stirred by hand. The formulation was stored in refrigerator (4-8 ℃) prior to further use.

4.3.3 Texture analysis

The analysis was based on the method proposed earlier by Hurler et al. (Hurler *et al.*, 2012). The hydrogel was stored at room temperature prior to the test (~12 hours). Approximately 35 grams of hydrogel was filled in a beaker (100 mL) and a 40 mm disc was used to compress into the hydrogel. A texture analyser from Stable Micro systems was used to detect cohesiveness, adhesiveness and hardness of the hydrogels (Figure 11). The force and the height of the apparatus was calibrated each time before testing. The analysis settings were chosen according to type of hydrogel (speed rate: 4 mm/sec, distance: 10 mm). Six parallels of each hydrogel were measured. The first parallel was excluded from calculation to obtain the same conditions for all measurements because hydrogel remained on the probe disk after the first measurement.

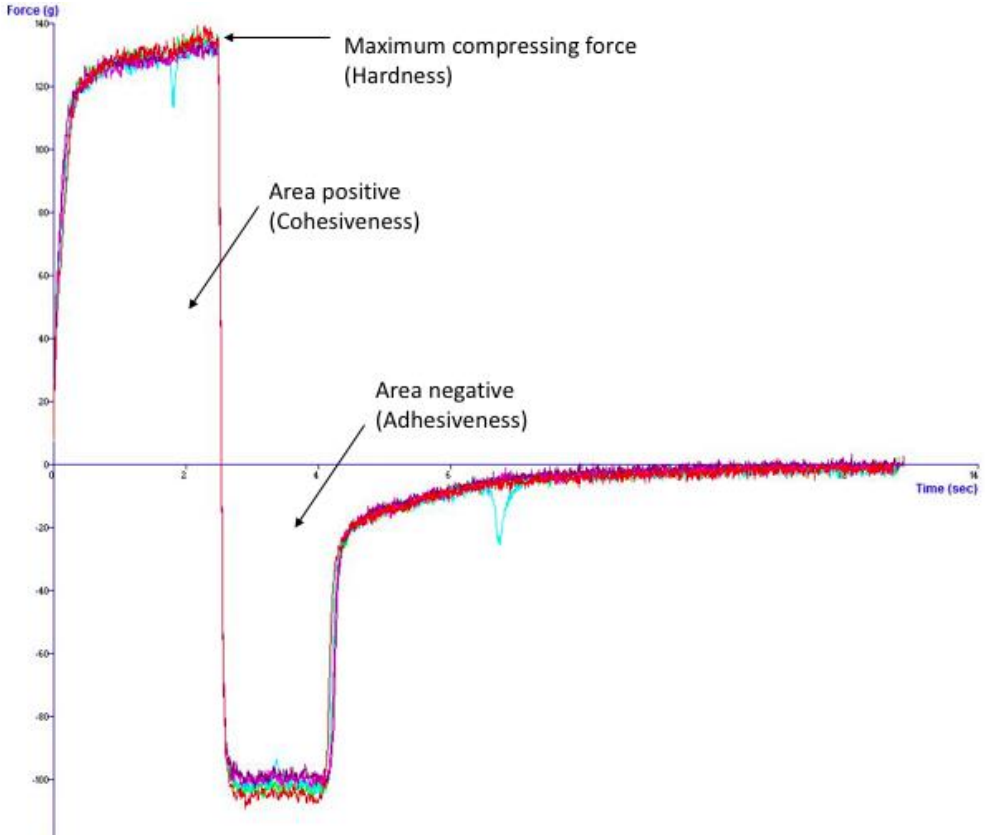

Figure 11: An example of a plot generated from texture analysis showing a force (g) versus time (sec) plot.

4.4 Mucoadhesive properties of hydrogel

4.4.1 Preparation of phosphate buffer

Sodium chloride (8 g/L), potassium dihydrogen phosphate (0.19 g/L) and disodium phosphate (2.38 g/L) was dissolved in d-water. The pH was measured and adjusted to 7.4 with sodium hydroxide (Jøraholmen *et al.*, 2014).

4.4.2 Preparation of cow and sheep vaginal tissue

The vaginal mucosa was separated from the rest of the tissue and cleaned and kept moisturized by using phosphate buffer (pH 7.4). The thickness of the cow tissue was approximately 1.5 mm and the sheep tissue approximately 1.1 mm. The tissue was packed in a clinging film and frozen (-20 °C). The vaginal tissue originated from cow was a generous gift from Nortura SA, Målselv.

4.4.3 *Ex vivo* mucoadhesive properties

The mucoadhesive properties of the hydrogel were tested by using two different methods (Hurler and Škalko-Basnet, 2012). The tissue was defrosted in phosphate buffer (pH 7.4) for about 30 minutes prior to experiment and cut to appropriate sized pieces. The tissue pieces were placed on a membrane holder intended for the Texture analyzer. In the first method the detachment force was recorded. Hydrogel sample (150 µL) was applied onto the probe by using a 1 mL syringe. The compression time was set to 10 sec., the compression force was set to 25.0 g and the redrawn speed was set to 0.1 mm/sec. Between each measurement the tissue was cleaned with EtOH and phosphate buffer (pH 7.4). In the second method the amount of hydrogel remaining on the tissue was calculated by measuring the weight of the probe with hydrogel before and after the tests.

4.5 *In vitro* release study

4.5.1 Preparation of acetate buffer

Ammonium acetate (38.55 g) was dissolved in d-water in in a 500 mL volumetric flask. Glacial acetic acid (35 mL) was then added. The volumetric flask was then filled ad 500 mL with d-

water. The pH was measured and adjusted to 4.6, with a few drops of hydrochloric acid (Jøraholmen *et al.*, 2015). The buffer was stored at room temperature.

4.5.2 *In vitro* RES release

The test was performed as described earlier by Jøraholmen et al. (Jøraholmen *et al.*, 2015). The Franz diffusion cells were cleaned with MeOH for 30 minutes, then twice with d-water for 30 minutes. Cellophane was cut to fit the cells and soaked in acetate buffer (pH 4.6) for at least 30 minutes prior use. Acceptor chambers (12 mL) were filled with acetate buffer (pH 4.6). Cellophane was put on top of acceptor chamber and the donor chambers were placed on top of the membrane (Figure 12). The temperature was set to 37 °C, with circulating heat. A stir bar was placed in each acceptor chamber and each donor chamber was filled with 600 µL of the test samples RES in propylene glycol served as a control, and the release was determined for RES-liposomes, hydrogel containing RES in propylene glycol and RES-liposomes-in-hydrogel.

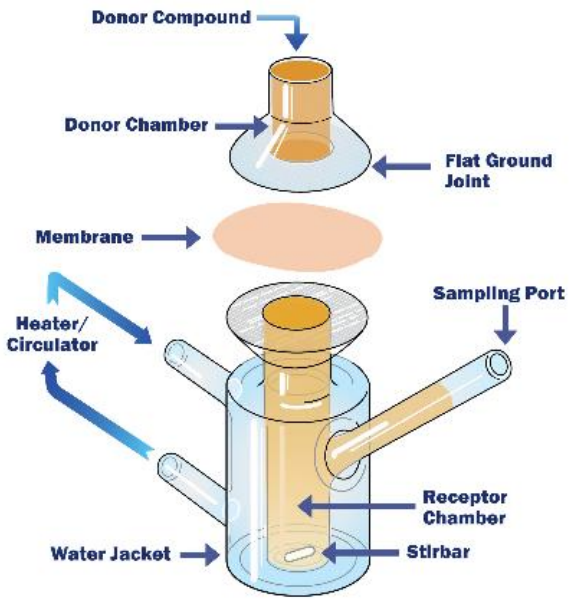

Figure 12: Illustration of the Franz cells (Picture from: http://permegear.com/franz-cells/)

Samples (500 µL) were collected from the acceptor chamber after 1, 2, 3, 4, 6 and 8 hours, and replaced with an equal amount of fresh acetate buffer (pH 4.6). After 8 hours of experiment, the remains in the donor chambers were collected and the volume was determined by micropipette. The membrane was soaked in 1000 µL of MeOH. All samples were diluted in

MeOH, as well as a control for all tested samples to determine the actual amount of RES added in each donor chamber. Samples were measured spectroscopically at 306 nm and RES content determined.

4.6 *Ex vivo* penetration study

4.6.1 Preparation of vaginal fluid simulant

Sodium chloride (3.51 g/L), potassium hydroxide (1.40 g/L), calcium hydroxide (0.22 g/L), bovine serum albumin (0.018 g/L), lactic acid (2 g/L), acetic acid (1 g/L), glycerol (0.16 g/L), urea (0.4 g/L) and glucose (5 g/L) were dissolved in d-water. The pH was measured and adjusted to 4.5 with 1 M hydrochloric acid (Owen and Katz, 1999).

4.6.2 *Ex vivo* RES penetration

The test was performed on the vaginal tissue from pregnant sheep and based on the method described earlier by Jøraholmen et al. (Jøraholmen *et al.*, 2014). The tissue was defrosted in phosphate buffer (pH 7.4) for about 30 minutes before performing the test. The acceptor chambers were filled with phosphate buffer (pH 7.4). Appropriately sized pieces of tissue were placed on the top of the acceptor chamber. The temperature was set to 37 °C, with circulating heat. A stir bar was placed in each acceptor chamber and each donor chamber was filled with 550 μL of the test samples and vaginal fluid simulant (50 μL). RES in propylene glycol served as a control, and the RES release was determined for RES-liposomes and RES-liposomes-in-hydrogel.

Samples (500 μL) were collected from acceptor chamber after 1, 2, 3, 4, 6 and 8 hours, and replaced with equal amount of fresh phosphate buffer (pH 7.4). After 8 hours of experiment, the remain formulation in the donor chambers were collected and the volume was determined by micropipette. The tissue was soaked in 3 mL MeOH to extract remaining RES. All samples were diluted in MeOH, as well as a control for all tested samples, to determine the actual amount of RES found in each donor chamber. The RES content in samples was determined by high performance liquid chromatography (HPLC) (method described in 4.6.3.).

4.6.3 HPLC method

The mobile phase consisted of 75 % (v/v) MeOH, 22.5 % (v/v) acetonitrile, 2.4 % (v/v) Milli-Q water and 0.1 % (v/v) of acetic acid (Kristl *et al.*, 2009). The RES amount in the samples was detected at the wavelength of 306 nm by the use of UV-HPLC from Waters and a C18 column. An injection volume of 20 μL was used, and the flow rate was set to 0.8 mL/min with a run time of 5 minutes for each sample.

4.7 Radical scavenging activity

4.7.1 DPPH radical scavenging activity

DPPH was diluted with EtOH to a final concentration of 134 μM. RES was also diluted in EtOH to the concentrations of 5, 10, 25, 50 and 75 μg/mL. Corresponding samples were prepared for vitamin C and vitamin E. Aliquots (0.3 mL) of each of the test samples were mixed with 0.3 mL of DPPH solution. The samples were shaken and kept in dark at room temperature for 30 minutes. The scavenging activity of RES was measured spectrophotometrically at 519 nm and expressed by the decrease in the absorbance intensity. The activity was compared to those of vitamin C and vitamin E.

4.7.2 $ABTS^{\bullet+}$ radical scavenging activity

ABTS (7.4 μM) was mixed with potassium persulfate (2.6 μM) and kept dark overnight. The following day, the $ABTS^{\bullet+}$ solution was diluted in EtOH. RES was also diluted in EtOH to the concentrations of 5, 10, 25, 50 and 75 μg/mL. The same concentrations were prepared for vitamin C and vitamin E. Aliquots of 0.3 mL of the test samples were mixed with 0.3 mL of the $ABTS^{\bullet+}$ solution. The samples were shaken and kept in dark, at room temperature for 30 minutes. The scavenging activity of RES was measured spectrophotometrically at 757 nm and expressed by the decrease in the absorbance intensity. The activity was compared to those of vitamin C and vitamin E.

4.8 Statistical evaluation

Students *t*-test was used to compare means. A significant level at $p \leq 0.05$ was considered acceptable.

5 Results and discussion

5.1 Liposome characterization

To overcome the poor water solubility of RES and to exploit its favorable pharmacological effects, RES was entrapped in liposomes. The RES-liposomes was made by the film-hydration method, one of the most common liposome preparation method (Jøraholmen *et al.*, 2015). This production method yields large heterogeneous MLVs (Bozzuto and Molinari, 2015). Because molecules/particles in the size range of 0.2–0.5 μm have shown the ability to penetrate mucus network, it is desirable to reduce the original liposomal size by some of the size reduction methods available (Wong *et al.*, 2014). The polydispersity index (PI) also has to be taken into consideration. It tells us how homogenous the size distribution of liposomes is. The value ranges from 0 to 1, where 1 is a totally heterogenous suspension of liposomes, which would be difficult to control (Isailović *et al.*, 2013). An accepted PI value is below 0.7. Lower PI values, indicates a more homogenous liposomal dispersion, which would also permit more controllable release of active ingredient, in our case RES.

To obtain a desirable size and a more homogeneous distribution there are different available methods, the most common are; extrusion, sonication and high-pressure homogenization (Bozzuto and Molinari, 2015). Extrusion has shown to be a suitable method to produce smaller liposomes with a low PI and is well established in our group, (Jøraholmen et al., 2015), hence, this method was applied.

Table 1: Liposomal characteristics (n=3).

Size (nm)	PI*	Zeta potential (mV)	Entrapment (%)
188.27 ± 23.01	0.06 ± 0.03	-4.27 ± 2.18	93.99 ± 1.72

*PI = polydispersity index.

The size of liposomes after extrusion was found to be close to the desired vesicle size of 200 nm (Table 1), this means that the liposomes would be able to penetrate through the mucus network and reach the underlying epithelium. Further, the very low PI indicates a rather homogenous liposomal size distribution. The similar liposomal size was reported by Jøraholmen *et al.*; their liposomes were of a mean size of 206 nm after extrusion (Jøraholmen *et al.*, 2015). The size of RES-liposomes produced by the same method and same size reduction

method was also reported by Isailović *et al.* (Isailović *et al.*, 2013). They produced liposomes in the size range between 120 and 290 nm after extrusion. However, a difference is that they used Phospholipon 90G in their preparation, whereas in this study there was used Lipoid s100.

The zeta potential of liposomes was determined, indicating a slightly negative charge (Table 1). Zeta potential can be used as an indicator of the surface charge of the liposomes. Zeta potential is also of importance when evaluating the stability, permeability and biocompatibility of liposomal formulation (Smith *et al.*, 2017). A slightly negative zeta potential indicates that the surface of the liposomes has anionic properties. Jøraholmen *et al*. have previously reported RES-liposomes with a zeta potential value of -3.17 mV (Jøraholmen *et al.*, 2015).

To determine how much of RES was entrapped in the liposomes, free RES was removed by a dialysis. A calibration curve was made by dilution of RES in MeOH and the absorbance of standard solutions with concentrations of 0.5, 1, 2, 4, 6, 10 and 12 µg/mL, as well as the liquots of liposomal dispersions and dialysis medium were measured spectroscopically at 306 nm.

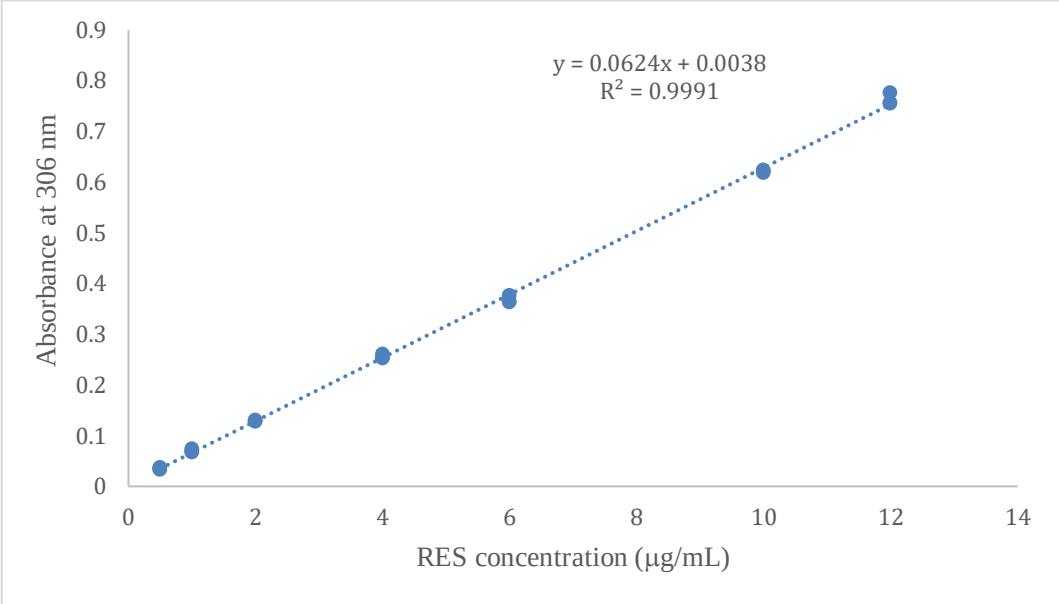

Figure 13: Calibration curve (Concentration vs. absorbance of resveratrol (RES) diluted in methanol)

The entrapment efficiency was thereby calculated from the calibration curve (Figure 13). Liposomal entrapment efficiency can be affected by size reduction; the size reduction may lead to the loss of originally entrapped drug or active molecule. However, the results after dialysis indicate that a high degree of entrapped RES (Table 1) was retained within liposomes. Liposomes made by the film-hydration method shows relatively low entrapment efficiency for

hydrophilic molecules, and the method is therefore probably more suitable for lipophilic molecules (Bozzuto and Molinari, 2015). RES is a highly lipophilic compound with a logP value of 3.1, therefore this method is suitable for the preparation of RES-liposomes (Amri *et al.*, 2012). A high and reproducible entrapment efficiency was observed with a mean of 93.99 ± 1.72 % RES entrapped in the liposomes. A high entrapment efficiency was also previously reported by Isailović *et al.* and by Jøraholmen *et al.* which reported an entrapment efficiency of 92 ± 0.82 % and 80 ± 4 %, respectively (Isailović *et al.*, 2013; Jøraholmen *et al.*, 2015). The liposomal size would also contribute to the entrapment efficacy (Pavelić *et al.*, 2005), therefore direct comparison can be done only for liposomes of similar sizes.

5.2 Liposomes-in-hydrogel characterization

As mentioned previously, liposomal suspension as such is not suitable for vaginal application because of the liquid nature of the suspension and the rapid clearance from the vagina (Pavelić *et al.*, 2005)Therefore, liposomes were incorporated in chitosan hydrogels to assure their retention at vaginal site.

5.2.1 Surface charge of hydrogel

The zeta potential of the hydrogel was also determined to evaluate the effect of chitosan concentration on the overall zeta potential of the formulation.

Table 2: Zeta potential of hydrogels with different chitosan concentrations and 10 % w/w liposomes incorporated within hydrogel (n=3).

Concentration of chitosan in hydrogel (%)*	Zeta potential (mV)
2.5	71.93 ± 2.09
2.5 (final concentration)	72.97 ± 2.03
3	75.64 ± 1.93

* 3 % and 2.5 % chitosan amount calculated without liposomes incorporated. 2.5 % (final concentration) chitosan concentration calculated taken into account the incorporated liposomes.

The RES-liposomes exhibited a slightly negative zeta potential, while when incorporated in hydrogel the value of RES-liposomes-in-hydrogels was positive (Table 2) as expected. This is due to the cationic nature of chitosan (Szymańska and Winnicka, 2015). Moreover, the charge was increasing with the increasing chitosan concentration.

5.2.2 Texture properties of hydrogels

Texture properties of hydrogels are important and of a high relevance to determine the applicability of the formulation at the administration site, in our case vaginal cavity. It can function as an in-process control which allows optimizing the formulation (Hurler *et al.*, 2012). From texture analysis, there will be generated a plot showing force (g) versus time (sec) (Figure 11). From this plot, cohesiveness (area positive), adhesiveness (area negative) and hardness (peak positive) of the hydrogel can be calculated. The hardness parameter indicates the applicability of the hydrogel, especially considering storage and packing, whereas the adhesiveness is an indicator of how well the formulation will adhere to the mucus. The cohesiveness is defined as the force required to deform the hydrogel when the probe is compressed into it (Hurler *et al.*, 2012).

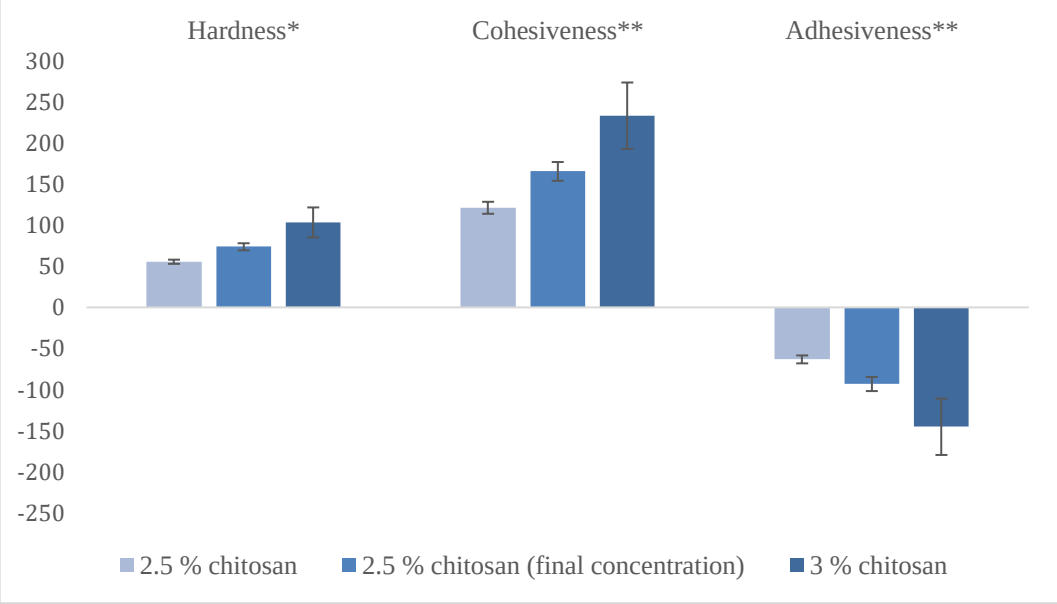

*Figure 14: Texture properties (hardness, cohesiveness and adhesiveness) of hydrogels containing different chitosan concentrations. *Unit= Force (g), **Unit=Force•sec (n=3).*

All formulations comprised 10 % (w/w) liposomes and 10 % (w/w) glycerol. The hydrogels with 3 % (w/w) chitosan showed the best properties in adhesiveness, cohesiveness and hardness (Figure 14), however they also exhibited a relatively high standard deviation. Due to difficulty to work with the hydrogel containing the highest chitosan concentration (3 % w/w), namely gel stiffness, this hydrogel was not used in further experiments, and the hydrogel with 2.5 % (w/w) chitosan as final concentration was selected to work with. Additionally, the hydrogels containing 2.5 % (w/w) chitosan exhibited a higher degree of reproducibility as observed in reduction of standard deviation. It is also important to consider that the viscosity of hydrogel to be applied to the vagina should be suitable for assuring spreading within vaginal cavity (Vanić and Škalko-Basnet, 2014) whereas for skin application, the viscosity could be higher.

Texture analysis of MMW chitosan hydrogel (3.5 %, w/w) with liposomes containing chloramphenicol destined for to use in the treatment of burns has been previously reported (Hurler and Škalko-Basnet, 2012). All of the mechanical properties of the hydrogels were reported to be higher as compared to the findings found in this project. This is also to be expected since the authors used a higher concentration of chitosan in their formulation. It seems that there is a trend where the mechanical properties of the hydrogels increase with an increased concentration of chitosan in the formulation.

5.2.3 Stability testing

The stability of the hydrogel expressed through mechanical properties (hardness, adhesiveness, cohesiveness) of hydrogels was determined by re-testing the hydrogels after 1 and 2 months (stored in refrigerator at 4-8 C) for hydrogel containing 2.5 % chitosan (as final concentration after incorporation of liposomes). The first measurement was done for the freshly made formulation and is expressed as reading 0.

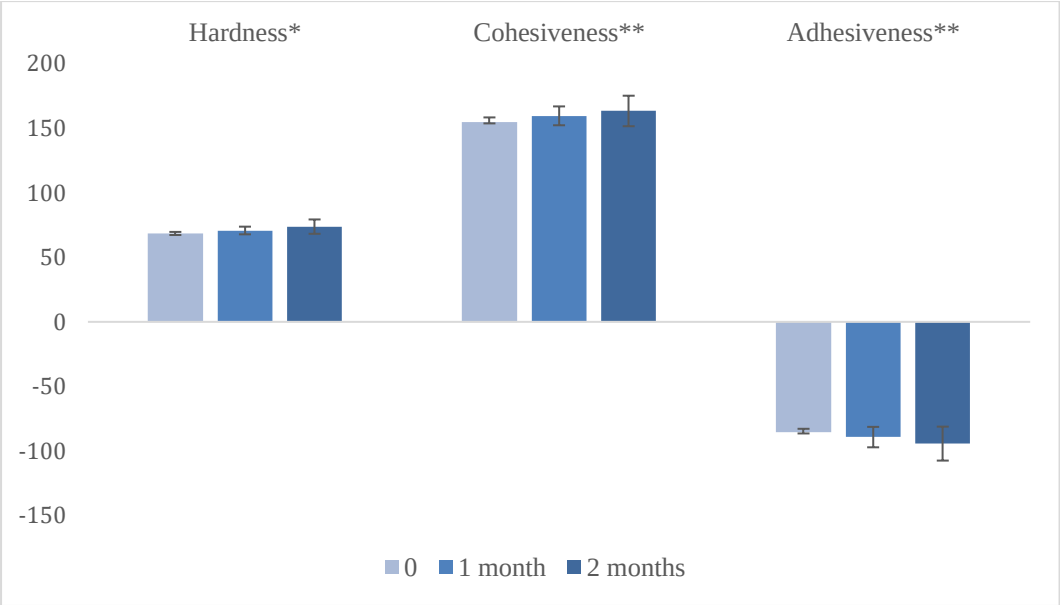

*Figure 15: Stability testing of the mechanicals properties (hardness, cohesiveness, adhesiveness) of 2.5 % (final concentration) hydrogel after 1 and 2 months storage. *Unit= Force (g), **Unit=Force•sec (n=3).*

The results indicate that there are only slight changes in the mechanical properties of the hydrogel (Figure 15) that are not significant ($p>0.05$). A slight increase in standard deviation was detected. One possible explanation could be that the hydrogel contains a high amount of water from the acetic acid solution used in the formulation. Comparing the freshly made hydrogel to the hydrogel measured after 1 month of storage, some of the water could have evaporated, contributing to the variation in reading.

There are reported stability problems related to the storing of chitosan hydrogels over a period of time due the degradation of the polymer, however, it is shown that the addition of glycerol could increase the stability of chitosan hydrogels (Szymańska and Winnicka, 2015). These formulations contained 10 % (w/w) of glycerol which could assist in maintaining the stability of the hydrogels.

As mentioned previously, the formulations were kept in a refrigerator between measurements. Therefore, this result is only an indication of the stability of the formulation at this storing situation. This is an important parameter, but for further investigation the stability of the formulation should possibly also be assessed after storing at room temperature and over longer period of time.

5.3 *Ex vivo* mucoadhesion properties

The texture analysis provides an indication of the formulation mucoadhesiveness, however, to obtain further insight in the mucoadhesive properties of the formulation, an *ex vivo* mucoadhesion test was applied to confirm the textural properties. The test was performed by the use of two different methods as previously reported by Hurler and Škalko-Basnet (Hurler and Škalko-Basnet, 2012). In our study the tests were performed on vaginal tissue obtained from cow. In the first test the detachment force was measured. The detachment force is defined as the force required to overcome the adhesive bond between the hydrogel and the skin, in our case vaginal mucosa (Hurler and Škalko-Basnet, 2012). Three different hydrogels with the same chitosan concentration (2.5 % w/w as final concentration) and same amount of liposomes incorporated (10 % w/w) were tested. A formulation containing 3 % chitosan was also tested and showed a very similar properties to 2.5 % chitosan hydrogel (results not shown).

Although reproducible results were to be expected since the hydrogels contained the same amount of chitosan (2.5 %, w/w, chitosan as final concentration), we determined the differences in the detachment force (Figure 16). When we measured the detachment force the probe with hydrogel was weighed before and after the test, and the amount of hydrogel retaining on the tissue was calculated (Figure 17), the results were more reproducible.

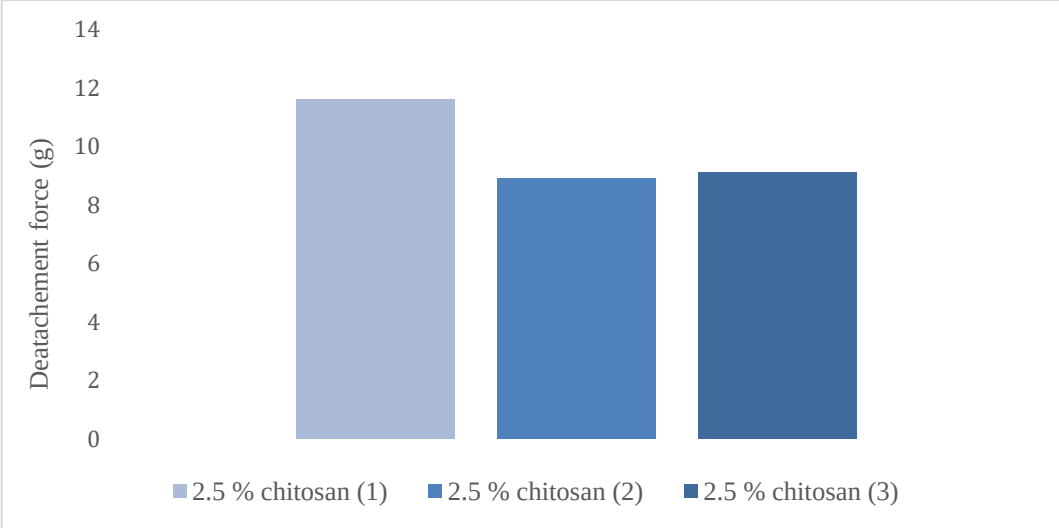

Figure 16: Detachment force of three different hydrogels applied to vaginal tissue (containing 2.5 % chitosan as a final concentration.

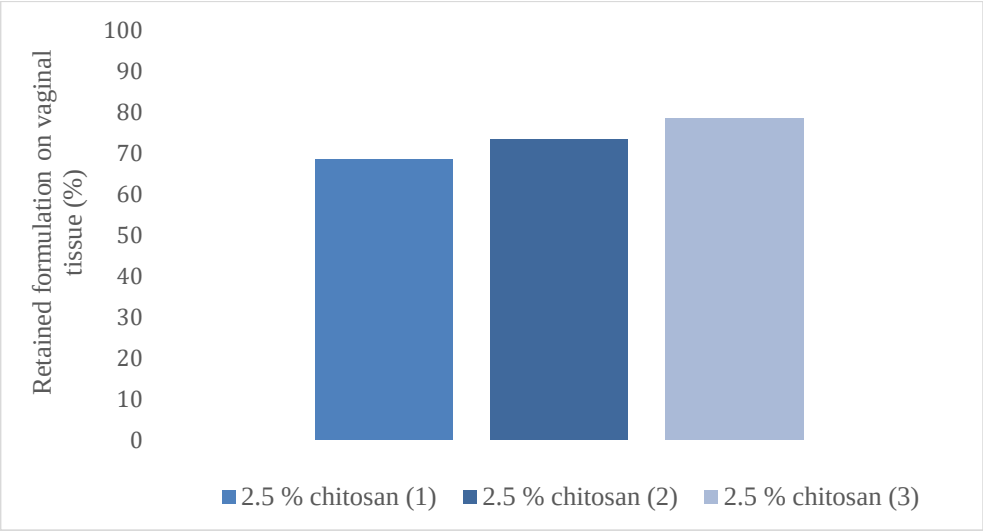

Figure 17: Amount of hydrogel retained on the tissue after mucoadhesion test.

The results indicate that the formulation containing 2.5 % chitosan as final concentration possesses good mucoadhesive properties, with 68–78 % of the formulation retained onto the tissue. There were also performed a test using the tissue attached to probe and lowered into the formulation, we found the method not to be suitable for this type of tissue, and more suitable for testing of formulations applied onto the skin (results not shown).

Hurler and Škalko-Basnet tested the bioadhesion of chitosan (MMW chitosan, 3.5 % w/w) hydrogel, however, these results are not directly comparable since their test was conducted on pig ear skin that is different to the mucosal tissue (Hurler and Škalko-Basnet, 2012). It is also important to take into account that there is a vaginal fluid present in the vaginal environment, which might affect the viscosity of the hydrogel (das Neves and Bahia, 2006). We also tried to perform the bioadhesion study in the presence of vaginal fluid simulant, however, the results were not reproducible, and we opted not to include them. The reason is that the validated method for "dry" tissue could not be adjusted for the interference from fluid. We would have required a specific adapter to the texture analyzer, which was not available during the project. This should be assessed in further studies when optimizing the formulation. Moreover, another limitation to consider is that the test was performed at room temperature (~22–24 °C) which is not directly comparable with the environment temperature of the vagina. However, testing of bioadhesion at the room temperature is a common practice.

5.4 *In vitro* RES release

In vitro RES release study from chitosan-coated RES-liposomes intended for the topical treatment of vaginal infections was reported by our group (Jøraholmen *et al.*, 2015). The findings suggested that the coating of liposomes with chitosan assured a sustained release profile of RES. In this study, RES-liposomes was incorporated in chitosan hydrogel instead of coating the liposomes. This was done to further enhance the retention of RES-liposomes at vaginal site. To confirm the sustained release profile of the formulation, an *in vitro* RES release test, using the Franz diffusion system and cellophane membrane was conducted. The use of an artificial membrane eliminates the disturbance of variation in biological material that occurs when using vaginal tissue, still providing usable preliminary permeation data (Ng *et al.*, 2010). The Franz diffusion system is regarded as an appropriate method for testing of release properties of drug/active ingredient from a formulation destined for topical vaginal administration (das Neves and Bahia, 2006). Acetate buffer (pH 4.6) was used as acceptor medium to mimic the pH of a healthy vaginal environment (pH 4–7) (Sahoo *et al.*, 2013). The RES-liposomes and RES-liposomes-in-hydrogel were compared with respective controls (RES in propylene glycol and RES in propylene glycol in hydrogel, respectively). The amount of RES released was determined by UV spectroscopy at 306 nm. The results were expressed as a mean of 3 experiments ± standard deviation and are summarized in Figure 18.

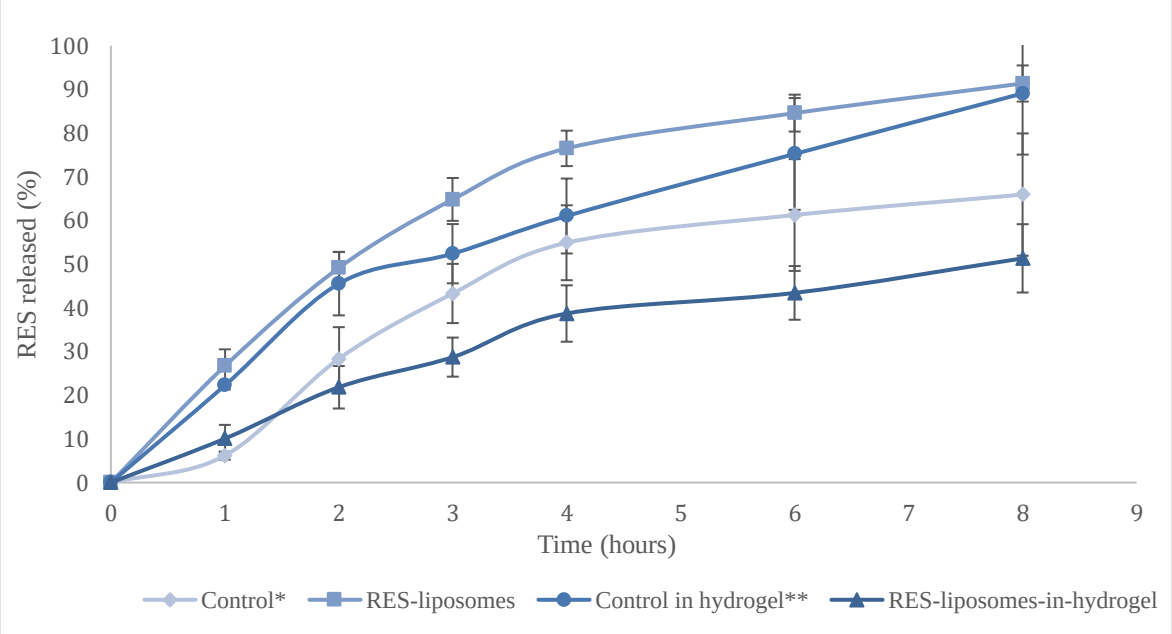

*Figure 18: Resveratrol (RES) release from different formulations. *=RES in propylene glycol. **=RES in propylene glycol in hydrogel (n=3).*

RES-liposomes showed a faster release profile compared to the RES-liposomes-in-hydrogel, as expected. This indicates that the chitosan hydrogel has the ability to further prolong the release of RES from the formulation. After 8 hours, approximately 90 % of RES was released from the plain liposomes compared to approximately 50 % from the RES-liposomes-in-hydrogel formulation. To compare the release pattern from the RES-liposomes-in-hydrogel, a control (hydrogel containing RES in propylene glycol) was also measured. As expected, the RES release was slower from the RES-liposomes-in-hydrogel formulation compared to the control in hydrogel. This indicates that both liposomes and liposomes-in-hydrogel contribute to sustained release of RES (Jøraholmen *et al.*, 2014)

It was unexpected that the control (RES in propylene glycol) exhibited a slower release profile than the RES-liposomes. This could be due to the solubilization of RES by the liposomes. Also, a possible explanation could be that more of the control was retained in the membrane compared to the RES-liposomes. This was confirmed by extracting the membrane in MeOH and thereafter analyzing the sample by UV spectroscopy (Figure 19).

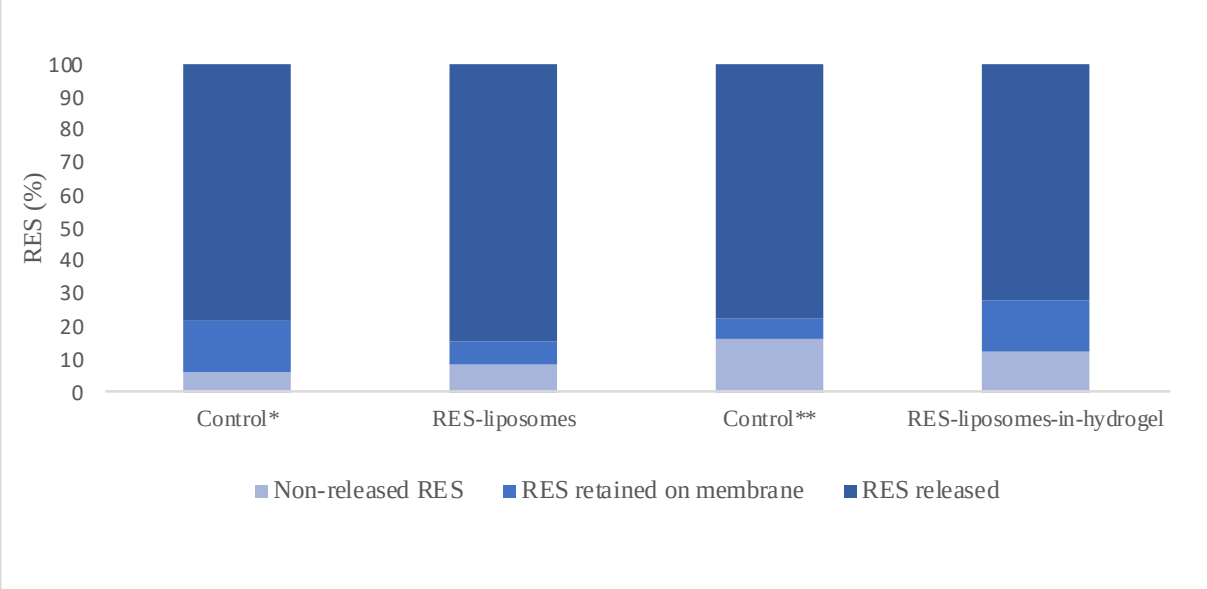

*Figure 19: Distribution of resveratrol (RES) after 8 h of release experiment. *=RES in propylene glycol. **=RES in propylene glycol in hydrogel (n=3).*

Similar findings (Jøraholmen *et al.*, 2015; Isailović *et al.*, 2013) are previously reported. One study reported a release of ~80 % of RES from the liposomes during the first 100 minutes of the experiment (Isailović *et al.*, 2013). In comparison, these levels was reached only after 6 hours in our experiments, indicating sustained release. However, it is noteworthy to point out

that these results are not directly commperable since different artifficial membranes were used in the experiments, expected to affect the release profile. A slower release from the liposomes, reaching a maximum release of RES (~40 %) after 8 hours into the experiment has been reported in our group earlier (Jøraholmen *et al.*, 2015). These results are however more comparable, since the same membrane and experiment setting were used. In both of the mentioned studies the RES-liposomes showed slower release compered to the controls (RES in propylene glycol in study by Jøraholmen *et al.*, RES in EtOH in study by Isailović *et al.*). No reported studies testing the RES release from chitosan hydrogel were found for comparison.

5.5 *Ex vivo* RES penetration

The RES penetration was tested on sheep vaginal tissue. RES dissolved in propylene glycol, which served as a control, RES-liposomes and RES-liposomes-in-hydrogel were tested. A rather low amount of RES was detected in the acceptor chamber, which indicates that there was close to no RES that had penetrated through the tissue. The analysis of the samples taken at the different time intervals, gave rather inconsistent data when using UV spectroscopy as an analysis method. This was probably due to interference of proteins and other remains from the tissue. However, after determining the remaining formulation in the donor chamber after completion of the experiment (8 hours), the amount of RES remaining was lower than the starting amount. This indicates that RES must have penetrated into the tissue; however, RES did not penetrate through the tissue. Hence, to support this indication, the samples were filtered and analyzed by the HPLC method expected to provide more sensitive data. A control test with pure phosphate buffer (pH 7.4) in the donor chamber under the same settings as for the formulation, was also assessed to possibly exclude the interference for the tissue. However, the same interference was observed, which indicated that proteins and other molecules originating from the tissue interfere with the determination. Due to the limited time frame, we could not invest more time in the optimization of the HPLC method.

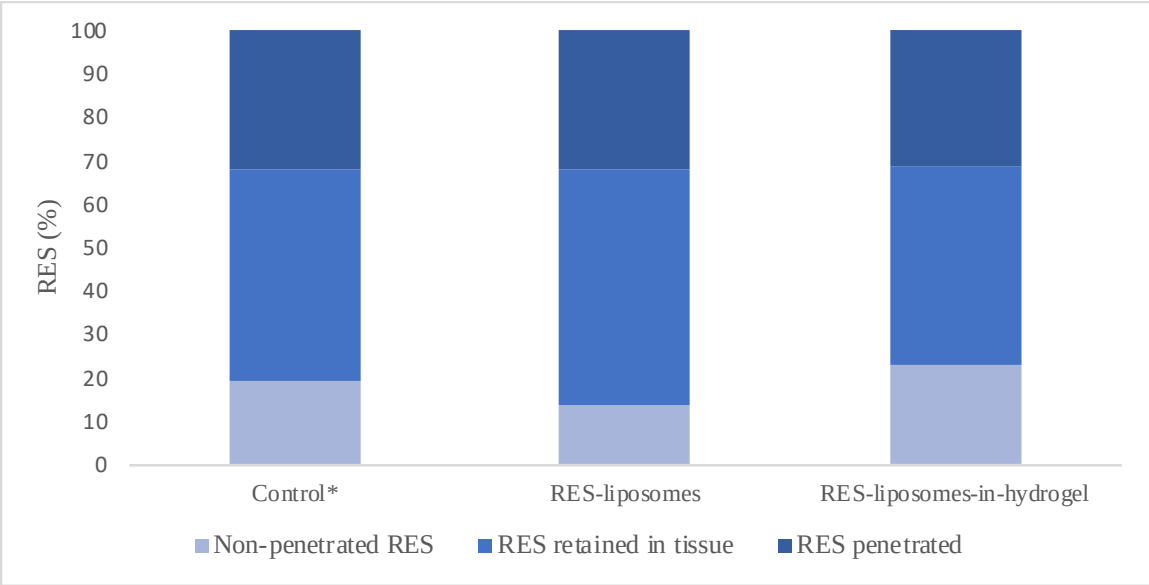

*Figure 20: Distribution of Resveratrol (RES) in ex vivo vaginal tissue. *=RES in propylene glycol (n=3)*

The results showed that only a small amount of RES penetrated the tissue (Figure 20). This indicates that the formulation prevented systemic delivery of RES which is highly favorable since *C. trachomatis* bacteria infect mucosal cells (Malhotra *et al.*, 2013) and mucosal site is the targeted site for RES action. Most of the formulation was indeed found in the tissue. Because the aim was to develop and optimize a formulation of RES-liposomes for local treatment of vaginal infections, these results were found highly favorable. This can be interpreted by the fact that the hydrogel provided a sustained release of RES from the formulation. The different samples tested for *in vitro* RES release showed similar distribution of released RES. As discussed previously, this might be inconsistent with what one would expect. One possible explanation could be that the applied method needs further optimization to be able to determine even small differences between release patterns from different formulations.

Even though the vaginal physiology is well known, there are still only a few studies on the potential of liposomes in vaginal delivery (Vanić and Škalko-Basnet, 2013). For example, Scalia *et al*. conducted a study where they tested RES-lipid microparticles coated with chitosan and incorporated it in a cream formulation that were applied onto human skin for the purpose of treatment of skin diseases (Scalia *et al.*, 2015). This is not a study that could directly be compared with the work in this thesis, since the study was conducted on human skin and RES was encapsulated in lipid microparticles. However, the reported study deals with RES in delivery system destined for localized therapy and their findings suggested that the formulation

enhanced the penetration of RES in stratum corneum compared to the control (carrier-free formulation). They suggest that the mechanism behind the action is the bioadhesion of the chitosan formulation. This could be related to the findings reported in Figure 20 that indicates that most of the RES from our novel formulation is retained within the tissue.

An *ex vivo* study on sheep vaginal tissue, using chitosan-coated liposomes containing clotrimazole for the treatment of vaginal infections reported by our laboratory (Jøraholmen *et al.*, 2014), showed similar results; the chitosan-coated liposomes prevented clotrimazole from penetrating through the vaginal tissue. Most of the drug was retained on or into the tissue, suggestion localized drug action.

5.6 Antioxidative potential of resveratrol

RES is reported to have antioxidant potential and the mechanism behind its activity is associated to the potential of RES to promote activity of different antioxidant enzymes (de la Lastra and Villegas, 2007). DPPH and $ABTS^{\bullet+}$ scavenging assays are commonly used methods to evaluate the antioxidative effects of phenolic compounds (Noreen *et al.*, 2017). The radical scavenging test was performed by testing the radical scavenging activity of RES against the DPPH and $ABTS^{\bullet+}$ radicals. The activity was compared with the activity of well-known antioxidants vitamin C and vitamin E. The scavenging activity was calculated by the equation:

Equation 1:

$$\text{Inhibition (\%)} = (100 \times (A_{Control} - A_{Sample})/A_{Control}).$$

Were $A_{Control}$ is the absorbance for the control/radicals without samples and A_{Sample} is the absorbance where samples are present (Jøraholmen *et al.*, 2015; Hangun-Balkir and McKenney, 2012). The test was performed in triplicates, and the results are expressed as mean ± S.D. All samples showed a concentration-independent scavenging activity, corresponding to the results published by Jøraholmen *et al.* (Jøraholmen *et al.*, 2015). It is evident that RES exhibited a greater scavenging activity against $ABTS^{\bullet+}$ as compared with DPPH (Figures 21 and 22.).

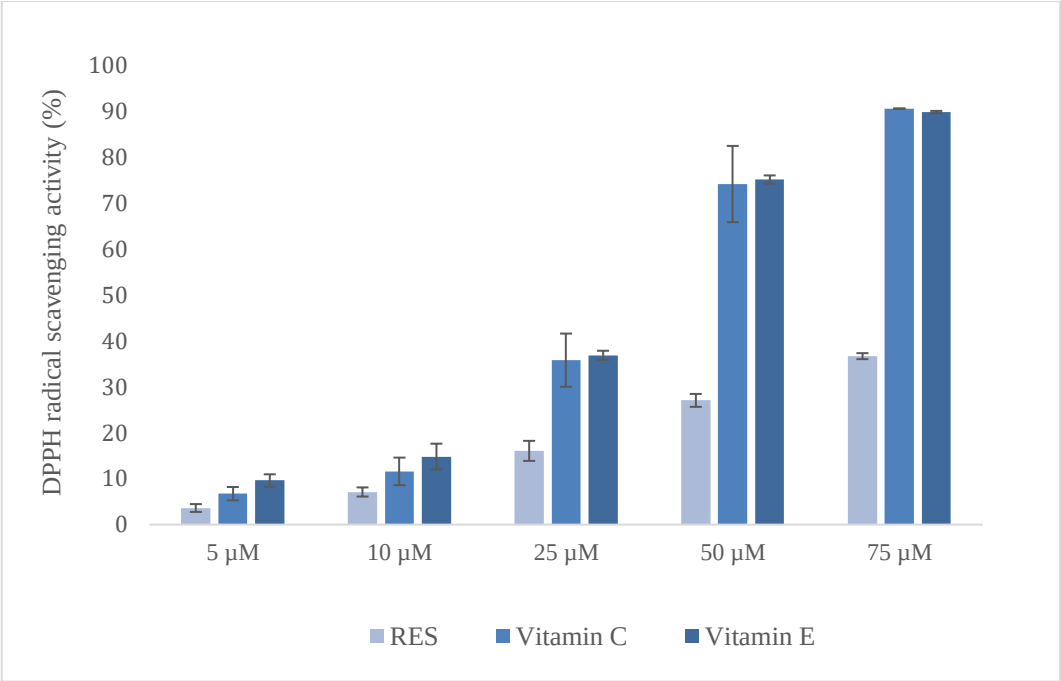

Figure 21: DPPH radical scavenging activity (%) of resveratrol (RES), vitamin C and vitamin E (n=3).

The results indicate that RES possess antioxidative properties against DPPH radicals; the by reduction mechanism of action can be simplified as:

$$DPPH^{\bullet} + AH \rightarrow DPPH\text{-}H + A^{\bullet}.$$

Where A represent the antioxidant. The radical scavenging activity is also visually observable since DPPH in EtOH is purple color solution which will change accordingly to the amount of radical scavenged. At the highest concentration RES showed the ability to scavenge approximately 45 % of the radicals (Figure 21). Even though the antioxidative effect of RES was confirmed, the effect was significantly lower ($p<0.05$) compared to the activity of both vitamin C and E.

The $ABTS^{\bullet+}$ is also unstable, therefor it has to be prepared prior to the scavenging assay is performed (Opitz *et al.*, 2014). $ABTS^{\bullet+}$ is created by mixing of ABTS with potassium persulfate. It is also visually observable since $ABTS^{\bullet+}$ is of a blue-greenish color which will change accordingly to the amount of radical scavenged (Opitz *et al.*, 2014).

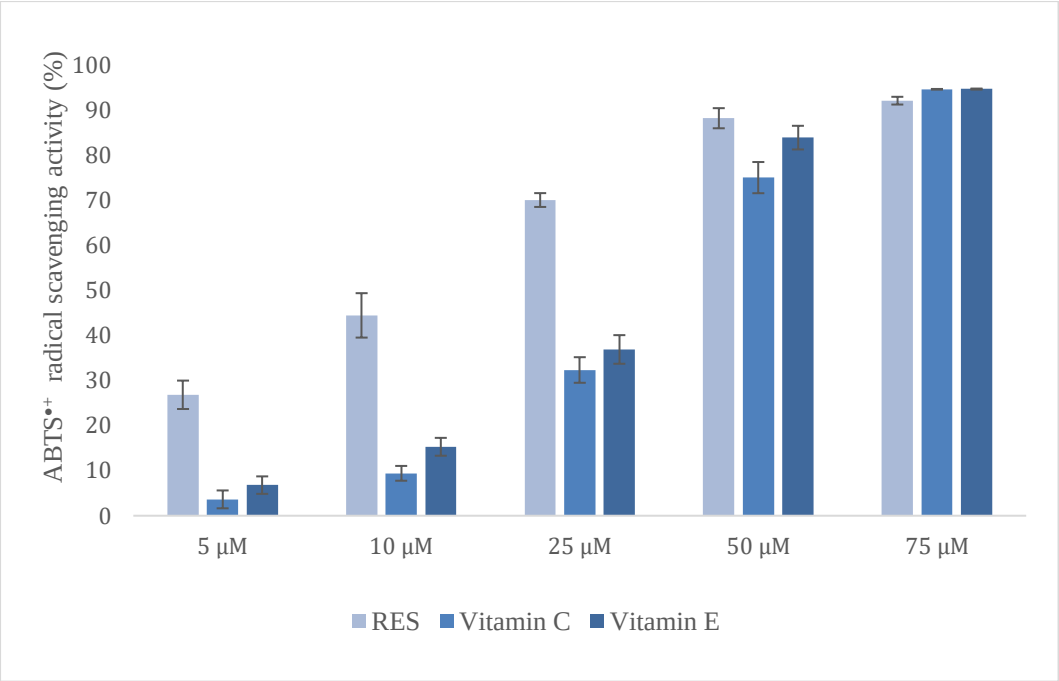

Figure 22: ABTS•+ radical scavenging activity (%) of resveratrol (RES), vitamin C and vitamin E (n=3).

Regarding to the ABTS•+ scavenging activity, the results show that RES exhibited a significantly ($p>0.05$) higher scavenging activity in the lower concentrations (5–25 µM) compared to the activity of both vitamin C and vitamin E (Figure 22). In the higher concentrations (50–75 µM) RES exhibited a close to equal scavenging activity ($p<0.05$) compared to vitamin C and vitamin E. The findings are important considering that the aim of the therapy is to achieve higher outcome at lower doses applied. Moreover, the results correspond to previously reported literature (Jøraholmen *et al.*, 2015). The results clearly justify the proposed use of RES in localized vaginal therapy of infections and inflammation.

6 Conclusions

A formulation comprising RES-liposomes-in-hydrogel system for the local treatment of vaginal infections was developed. RES-liposomes prepared by the film-hydration method produced liposomes with high and reproducible entrapment efficiency. Extrusion enabled a controlled size reduction of liposomes to the desirable size-range and a very low PI value indicated a rather uniform size distribution, confirming extrusion as a suitable size reduction method for liposomes.

The liposome-in-hydrogel formulation expressed mucoadhesive properties and good mechanical properties that confirm its potential for local administration at vaginal site. Additionally, these properties were shown to be maintained after two months of storage. Liposomes provided a sustained release of entrapped RES *in vitro*, and the release was prolonged when liposomes were incorporated in hydrogel. Further, only a small amount of RES was able to penetrate through vaginal tissue and most of the RES remained on the top and within the tissue. The systemic absorption is not desirable in the local treatment of vaginal infection; thus, RES-in-liposomes-in-hydrogel assured localized RES delivery.

The antioxidative effect of RES was confirmed. RES expressed higher or equal ABTS scavenging activity compared to other well-known antioxidants and shows promise as an antimicrobial substance.

7 Perspectives

Liposomes-in-hydrogel shows the potential as a delivery system for RES in local vaginal therapy, however, further investigation is required to confirm the promise. An *ex vivo* mucoadhesion study needs to be optimized and further evaluation of the delivery system and its interaction with soft tissue is needed. Further, the analysis method for determining the *ex vivo* penetration of RES needs further optimizing to enable an accurate and reproducible quantification of RES. The analysis method needs to be sensitive and able to adjust for interference from proteins and tissue remains from the vaginal tissue. The developed delivery system need to be evaluated for its stability in the simulated vaginal environment and overall safety in vaginal application. Further studies on the antibacterial effect of the RES in the novel system are required to confirm real potential in the local treatment of chlamydia infection.

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