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Surface modification of liposomes increases drug efficacy in local vaginal therapy

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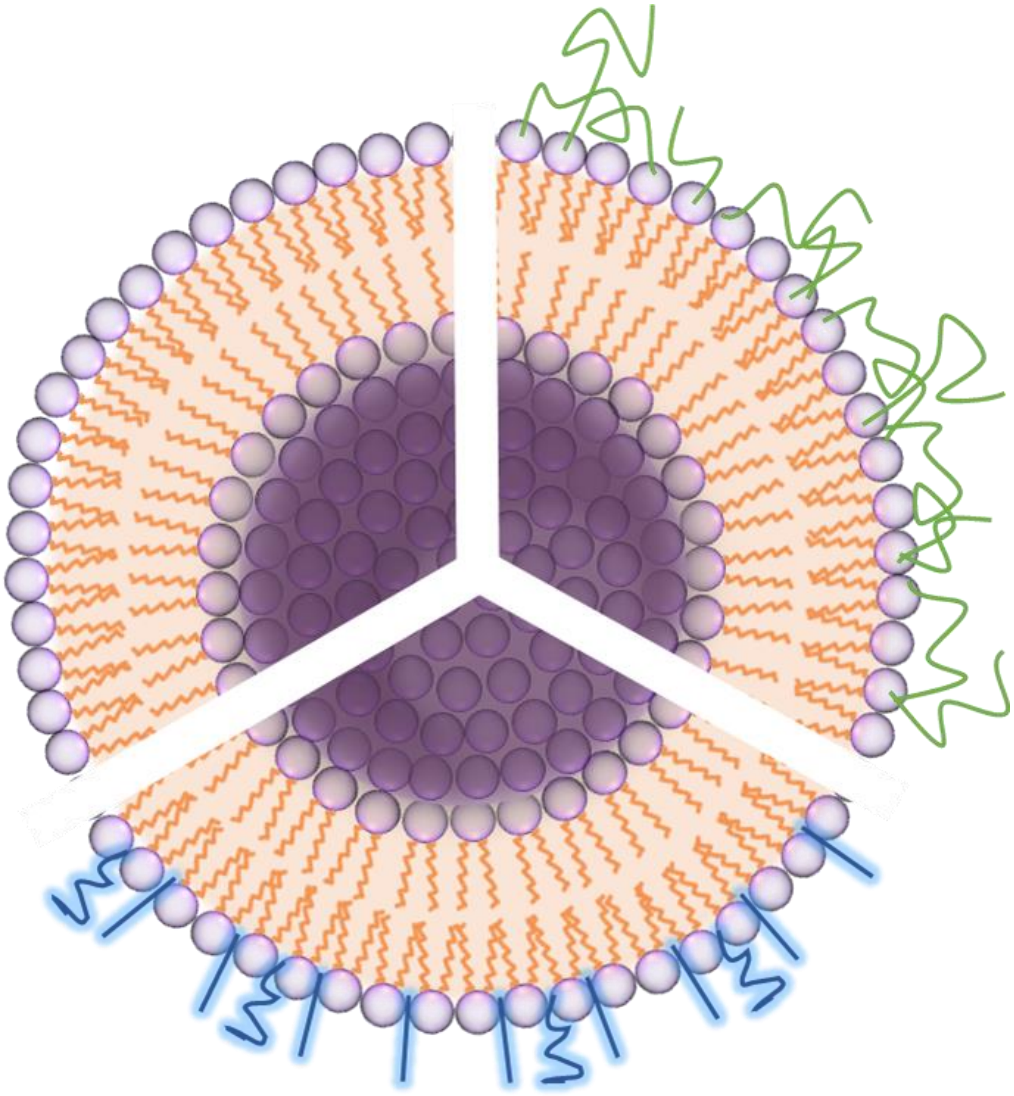

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May Wenche Jøraholmen

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List of abbreviations

ABTS	2,2’-azino-bis(3-ethyl benzothiazoline)-6-sulfonic acid diammonium salt
CLZ	Clotrimazole
DIC	Differential interference contrast
DMSO	Dimethylsulphoxide
DPPH	1,1-Diphenyl-2-picrylhydrazyl
EC_{50}	Effective concentration for 50 % decrease
ELISA	Enzyme-linked immunoassay
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
HPV	Human papilloma virus
HSV	Herpes simplex virus
IC_{50}	50 % inhibitory concentration
IFN	Interferon
IL-1β	Interleukin-1β
IL-6	Interleukin-6
LPS	Lipopolysaccharide
LUV	Large unilamellar vesicles
MUV	Multilamellar vesicles
NAME	L-nitro-arginine methyl ester

NO	Nitric oxide
PC	Phosphatidylcholine
PEG	Polyethylene glycol
PI	Polydispersity index
PM	Pig mucin
RES	Resveratrol
ROS	Reactive oxygen species
SFS	Semen fluid simulant
SOD	Superoxide dismutase
STD	Sexual transmitted disease
STI	Sexual transmitted infection
SUV	Small unilamellar vesicles
S-100	Soybean lecithin, phosphatidylcholine
TNF-α	Tumor necrosis factor-α
VFS	Vaginal fluid simulant

Abstract

The vaginal inflammation and infection are one of the major female health issues, and unfortunately rather neglected. The anatomical structure and physiological conditions make vagina vulnerable to inflammation and infection, which if not successfully treated, can lead to deteriorating female health conditions. In pregnant patients, the pregnancy outcome can be severely affected. Although a standard treatment for vaginal infection is available, it is often not successful and recurrence rates are high. Therefore, a patient-focused drug development targeted at the vaginal inflammation and infection is the current social demand. Research and practice have shown that the topical treatment by the drugs against vaginal inflammation and infection can be superior comparing to the classical oral drug administration. However, the thick vaginal mucus lining the luminal surface of vagina and cervix, which protects the underlying tissue, limits the ability of a drug to reach vaginal mucosa. The success of mucosal delivery is highly dependent on a suitable drug carrier. Current dosage forms suffer from limited residence time at administration site and unpleasant leakage of dosage forms residues due to the self-cleansing action of the vagina, resulting in a reduced therapeutic effect. Therefore, liposomal drug delivery systems, with the ability to incorporate poorly soluble drugs and assure their stability, would be suitable for this purpose. Moreover, the modification of liposomal surface with mucoadhesive or mucoresistant polymers, might further enable improved mucosal drug delivery by providing prolonged residence time or rapid mucus-penetration, respectively.

We selected chitosan as a mucoadhesive polymer due to its biocompatibility, low toxicity and intrinsic anti-microbial potential. By combining the liposomal carrier and the mucoadhesive chitosan, an optimized vaginal drug delivery system with specific, prolonged and controlled drug release properties might be developed. Alternatively, improved drug delivery to vaginal mucosa can be provided through mucoresistant properties of the delivery system. Polyethylene glycol (PEG) plays an important role in this approach, and PEGylated liposomes enable controlled drug release in close proximity to the vaginal epithelium. Three model drugs/active ingredients were tested in mucoadhesive/mucoresistant liposome-based delivery systems, namely clotrimazole, resveratrol and interferon. Particularly interesting were the anti-oxidative and anti-inflammatory properties of resveratrol, which has a great potential in the treatment of vaginal inflammation and infection, however, its low solubility and poor bioavailability

accompanied by poor stability limit its therapeutic effects. Liposomal system enhanced its activities and confirmed its potential.

The *in vitro* drug release and *ex vivo* penetration confirmed a sustained release of all liposomally-associated drugs/active molecules. PEGylated liposomes (mucoresistant) assured improved penetration of interferon. By modulation of liposomal surface properties to be either mucoadhesive or mucoresistant it is possible to achieve prolonged residence time or deeper penetration of drug within vaginal epithelium, respectively. Moreover, the system can be modified for the different drugs, regardless of their molecular size and physicochemical characteristics.

List of publications

Paper I:

Jøraholmen, M.W., Vanić, Ž., Tho, I., Škalko-Basnet, N., 2014. Chitosan-coated liposomes for topical vaginal therapy: Assuring localized drug effect. *International Journal of Pharmaceutics*. 472, 94-101.

Paper II:

Jøraholmen, M.W., Škalko-Basnet, N., Acharya, G., Basnet, P., 2015. Resveratrol-loaded liposomes for topical treatment of the vaginal inflammation and infections. *European Journal of Pharmaceutical Sciences*. 79, 112-121.

Paper III:

Jøraholmen, M.W., Basnet, P., Acharya, G., Škalko-Basnet, N. PEGylated liposomes for topical vaginal therapy: Mucus-penetration improves delivery of interferon alpha. *Manuscript*

1 Introduction

Recently released data (the 2014 STD Surveillance Report) from the U.S. Centers for Disease Control and Prevention indicate a significant increase in the sexually transmitted infections (STIs) in 2014, the first rise in sexual transmitted diseases (STDs) incidents since 2006. Although human immunodeficiency virus (HIV) infections received relatively lot of attention, non-HIV genital infections are clearly a neglected area of biomedical research. Yet, genital infections represent a major public health problem with increasing incidence, affecting both female and male population. Due to the anatomical structure and physiological functions of the vagina, women are predisposed to pathogenic vaginal infections. Since vaginal infections are readily transmitted to men, a more appropriate terminology would be to use the term genital infections. However, this project focused on the optimization of local vaginal therapy and the term vaginal infections is applied throughout the thesis.

Vaginal infections are common in women of all ages and if not treated adequately, are associated with serious consequences such as infertility, preterm delivery, increased neonatal mortality and morbidity as well as induction of malignancy (Fenton and Lowndes, 2004). In spite of the concerns and the serious consequences, the therapeutic options offered by conventional treatment remain to be limited. Better understanding of the healthy and diseased conditions, and design of advanced drug delivery systems to assure improved therapy outcome are the prerequisites for successful treatment of vaginal infections and inflammation.

Any condition causing an inflammation of the vagina is generally referred to as vaginitis, one of the most frequent gynecological problems faced by women of all ages (Hainer and Gibson, 2011). Vulvovaginal candidiasis, trichomoniasis and bacterial vaginosis are the most common causes of vaginitis (Owen and Clenney, 2004). In general, the topical treatment is found to be as effective as oral drug therapy in the treatment of vaginitis, and is preferential due to minimal adverse effects. However, the local characteristics of infected vaginal tract might influence the efficacy of topically administered dosage forms and limit the efficacy of the treatment. These characteristics need to be considered in the design of effective topical vaginal therapy (Palmeira-de-Oliveira *et al*., 2015). Moreover, the *Herpes simplex* virus (HSV) and Human papilloma virus (HPV), the most common vaginal viral infections, represent a specific therapeutic challenge (Viera *et al*., 2010). The elimination of virus is a tough challenge for current treatment options resulting in a high recurrence and increasing number of infections. Thus, the search for optimal treatment is urgent, including the search for potential therapeutic agents within natural products

and biologicals. Nanomedicine has the potential to address some of the challenges of the limited therapy success and improve the therapy outcome (Vanić and Škalko-Basnet, 2013).

1.1 Common vaginal infections

1.1.1 Vulvovaginal candidiasis

Candida albicans is a natural part of the normal vaginal microflora of women in reproductive age, yet it is a pathogenic fungus potentially causing mucosal infection and can be referred to as an opportunistic pathogen. It is accountable for up to 92 % of vulvovaginal candidiasis incidents (Sobel, 1997). Disturbance of the normal microbial balance or a weakened host defense can result in infection caused by *Candida* (Pavelić *et al*., 2005). The infection is initiated by the adhesion of *Candida* to epithelial cells (Johal *et al*., 2014). Definite factors that cause the virulence of *Candida* are not clear; however, pregnancy, diabetes and the use of antibiotics or oral contraceptives can increase the perceptibility to infection. Although it is not considered a sexually transmitted disease, sexual transmission do occur. Up to 75 % of women will experience a vaginal candidiasis infection at least once in their lifetime and several endure recurrent infections (Palmeira-de-Oliveira *et al*., 2015). The conditions can be treated both orally and locally, and the efficacy of the two approaches is considered equivalent. Nevertheless, local drug therapy presents several advantages such as reduced incidence of adverse effects and avoidance of extensive drug absorption (das Neves *et al*., 2008). Mechanisms involved in the pathogenicity of candidiasis, such as the ability to form biofilms is possibly also relevant in the explanation of treatment failure (Palmeira-de-Oliveira *et al*., 2015). Imidazole-derivative anti-fungal agents, including clotrimazole, are main topical treatment regimens of candidiasis (Owen and Clenney, 2004).

1.1.2 *Herpes simplex* virus (HSV)

HSV is highly contagious, and oral or genital sites are easily infected via direct contact with infected individuals. Damaged skin or mucosal surfaces permit entry of the virus that replicates within the epithelial cells (Cortesi and Esposito, 2008). In general, facial infection is caused by HSV-1, while HSV-2 induces genital herpes (Devi Kusum and Bhosale, 2009). Due to the virulence and high frequency of unrecognized and undiagnosed infections, HSV is one of the most common STIs. Furthermore, recurrent episodes will follow once infection occur. Serious consequences such as neonatal transmission and increased

susceptibility to HIV infection make HSV a significant public health concern (Gupta *et al*., 2007; Gardella, 2011). Systemic treatment by anti-viral agents, such as acyclovir, are usually standard treatment of symptomatic genital HSV infection. In addition to the attached systemic adverse effects, anti-viral treatment merely subside symptoms and duration of lesions, and does not eradicate infection or prevent reoccurrence (Gupta *et al*., 2007; Viera *et al*., 2010). The number of infections are increasing, and strategies to prevent HSV transmission such as topical viracides and the development of new anti-HSV products is ongoing. One approach has been to examine natural products isolated from plants for anti-HSV activity (Docherty *et al*., 2005).

1.1.3 Human papilloma virus (HPV)

HPV infections are considered the most common STIs (Forcier and Musacchio, 2010; Foldvari, 2012). Similar to HSV, HPV is transmitted via mucosal surfaces (Patel *et al*., 2012). Currently, more than 120 subtypes of HPV have been identified, of which approximately 40 can infect the human genital tract (Chinchai *et al*., 2012; Patel *et al*., 2012). HPV, primarily type 16 and 18, is a major cause of cervical cancer. Further, HPV is associated with other cancers and is principally responsible for genital warts (Forcier and Musacchio, 2010). To date, there is no available anti-viral therapy (Rodríguez-Gascón *et al*., 2015). The common treatment of genital warts is removal of growth, potentially combined with topical treatment. This is associated with pain and systemic and local adverse effects, yet not elimination of the latent viral infection (Fathi and Tsoukas, 2014). Developed vaccines prevent HPV infection, but only if administered before the sexual debut, and a curative therapy is yet to be found. One approach with great potential is the further development of biologicals, of which interferon alpha (IFN-α) is used as intralesional injection in the treatment of HPV infection (Foldvari *et al*., 2011).

1.2 Current and perspective therapeutic approaches to vaginal infections

1.2.1 Candidiasis: Clotrimazole

The anti-fungal agent clotrimazole is known to be effective in the local treatment of candidiasis and is commonly used due to its potent local action with no major side effects (Kast *et al*., 2002). Clotrimazole formulations such as vaginal tablets (Canesten®) and gels (Candid V Gel®) are available for the local therapy of candidiasis (Wong *et al*., 2014). However, the conventional clotrimazole formulations aimed for vaginal

administration suffer from limited residence time at the site of administration, impairing the therapeutic outcome and resulting in a need for frequent drug administration (Bilensoy *et al*., 2006).

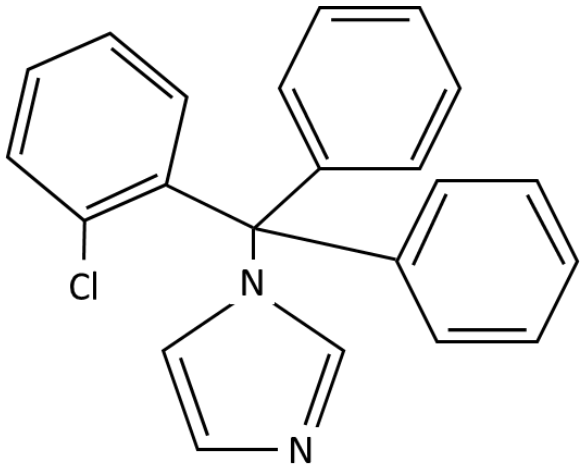

Figure 1.1: Structure of clotrimazole.

Clotrimazole (Figure 1.1) is a highly lipophilic drug (log P of 3.5), and presents challenges in the formulation of novel dosage forms due to its poor aqueous solubility. Hence, clotrimazole was found to be a suitable choice of model drug for the development of optimal vaginal drug delivery system. Its incorporation in mucoadhesive liposomes is expected to improve the efficacy of its therapy.

1.2.2 HSV infections: Resveratrol

Resveratrol might be the active substance with unexplored potential in topical treatment of vaginal infections. It is a substance of natural origin, with strong anti-oxidant and anti-inflammatory effect. It occurs in *cis* and *trans* isomers, of which the *trans*-resveratrol (Figure 1.2), with the chemical name *trans*-3,5,4'-trihydroxy stilbene, is the most biologically active (Mukherjee *et al*., 2010). Resveratrol is highly photosensitive and when exposed to light *trans*-resveratrol will convert to the *cis*-isomer, which is stable only at neutral pH and when completely shielded from light (Amri *et al*., 2012).

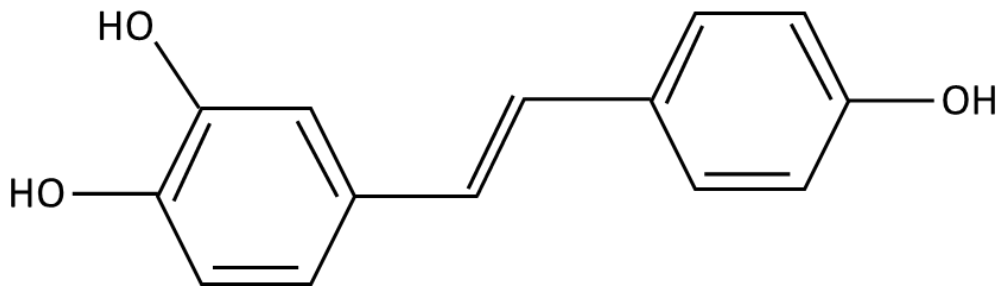

Figure 1.2: Structure of resveratrol.

The many beneficial effects of resveratrol have recently gained great attention among scientists, nutraceutical and food industries, especially its anti-oxidant effects. Pharmacological activities of resveratrol such as anti-aging, anti-cancer, cardio-protective, anti-inflammatory, anti-microbial, anti-oxidant, anti-inflammatory and anti-viral effects have been reported (Baur and Sinclair, 2006). Considering the local vaginal therapy, its strong anti-oxidant and anti-inflammatory effects are of great interest for the treatment of vaginal inflammation and infections (Bhat *et al*., 2001; Houillé *et al*., 2014). Further, the local administration of resveratrol has shown reduced replication of HSV in both skin (Docherty *et al*., 2004) and vagina (Docherty *et al*., 2005). However, resveratrol is poorly water-soluble (log P of 3.1) and its bioavailability and stability is rather limited (Amri *et al*., 2012). Hence, there is a need for a suitable drug delivery system that not only enables controlled drug delivery, but also protects the drug from environmental and chemical changes. Liposomal delivery system is expected to serve both purposes.

1.2.3 HPV infections: Interferon (IFN)

In a search for therapeutic options to treat HPV infections, recent attention has been focused on biologicals. Among biologicals, IFNs, naturally occurring cytokines produced by leukocytes mainly in immunomodulating response of viral infection, are of particular interest (Bergman *et al*., 2011). IFNs are involved in the body's natural defensive responses to foreign components such as microbes, tumors and antigens, and presents one of the most widely investigated and clinically used biopharmaceuticals (Baron *et al*., 1991). Their anti-viral, anti-proliferate and immunomodulatory effects have been confirmed and several subtypes are clinically approved for various indications. Among these subtypes IFN α-2b, with the trade name Intron A, is FDA-approved for indications such as chronic hepatitis B and C, malignant melanoma and condylomas (Hamidi *et al*., 2007). For visible condylomas, intralesional IFN α-2b appear to

be applicable treatment, yet this includes pain and side effects due to systemic exposure. Further, this is not suitable treatment for latent or subclinical infections and a non-invasive approach is desirable (Foldvari and Kumar, 2012). Foldvari and Moreland introduced the vaginal administration of IFN α-2b, for the treatment of HPV infections (Foldvari and Moreland, 1997). Topical treatment provides advantages such as drug delivery directly to site of infection and increased efficiency at lower doses, reduced adverse effects due to decreased systemic levels and a more adequate treatment that include non-visible lesions (Hamidi *et al*., 2007; King *et al*., 2013). Like other biologicals, the IFNs are labile drugs prone to alterations (Karau *et al*., 1996). A suitable delivery system might alter the bioavailability, increase the stability and provide controlled release of IFN α-2b (Yang *et al*., 2006; Li *et al*., 2011).

1.3 The vagina

The human vagina is a tubular, fibromuscular organ that connects the cervix and the vulva and plays a major role in reproduction. It is slightly S-shaped with dimensions of 8.4 - 11.3 cm in length and 2.1 - 5.0 cm in diameter (de Araújo Pereira and Bruschi, 2012). Histologically, the vaginal wall consists of three layers; the epithelial layer (stratified squamous epithelium resting on *lamina propria*), the muscular layer and the *tunica adventia* (Figure 1.3).

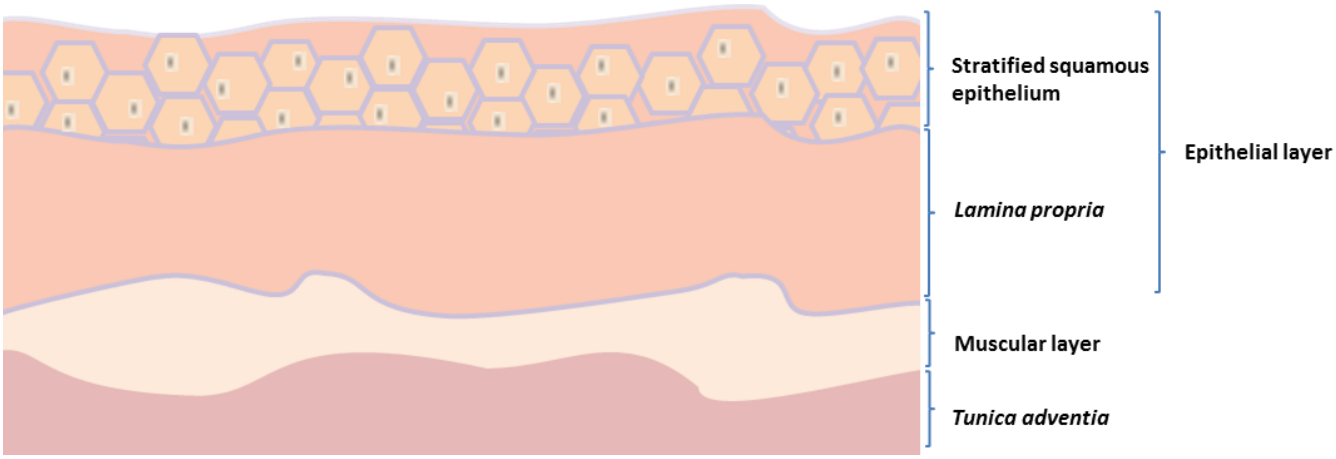

Figure 1.3: Schematic drawing of the vaginal wall.

The stratified squamous epithelium contains numerous folds, or rugae, providing distensibility and an increased surface area for absorption. The thickness of the vaginal epithelium is age dependent. With hormonal activity the thickness increases, hence, the epithelium is relatively thin before puberty (except in newborns), increasing in thickness after puberty and then retrieving a state similar to pre-puberty after menopause. Additionally, the hormone levels during the menstrual cycle also affect the epithelial thickness (Hussain and Ahsan, 2005).

Although considered a mucosal tissue, vagina does not have secretory glands. However, a certain amount of fluid is secreted, mainly constituted of transudate from vaginal and cervical cells. Studies suggest that the daily production of fluid is approximately 6 g (Marques *et al*., 2011). The vaginal fluid is a mixture of several components including enzymes, enzymatic inhibitors, proteins, carbohydrates, amino acids and cervical mucus composing a moist surface film covering the vaginal wall. The cervicovaginal mucus forms a continuously renewed, semipermeable viscoelastic barrier that protects the underlying tissue from infection. Vaginal mucus consists mainly of water (90 - 95 %) and mucins (1 - 2 %) and has a pH around 4.5 in healthy women of reproductive age (das Neves *et al*., 2015). The mucus barrier consists of two layers, namely; the luminal mucus layer (soluble secretory mucin) which is rapidly cleared and the unstirred mucus layer (membrane bound mucins) which is adherent to vaginal epithelium and undergo a slower physiological turnover (Wong *et al*., 2014; Edsman and Hägerström, 2005). Foreign particles are sterically trapped and effectively removed by mucus clearance, preventing pathogens from reaching the mucosal tissue. Nevertheless, virus has the ability to overcome this barrier and cause mucosal infection. Environmental changes such as changes in pH due to vaginal infections or changes in viscosity of vaginal fluid affect the properties of mucus. When exposed to infection, a reduction in the viscosity of vaginal fluid causes reduced barrier properties and an increased risk of secondary infections (Palmeira-de-Oliveira *et al*., 2015). The mucus viscosity varies also during the different phases of the menstrual cycle. A more watery mucus that facilitates penetration occur during the first half of the cycle, due to an increase of estrogen level, while progesterone originate a more viscous mucus during the second half of the cycle (Caramella *et al*., 2015).

Vaginal pH also attributes to the protection against infections (de Araújo Pereira and Bruschi, 2012). Normal vaginal microflora is dominated by lactobacilli, creating a natural acidic environment by converting glycogen into lactic acid resulting in a pH of 3.8 - 4.2 in healthy, pre-menopausal women (Alexander e*t al*., 2004). The dynamic system of the vaginal flora can be affected by the presence of semen, menstrual blood or disease, such as bacterial infections, that will temporarily elevate the pH level. A maintenance of a low

vaginal pH is necessary for the prevention of microbial growth and vaginal infections (Mallipeddi and Rohan, 2010a). Exposure to unnatural products such as soap and sanitary towels or tampons might also threaten the healthy vaginal environment. Close contact with these foreign materials can lead to elevated pH, dryness and irritated mucosal tissue, thus facilitate the establishment of infection. Additionally, the local application of vaginal dosage forms could disturb the vaginal flora (Fichorova *et al*., 2011).

1.4 Vaginal drug delivery

The vagina provides a suitable site for local as well as systemic drug delivery due to its large surface area, rich blood supply, avoidance of first-pass metabolism, relatively high drug permeability and the possibility of self-insertion and removal of dosage form. Local application further provides a more concentrated delivery at the site of action enabling lower dose regimens. However, the vagina is often not prioritized as route of administration, due to factors such as gender specificity and individual or cultural sensitivities. Traditionally, vagina as a site for drug delivery has been considered for locally acting drugs, such as anti-microbial, anti-viral and contraceptives. Advances in the development of vaginal drug delivery include both local and systemic therapy with a wide range of compounds (Baloglu *et al*., 2009; Srikrishna and Cardozo, 2013). Despite the interest for this non-invasive route of administration in both the scientific community and pharmaceutical industry, only a few conventional vaginal dosage forms are currently available. Further, current dosage forms such as creams, foams, gels and tablets suffer from leakage and limited residence time at administration site due to the self-cleansing action of the vagina resulting in reduced therapeutic effect (Baloglu *et al*., 2009).

The basic physiology of the vagina must be considered in the development and optimization of vaginal delivery systems. In order to obtain a suitable dosage form and delivery system for successful vaginal therapy it has to be modified for this specific route of administration. Affecting factors such as anatomy, presence of mucus and the changes in vaginal fluid, pH and the thickness of epithelium influence the efficacy of administered drug (das Neves *et al*., 2011b; Machado *et al*., 2015). Increased vaginal pH due to menopause, the presence of semen, menstrual blood or disease may influence the solubility and stability of the drug. The composition, volume and viscosity of vaginal fluids are influenced by age, the menstrual cycle and sexual arousal, and affecting the release pattern of the vaginal delivery systems. The vaginal mucus has several important physiological functions and the distribution, penetration and residence time of locally applied delivery systems is greatly influenced by the mucus. The ability of a drug to overcome

the mucus barrier and reach the vaginal mucosa is highly dependent on a suitable drug carrier (Gupta *et al*., 2011; Vanić and Škalko-Basnet, 2013; Ensign *et al*., 2014). In parallel to the local physiological conditions in the vaginal cavity, the physiochemical properties of a drug such as its molecular weight, lipophilicity and the degree of ionization affect the possible absorption across the vaginal epithelium (Cicinelli, 2008). Considering local therapy, the absorption of drug is not desirable, especially in pregnant patients. The intended effect of the therapy (local or systemic) contributes to the choice of drug formulation (Srikrishna and Cardozo, 2013).

Ideally, a vaginal delivery system should be easy to use, discreet, offer a reversible application, painless to the patient, cost-effective, non-toxic, non-irritating to the mucus membrane, widely available and safe for continuous administration (Srikrishna and Cardozo, 2013; Hussain and Ahsan, 2005; das Neves and Bahia, 2006). All earlier mentioned challenges related to the local therapeutic effect need to be overcome to obtain an optimal local treatment of vaginal infections. A uniform distribution of drug throughout the vaginal cavity and maintenance of a sufficient drug concentration at vaginal site is necessary for a successful therapy. Drugs administered vaginally that have targets within the mucosa, must move through and penetrate the unstirred layer before it is shed or degraded. This can be achieved by adequate retention time at vaginal site or rapid diffusion through mucus.

Over the last two decades, mucoadhesive delivery systems, particularly nanopharmaceuticals have been extensively investigated as means of improving drug delivery in different mucosal tissues, including the vagina (Vanić and Škalko-Basnet, 2013; 2014). Recently, Lai and colleagues proposed a mucoresistant approach, which considers successful drug delivery obtained by mucus-penetrating particles (Lai *et al*., 2007). This approach was inspired by the evolved properties of viruses, enabling them to penetrate mucus. In this project, we focused on the development of liposomal delivery systems for local treatment of vaginal infections. To overcome the obstacles of vaginal drug delivery, such as the mucus barrier, we aimed to improve liposomal properties by surface modifications. Conventional (non-coated) liposomes were compared to mucoadhesive (chitosan-coated) and mucus-penetrating (PEGylated) liposomes (Figure 1.4).

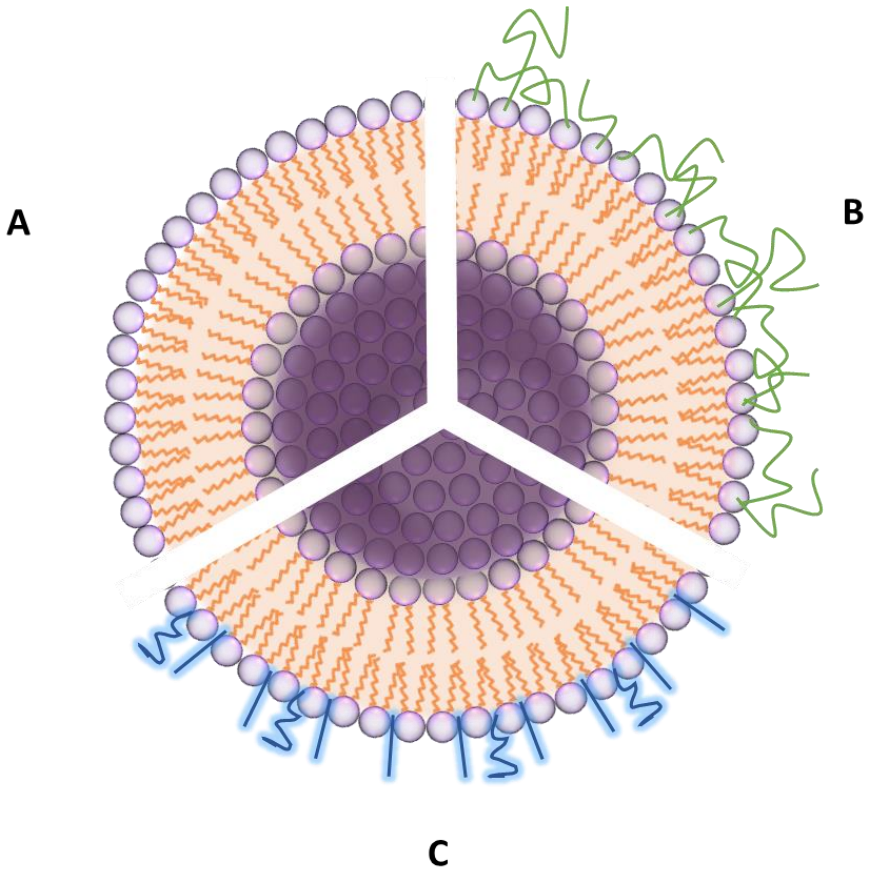

Figure 1.4: Schematic drawing representing liposomes with various surface properties; A) Non-coated, conventional liposome, B) Polymer-coated, mucoadhesive liposome, C) PEGylated, mucus-penetrating liposome.

1.5 Mucoadhesion

Mucoadhesion is a state in which a material adheres to a mucosal tissue and is held together for extended periods of time by interfacial forces (Smart, 2005). In respect to drug delivery, this interaction is between the surface of mucus and the surface of the drug delivery system. Mucoadhesion occurs in two steps (Figure 1.5); the contact stage (established contact) and the consolidation stage (formation of bonds).

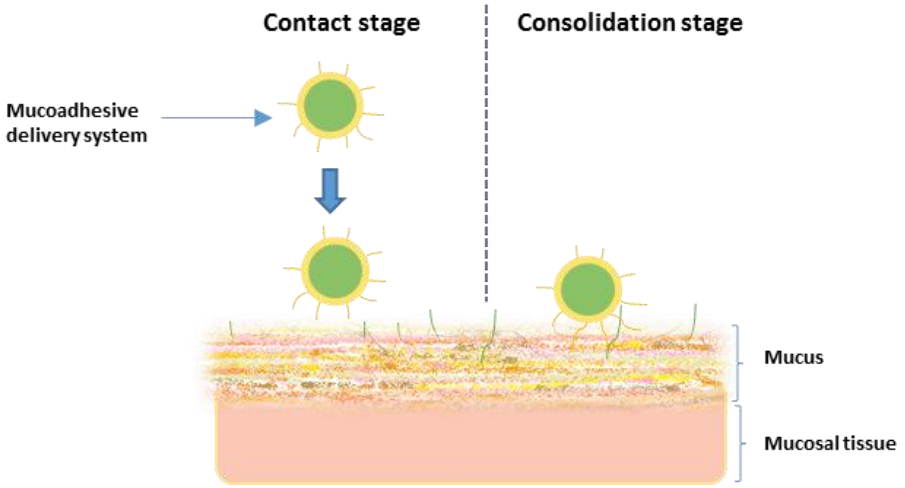

Figure 1.5: The two steps of mucoadhesion.

The event of mucoadhesion is complex and not yet fully understood. Six theories have been proposed to explain the mechanism (Smart, 2005), namely the electronic theory, the wetting theory, the adsorption theory, the diffusion theory, the fracture theory and the mechanical theory (Figure 1.6). The electronic theory explains adhesion as electron transfer between mucus and the mucoadhesive system forming an electrical double layer at the interface that generates attractive forces. The wetting theory relates to the ability of a mucoadhesive polymer to spread over a mucosal tissue and proposes adhesion that occurs in the presence of liquid, where the liquids may act as an adhesive at the interface depending on the contact angle between liquid and surfaces. The adsorption theory is generally the most accepted principle of adhesion (Vanić and Škalko-Basnet, 2014). It is based on adhesion due to the presence of intermolecular forces, hydrogen bonding and/or Van der Waal's forces between the surface of mucus and the mucoadhesive system. The diffusion theory discusses the interpenetration of glycoprotein mucin chain to sufficient depth to form a semi-permanent adhesive bond between the surfaces. The mechanical theory proposes that liquid adhesives diffuses into irregularities present on the surfaces developing an interlocked structure. The fracture theory describes the force required for detachment of the two involved surfaces after adhesion is established. Each of the proposed theories fails to independently explain mucoadhesion and the actual mucoadhesion process is probably achieved through a combination of several mechanisms (Edsman and Hägerström, 2005; Serra *et al*., 2009).

Physiological variables such as the changes in the amount of vaginal fluid may affect the mucoadhesiveness. An increased amount of fluid will facilitate the interaction between mucus and the

mucoadhesive molecules, nevertheless, the degree of consolidation and strength of bonding will be decreased (das Neves *et al*., 2011b).

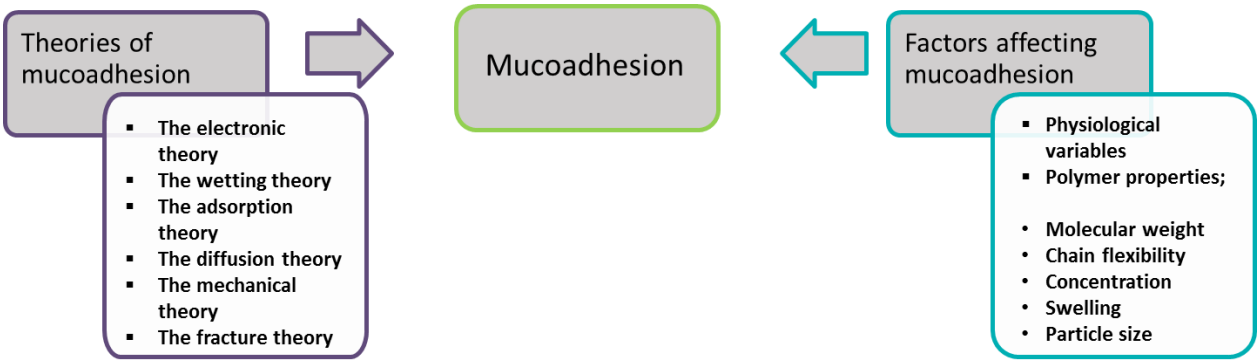

Figure 1.6: The proposed theories explaining the mucoadhesion phenomenon and the factors affecting mucoadhesion.

The use of mucoadhesiveness in vaginal drug delivery enables prolonged residence time, reduced administration frequency, desired distribution, controlled delivery and improved bioavailability (Vanić and Škalko-Basnet, 2014). The most common approach to obtain mucoadhesion in drug delivery is the use of mucoadhesive polymers (Smart, 2005).

1.5.1 Mucoadhesive polymers

It is generally accepted that the use of mucoadhesive polymers, either as a vehicle for delivery systems or as a surface-modifying material for a drug carrier, can prolong the residence time at vaginal site. A wide range of polymers, both synthetic and natural, has been studied for the potential use in mucoadhesive drug delivery. Chitosan, sodium alginate, pectin, cellulose derivatives, polyacrylates and carbomers are mucoadhesive polymers widely investigated in application to vaginal drug delivery (Gupta *et al*., 2011; Wong *et al*., 2014; Smart, 2005). An ideal polymer for a mucoadhesive drug delivery system should be non-toxic, non-irritant, and able to form adhesive bonds with mucus and provide controlled release of incorporated drug. The substantial properties of the polymers affect their ability to provide mucoadhesiveness to a system (Figure 1.6). Type of polymer and its molecular weight is essential. Ideally,

the chains should be small enough to allow easy penetration, however, still large enough for entanglements to occur. Chain flexibility also affects interpenetration and entanglement. Cross-linked polymers show less mobility, hence, less mucoadhesion due to reduced entanglement with mucus. A suitable polymer concentration is necessary to obtain adequate mucoadhesion, as high concentrations will reduce the flexibility and too low concentration will not present adequate chains available for interaction with mucus. The swelling of the polymer also depends on the concentration, in addition to the presence of water as over-hydration reduces the strength of bonding. Mucoadhesion will be affected by the charge of both mucus and the polymer; consequently, pH is an important factor. Further, greater surface area is more accessible for interaction with mucus, thus, the particle size will affect the mucoadhesiveness of the delivery system (Smart, 2005; Edsman and Hägerström, 2005).

1.5.1.1 Chitosan

Chitosan is the most widely used polymer in development of mucoadhesive nanoparticles (Mallipeddi and Rohan, 2010a). It is a natural cationic polysaccharide consisting of glucosamine and N-acetyl glucosamine units (Figure 1.7). Due to its biocompatibility, low toxicity and mucoadhesive properties, chitosan is attractive for many pharmaceutical applications, including those for vaginal use (Valenta, 2005).

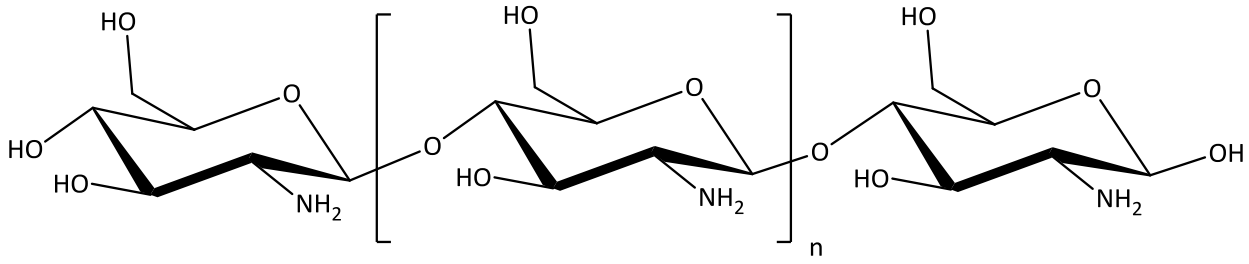

Figure 1.7: Structure of chitosan.

The mucoadhesive properties of chitosan can be explained by the electronic theory. Electronic interactions between the positively charged chitosan and the negatively charged mucin are considered the reason for its strong adhesion to mucosal surface (Vanić and Škalko-Basnet, 2014). Chitosan is additionally suited for repeated adhesion, as it does not become inactivated after the first contact with mucus and no reduction in mucoadhesion has been observed (Valenta, 2005). Moreover, chitosan (pKa 6.3 - 6.6) has a propensity

of expressing stronger mucoadhesiveness in acidic pH, which is favorable for drug delivery at vaginal site (Krajewska *et al*., 2011). Its mucoadhesiveness ensures localized drug release and the use of chitosan in drug delivery systems enables a controlled and sustained release of incorporated drug (Andersen et *al*., 2015). Additionally, chitosan enhances mucosal penetration by opening the tight junctions between mucosal epithelial cells allowing drug delivery to the deeper epithelial layers which is often desirable (Mallipeddi and Rohan, 2010a).

Another property of great interest considering the local treatment of vaginal infections is the intrinsic anti-bacterial and anti-fungal activity of chitosan. The anti-microbial activity against common pathogens associated with vaginal infections is confirmed. The mechanism of action include chitosan interaction with the fungal cell walls (Palmeira-de-Oliveira *et al*., 2015; Krajewska *et al*., 2011). Additionally, low molecular weight chitosan show anti-biofilm activity against *Candida*, interfering with the microbial virulence and potential recurrence (Silva-Dias *et al*., 2014). Its anti-biofilm activity is confirmed in both healthy and disease-influenced pH condition, which highlights chitosan as a suitable polymer for topical vaginal therapy (Kandimalla *et al*., 2013).

1.6 Nanosystems as vaginal drug delivery systems

Nanotechnology applied to drug delivery shows a great potential, and currently, numerous of nanocarriers are considered as suitable drug delivery systems. Among them, polymeric micelles, dendrimers, liposomes and solid nanoparticles are of great interest as novel approaches to successful drug delivery (Vauthier *et al*., 2013). As drug delivery systems, nanocarriers may enable improved solubility and delivery of poorly soluble drugs, more specific drug targeting, enhanced permeability through biological membranes and greater safety and biocompatibility (De Jong and Borm, 2008; Farokhzad and Langer, 2009). These qualities are due to nanosystems' unique physiochemical properties, such as their small size and consequent large surface area (Zhang *et al*., 2007).

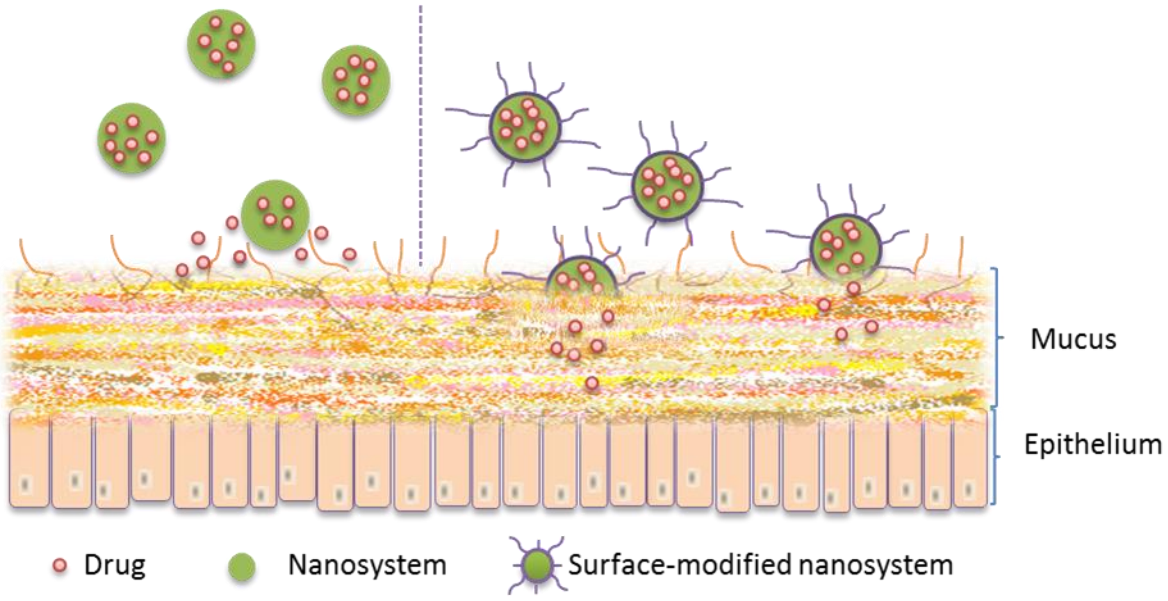

Figure 1.8: Schematic drawing of how surface modification can improve contact between drug and mucosal epithelium.

The cervicovaginal mucus is a major barrier for vaginal drug delivery, in terms of reaching the epithelium and avoidance of clearance. To achieve an effective drug delivery at mucosal site several requirements must be fulfilled; distribution and retention of the system on the mucosal surface, penetration into or through the mucus mesh and an adequate release profile of the drug (Vanić and Škalko-Basnet, 2013). Surface-modified nanoparticles may overcome this mucus barrier due to small size and improved surface properties (Mallipeddi and Rohan, 2010b) (Figure 1.8). Surface properties and the ability to involve in mucoadhesion or mucus-penetration, place nanoparticles as drug delivery systems in three categories, namely; conventional (non-mucoadhesive), mucoadhesive and mucus-penetrating (PEGylated) delivery systems. Two main approaches are used in the development of mucoadhesive nanosystems, namely designing mucoadhesive polymer-based nanocarriers and surface modification of preformed nanocarriers that are non-mucoadhesive originally, often applied concept (das Neves *et al*., 2011b).

The liposomes were the first nanosystems described as a drug carrier in vaginal therapy (Foldvari and Moreland, 1997) and their potential as vaginal drug delivery systems has been fully recognized (Ensign *et al*., 2014).

1.6.1 Liposomes

Liposomes are spherical vesicles that form spontaneously when phospholipids interacts with water (Akbarzadeh *et al*., 2013). The lipids form one or more bilayers around an aqueous core enabling the incorporation of drugs within or at the bilayer interface and in the inner core (Figure 1.9).

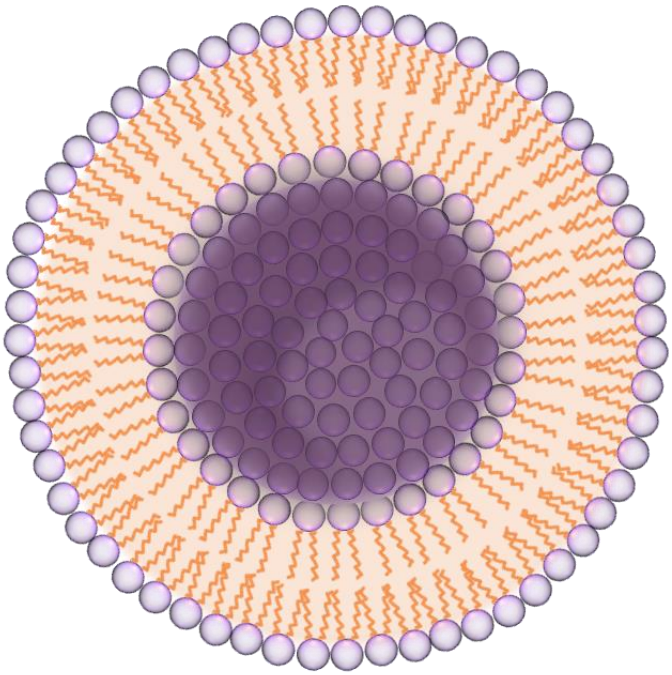

Figure 1.9: Schematic drawing of a unilamellar liposome.

Liposomes are characterized by their lipid composition, size distribution and lamellarity (Vanić and Škalko-Basnet, 2013). Depending on vesicle size and method of preparation, liposomes appear as small unilamellar vesicles (SUV) with size up to 100 nm, large unilamellar vesicles (LUV) in the size range of 100 - 800 nm or multilamellar vesicles (MLV) with a size of 500 - 5000 nm (Torchilin, 2012). The original method of Bangham and colleagues (Bangham *et al*. 1965), also referred to as the film method, is one of the most common methods of liposome preparation in addition to reverse phase evaporation, ultrasonication, detergent removal from mixed micelles by dialysis or gel filtration, freeze-thawing and extrusion (Torchilin, 2012). Phosphatidylcholine (PC) is a lipid commonly used for the manufacturing of conventional liposomes.

Liposomes are considered as biocompatible, biodegradable and non-toxic drug carrier. Additionally, their aptitude to trap both lipophilic and hydrophilic drugs, shield the functionality of unstable drugs and assure controlled release and site-specific delivery of the entrapped compound, make liposomes promising as pharmaceutical carriers (Akbarzadeh *et al*., 2013; Mufamadi *et al*., 2011). Various applications for liposomes are investigated, in particular as drug delivery systems, and a number of liposomal formulations are on the market and many more are in pipelines (Allen and Cullis, 2013). For example, AmBisone, DaunoXome and Doxil are liposomal formulations already approved for intravenous application (Torchilin, 2012). Adjustments of vesicle size, charge and composition can be performed to obtain desired properties for intended application. The stability of liposome membrane can be increased by the incorporation of cholesterol, which additionally will slow down liposomal disintegration and release of incorporated drug. Further, surface modification, such as coating or coupling, can increase their performance and suitability to the specific route of drug administration (Torchilin, 2012).

Foldvari and Moreland, who clinically evaluated the applicability of liposomes containing IFN α-2b in the topical therapy of genital HPV infections (Foldvari and Moreland, 1997), introduced liposomes as vaginal drug delivery system. Liposomes ability to increase solubility of poorly water-soluble drugs and protect drug from degradation in the presence of vaginal fluids make them suitable for vaginal application. Moreover, liposomes offer a sustained and controlled release of the entrapped drug at vaginal site (Vanić and Škalko-Basnet, 2014). However, the liquid nature of liposomal preparations is a challenge due to limited retention time at administration site. Assuring suitable viscosity of liposomal formulation by their incorporation in hydrogels, such as Carbopol (Pavelić *et al*., 2005) or coating the liposomal surface with polymers, such as chitosan (Andersen *et al*., 2015), enables desired formulation properties to overcome this challenge. When chitosan is used as a coating material for neutral PC liposomes, it is expected that hydrogen bonds be created between the phospholipid head groups and the cationic polysaccharide. Not only will this provide increased mucoadhesive properties; it will provide protection to liposomes and increase their stability (Filipović-Grčić *et al*., 2001). With a combination of liposomal carrier properties and the mucoadhesive properties of chitosan, development of an optimal vaginal drug delivery system with specific, prolonged and controlled drug release properties might be possible.

Mucoadhesion have drawn a great deal of attention in drug delivery at mucosal site, with emphasis on surface modification of liposomes; it was agreed that mucoadhesive polymers prolong the residence time at mucosal site (Vanić and Škalko-Basnet, 2014). As vaginal delivery systems, mucoadhesive liposomes has

shown great potential, assuring sustained drug release and prolonged residence time at vaginal site (Andersen *et al*., 2015; Berginc *et al*., 2014). Recent findings, however, indicated that new drug delivery systems that are able to penetrate mucus assure closer contact with the epithelium, and improved drug delivery to vaginal mucosa. This hypothesis needs to be further evaluated.

1.7 Mucus-penetration

The mucus barrier protecting the mucosal surface consists of two layers as described earlier. The luminal mucus layer with soluble secretory mucin has a high turnover and is rapidly cleared, while the unstirred mucus layer with membrane-bound mucin undergoes a lower physiological turnover and slower clearance (Wong *et al*., 2014; Edsman and Hägerström, 2005). Mucus efficiently entraps most foreign particles, both the pathogens causing infection and nanocarriers intended for mucosal drug delivery, preventing them from reaching the epithelial surface. Nevertheless, virus have the ability to overcome this barrier and establish mucosal infection. In order to achieve a successful local drug delivery to mucosal tissue the delivery system needs the ability to penetrate into or through the mucus mesh, uniformly distribute the drug to the underlying tissue and maintain a sufficiently high drug concentration at the site of action (Vanić and Škalko-Basnet, 2014). A recently proposed alternative approach to obtain these objectives is the imitation of the evolved properties of viruses, enabling them to penetrate mucus and cause infection (Lai *et al*., 2007; Cone, 2009).

Mucins, high molecular weight glycoproteins, form a tight mesh network and the mucin fibers in healthy vaginal mucus are found to have a diameter in the range of 50 - 1800 nm, with an average of 340 ± 70 nm (Lai *et al*., 2010). Particles larger than the pore size of the mucin fibers will be unable to enter mucus due to steric obstruction, however, this does not necessarily mean that smaller particles will be able to penetrate the mucus layer (Wong *et al*., 2014). The heterogeneous pore size of the mucus generates a sieving effect comparable to size-exclusion chromatography, hence, small particles are likely to enter smaller pores and retain in mucus, while larger particles unable to enter small pores will diffuse more rapidly (Lai *et al*., 2007).

In addition to the physiological characteristics of mucus and the size of nanocarriers, the surface properties are also essential for mucus-penetrating systems (Wong *et al*., 2014). It has been shown that HPV with the average size of 55 nm could rapidly diffuse through mucus, while HSV, with an approximate size of 180 nm remained trapped in the mucus mesh (Lai *et al*., 2010). The slow diffusion rate of HSV trough mucus was

shown to be due to adhesive interactions and not the steric obstruction. Despite the entrapment in mucus, HSV has the ability to reach mucosal surface and cause infection; this might be facilitated by compromised physiochemical properties of mucus (Lai *et al*., 2010). The carrier size controls to which extent carriers will fit in the pores of mucus, while the surface charge determines the interaction with the negatively charged mucin fibers. In contrast to the target of the mucoadhesive drug delivery systems which is the prolonged contact time with mucus, the mucoresistant carriers surface is desirable for successful drug delivery by mucus-penetrating particles. In the approach based on the administration of mucoresistant systems, polyethylene glycol (PEG) plays an important role.

1.7.1 PEG

Polyethylene glycol (PEG) is an uncharged hydrophilic polymer (Figure 1.10) widely applied in pharmaceutical formulations, including those for topical vaginal therapy. It is non-toxic and known to increase the *in vivo* circulation time of proteins and liposomes (Allen and Cullis, 2013). Surface modification by PEG (PEGylation) can enable liposomes to avoid interception by the immune system and improve their steric stability (Mufamadi *et al*., 2011). PEGylated liposomes are often referred to as stealth liposomes.

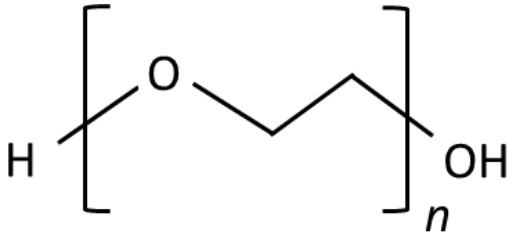

Figure 1.10: Structure of polyethylene glycol (PEG).

Extensive amounts of literature describes PEG as a polymer with strong mucoadhesive properties supported by the diffusion theory (Wang *et al*., 2008). Recent findings, however, show that PEG possess mucoresistant properties. When used as a coating material, PEG generate mucus-penetrating particles by minimizing the adhesive interactions with vaginal mucus allowing particles to efficiently penetrate the cervicovaginal mucus (Tang *et al*., 2009; Lai *et al*., 2007; Cu and Saltzman, 2008). The surface density and the molecular weight of PEG affect the diffusion rate through mucus and the recent findings indicate that when densely coated with low molecular weight PEG, nanoparticles avoid adhesive interactions with

mucus (Ensign *et al*., 2012; Lai *et al*., 2007; Mert *et al*., 2012). This enables a closer contact with the mucosal surface, improved drug effectiveness and a successful therapeutic effect is possible.

The development of an optimal vaginal drug delivery system might be possible by utilizing the carrier properties of liposomes combined with the mucoresistant properties provided by PEG, enabling a controlled drug release in close proximity to the vaginal epithelium.

To optimize the vaginal drug therapy of common vaginal infections, we have focused on two approaches; the modification of liposomal surface and use of natural-origin anti-virals. The mucoadhesive polymer chitosan was used at coating material to obtain mucoadhesive liposomes, while PEGylation was applied to develop mucus-penetrating liposomes.

2 Aims of the study

The overall aim of this project was the development and optimization of a drug delivery system for improved topical therapy of vaginal infections. To achieve this goal, we selected three drugs/active ingredients with high potential for improved therapy, namely clotrimazole (contemporary registered drug for vulvovaginal candidiasis), resveratrol (a potential candidate for treatment of genital HSV) and interferon α-2b (a potential drug for genital HPV). To optimize their delivery and assure their stability in vaginal environment, we separately incorporated the three drugs/candidates in surface-modified liposomes.

The specific aims can be presented as following:

- To demonstrate the capacity of liposomes to entrap both lipophilic and hydrophilic substances of different molecular size, confirming their suitability as nanocarrier
- To develop mucoadhesive liposomes enabling a prolonged retention time at vaginal site as well as providing localized effect avoiding systemic absorption, an important feature considering therapy of pregnant patients
- To optimize the mucoadhesiveness of the developed system and the effect of polymer concentration on the mucoadhesive properties
- To develop mucus-penetrating liposomes providing drug release in close proximity to vaginal epithelium
- To demonstrate the sustained release of liposomally-associated drug
- To confirm the stability of the formulations in the vaginal environment
- To confirm the safety and non-irritability of the delivery system *in vivo*
- In the case of resveratrol; to confirm that liposomal system enhances the anti-oxidative and anti-inflammatory activities of resveratrol

3 Summary of papers

3.1 Paper I

In this paper, we developed mucoadhesive liposomes aiming to assure localized effect and efficient drug delivery at vaginal site.

Clotrimazole-containing liposomes were prepared by probe sonication to be of desired vesicle sizes and liposomal surface was modified by polymer coating to assure mucoadhesive properties. Low molecular weight chitosan was used as coating polymer in concentrations of 0.1, 0.3 and 0.6 % (w/v), respectively. Non-coated and chitosan-coated liposomes were characterized in regards to vesicle size, surface charge, entrapment efficiency and phosphatidylcholine content. Franz cell diffusion system was used to determine *in vitro* clotrimazole release and *ex vivo* penetration, using cellophane membrane and sheep vaginal tissue (from pregnant animals), respectively. Further, the *in vitro* mucoadhesiveness was measured by determination of mucin-binding potential in pH similar to the condition in healthy vaginal environment (pH 4.6).

Probe sonication reduced the liposomal size resulting in two distinguished distribution peaks, indicating a bimodal size distribution with a rather high polydispersity index (PI). With the increase in sonication time, the PI values decreased resulting in liposomes of the desired size range (111 ± 16 nm). When liposomes were coated with chitosan, an increase in size was seen in addition to an increase in zeta potential. The change in vesicle size and surface charge was corresponding to the increase in polymer concentration, indicating that coating actually took place. The *in vitro* release studies confirmed a prolonged release of liposomally-associated clotrimazole compared to clotrimazole solution (propylene glycol). Interestingly, the release of drug was slower from non-coated liposomes compared to chitosan-coated liposomes. The *ex vivo* penetration experiments, however, displayed a similar penetration of clotrimazole from non-coated and chitosan-coated liposomes. Nevertheless, liposomally-associated clotrimazole penetrated significantly less compared to clotrimazole solution, preventing undesired penetration through vaginal tissue. Chitosan coating on liposomal surface was shown to be available for close interaction with mucin, confirming the mucoadhesive potentials provided by chitosan. A superior mucin-binding potential was seen for liposomes coated with low polymer concentration (0.1 %).

Liposomal delivery systems provided sustained delivery of entrapped clotrimazole and the mucoadhesive properties of chitosan-coated liposomes indicate a prolonged residence time within the vaginal cavity, assuring high levels of drugs at mucosal site, yet a limited drug absorption, suitable for the therapy of pregnant patients.

3.2 Paper II

In the second paper, we aimed to demonstrate that the mucoadhesive liposomes are a suitable delivery system for resveratrol, enabling controlled drug delivery at vaginal site and protection of entrapped resveratrol from environmental and chemical changes, resulting in improved resveratrol activities.

Resveratrol loaded liposomes were brought to desired vesicle size by extrusion through polycarbonate membranes with defined pore size. Coating of liposomes was performed in the presence of unentrapped resveratrol with low molecular weight chitosan in concentrations of 0.1, 0.3 and 0.6 % (w/v), respectively. Vesicles were characterized for size, size distribution, surface charge, entrapment efficiency and surface-availability of chitosan. Mucin-binding potential was measured to determine the *in vitro* mucoadhesiveness of the system in pH conditions corresponding to both healthy vaginal environment (pH 4.6) and pH occurring during vaginal bacterial infection (pH 7.4). Franz cell diffusion system was used to determine *in vitro* resveratrol release. To confirm the *in vitro* anti-oxidative activities of resveratrol, the DPPH (1,1-Diphenyl-2-picrylhydrazyl) and $ABTS^{\bullet+}$ (2,2'-azino-bis(3-ethyl benzothiazoline)-6-sulfonic acid diammonium salt) radicals scavenging activities were measured and compared to the well-known anti-oxidants vitamin C and vitamin E. Superoxide dismutase (SOD) activities in lipopolysaccharide (LPS)-induced J774.1 cells were measured to compare the anti-oxidative activity of resveratrol in solution and liposomal resveratrol. To compare the *in vitro* anti-inflammatory activities, the effects on the nitric oxide (NO) production, tumor necrosis factor (TNF)-α and interleukin (IL)-β in LPS-induced macrophages were measured.

The chitosan-coated liposomes with a mean vesicle size 212 nm exhibited high resveratrol incorporation (77 %). The system exhibited necessary mucoadhesiveness regardless of pH, with superiority of coating with low chitosan concentrations (0.1 %), corresponding to the measured surface-available chitosan for the respective test samples. *In vitro* release study showed the ability of liposomes to provide sustained release of resveratrol over a period of 8 hours. Resveratrol expressed a stronger radical scavenging activity for $ABTS^{+\bullet}$ radicals compared to DPPH free radicals, with superior $ABTS^{+\bullet}$ scavenging activity in lower concentrations compared to vitamin C and vitamin E. Liposomal resveratrol showed a superior SOD activity (26 %) compared to resveratrol solution (20 %). A concentration dependent NO production inhibition with a 50 % inhibitory concentration of 9.6 and 13.5 µg/mL was found for liposomal resveratrol and resveratrol solution, respectively. Liposomal resveratrol (10 µg/mL) inhibited 70 % of TNF-α production compared to 52 % inhibition by the resveratrol solution. Liposomal resveratrol further inhibited the IL-β production by 62 % compared to a 60 % inhibition by the resveratrol solution.

Resveratrol was confirmed to be a strong anti-oxidant and anti-inflammatory compound with enhanced activity when in liposomal formulation. Mucoadhesive liposomes assuring prolonged resveratrol release and retention time at vaginal site might serve as improved delivery system for resveratrol-based localized treatment of vaginal inflammation and infection mainly caused by HSV.

3.3 Paper III

In the work presented in paper III we developed mucus-penetrating PEGylated liposomes containing interferon (IFN) α-2b aiming to improve localized drug delivery to vaginal mucosa targeting the HPV-induced inflammation and infections.

Liposomes coated with low molecular weight polyethylene glycol (PEG) and containing IFN α-2b of desired vesicle size were prepared by the extrusion through polycarbonate membranes. The PEGylated liposomes were characterized regarding vesicle size, size distributions, surface charge and entrapment efficiency. The *in vitro* IFN α-2b release and *ex vivo* penetration experiments were performed in Franz cell diffusion system in the presence of vaginal fluid simulant (VFS), using polyamide membrane and sheep vaginal tissue (from pregnant animal), respectively. PEGylated nanoparticles are expected to avoid interactions with mucin, hence, the *in vitro* mucin-binding potential was measured to confirm the mucoresistant properties of the PEGylated liposomes. The stability of the system in the presence of biological fluids was explored by determining possible drug leakage from liposomes when mixed with semen fluid simulant (SFS) and VFS.

PEGylated liposomes containing IFN α-2b exhibited a monodisperse size distribution with a mean vesicle size of 181 nm and an entrapment efficiency of 81 %. A retained release was seen for both IFN α-2b solution and liposomally-associated IFN α-2b, even after 8 hours. However, the penetration through the sheep vaginal tissue was prominent, and a distinct increased IFN α-2b release from PEGylated liposomes was seen compared to IFN α-2b solution (Intron A buffer). A significantly ($p < 0.001$) reduced mucin-binding efficiency within the different pH conditions was expressed by the PEGylated liposomes compared to both conventional non-coated liposomes and chitosan-coated liposomes (0.1 %, w/v). The stability of PEGylated liposomes in a simulated vaginal environment and in the presence of SFS was confirmed, with a minor leakage of merely 5.1 % of the liposomally entrapped IFN α-2b.

The absence of interactions between mucin and PEGylated liposomes and the ability of the liposomally-associated IFN α-2b to penetrate through vaginal tissue indicate that the system enables drug delivery in close proximity to vaginal epithelium. The stability of PEGylated liposomes in a simulated vaginal environment and in the presence of semen fluid simulant strengthens the potential of the system as local treatment of HPV infections.

4 Experimental section

Materials and methods applied in this work are thoroughly described in papers I-III and the following presentation of methods comprehend merely an overview of the procedures as a background for the discussion in Chapter 5. In addition, the methodology not included in the papers is explained in more details.

4.1 Preparation and characterization of liposomal formulations

The preparation, characterization and further evaluation of liposomal formulations were done according to the schematic presentation in Figure 4.1.

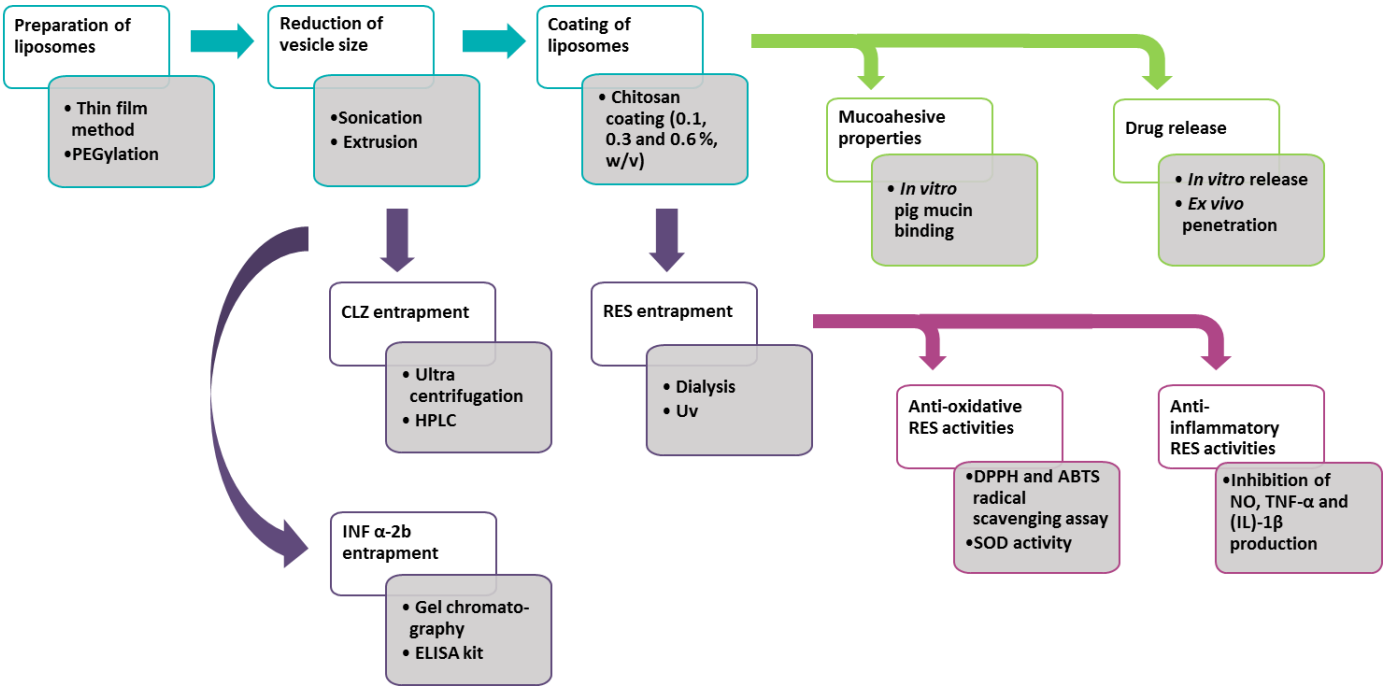

Figure 4.1: Schematic representation of the approach and methods used in the study.

CLZ: Clotrimazole, RES: Resveratrol.

In brief, liposomes made of phosphatidylcholine (Lipoid S-100) were prepared by the thin film method. Lipophilic drug was added to the lipid phase and dissolved prior to film formation, while hydrophilic drug was added to the aqueous phase applied for re-suspension of the lipid film. Polyethylene glycol (PEG) was added to the lipid phase in the preparation of PEGylated liposomes. Chitosan coating was performed after vesicle size reduction, either in the absence (clotrimazole) or in the presences (resveratrol) of free drug. Free drug was separated from liposomally-associated drug by suitable separation methods and appropriate drug detection method for the various preparations was applied. Vesicle size distribution and zeta potential measurements were performed both prior to and subsequently to chitosan coating. Formulations with desirable properties were further evaluated in respect to mucoadhesive/mucoresistant properties and drug release profile.

The anti-oxidative activities of resveratrol were examined and the effect of liposomal entrapment of resveratrol on the anti-oxidative and anti-inflammatory activities was investigated.

4.2 Differential interference contrast (DIC) microscopy

To explore a possible difference in the appearance of conventional non-coated, chitosan-coated and PEGylated liposomes, aliquots of the liposomal formulations were studied by differential interference contrast (DIC) microscopy (GE Healthcare, Buckinghamshire, UK). Non-coated, chitosan-coated (0.1 %, w/v), and PEGylated liposomes containing IFN α-2b were prepared as described above. Aliquots (5 µL) from respective samples, and highly diluted samples (1:1000 in distilled water) were applied to microscope slides (Gerhard Menzel GmbH, Braunschweig, Germany) and a cover slip (VWR International BHD Prolab, Leuven, Belgium) was placed onto sample. Cover slip was fixated to the microscope slip by nail polish to avoid unwanted motion.

All images were made at the following settings; channel: fluorescein isothiocyanate (FITC), exposure: 10 milliseconds and excitation: differential interference contrast (DIC).

4.3 Safety and non-irritability *in vivo*

The aim of the *in vivo* study was to detect any visual indication of local irritation of vaginal mucosal tissue due to application of the developed mucoadhesive drug delivery system. All formulations were prepared and modified (size reduction and chitosan coating) according to the methods described in paper I.

The local vaginal application of liposomal preparation was done in sheep undergoing experiments to study maternal and fetal hemodynamics in pregnant sheep (n=10). The animals were randomly divided into five groups, each receiving different formulation, namely; I) Non-coated liposomes containing clotrimazole, II) 0.1 % chitosan-coated liposomes containing clotrimazole, III) 0.6 % chitosan-coated liposomes containing clotrimazole, IV) empty non-coated liposomes and V) clotrimazole solution (propylene glycol). Samples of 10 mL were applied when animal was under general anesthesia. Photographical documentation of the mucosal vaginal tissue was made prior to application and approximately 3 hours after application. The application of respective samples (10 mL) was repeated after 6 days and additional before- and after-images were made.

The study was performed at the Laboratory Animal Center at the University of Oulu, and approved by the National Animal Experiment Board in Finland.

5 Results and discussion

The aim of this work was the development and optimization of liposomal drug delivery system for improved topical treatment of vaginal infections. Liposomes have the ability to incorporate a wide range of compounds, and provide protection to sensitive molecules such as resveratrol and IFN α-2b.

Successful delivery of drugs intended for mucosal vaginal tissue depends on the ability to overcome the obstacles related to the vagina as administration site. By modifications of liposomal size and surface properties, an adequate retention time at vaginal site or rapid diffusion through mucus can be achieved. Liposomal carrier properties were combined with the mucoadhesive properties of chitosan and the mucoresistant properties of PEG, respectively. To confirm the mucoadhesiveness/mucoresistance of the nanosystems, the interactions with mucin and the ability to provide sustained and controlled drug release were evaluated.

Where applicable, the properties of liposomes as a nanocarrier in respect to protection and enhanced efficacy of incorporated active molecule were explored and confirmed.

5.1 Characterization of liposomal size, surface charge and entrapment efficiency (Paper I - III)

The effectiveness of local drug delivery administered to the vaginal tissue is determined by the physicochemical characteristics of the delivery system. The vesicle size affects the ability to fit within the mucin pores, while the particles surface charge and surface properties manipulation may act on system's attraction or repulsion to mucus. Thus, the main focus in the development of a delivery system aiming for local therapy at the mucosal site is controlling the particle size, surface charge and surface properties (Vanić and Škalko-Basnet, 2013). However, to assure efficient drug therapy, a sufficient drug load within the nanocarrier is crucial.

Preparation of liposomes by the thin film method yields MLVs with a heterogeneous size population often larger than 1 μm (Samad *et al*., 2007). A variety of size reduction methods, including the probe sonication, gel chromatography and extrusion, can be applied to achieve desired vesicle size. Smaller liposomes with

a more uniform size distribution are preferred due to larger surface area and improved drug delivery properties. However, the decrease in vesicle size can affect the drug loading capacity and must be considered and often compromised.

When aiming for mucosal drug delivery at vaginal site, relatively little is known about the effect of vesicle size. das Neves and colleagues suggested that nanocarriers in the size range of 200-500 nm are superior compared to both smaller and larger nanosystems (das Neves *et al*., 2011b). Additionally, it was reported that the number of liposomes penetrating the intestinal mucous layer increased when the liposomal size was reduced to approximately 100 nm for both non-coated and chitosan-coated liposomes (Takeuchi *et al*., 2001).

5.1.1 Clotrimazole-containing liposomes

Probe sonication was chosen as size reduction method for the liposomes containing clotrimazole, and time of exposure was modified to achieve a desired vesicle size (around 200 nm). The sonication generated two distinct peaks indicating a bimodal size distribution with a relatively high, yet acceptable polydispersity index (PI below 0.7), in agreement with literature data on sonicated vesicles (Andersen *et al*., 2015). Due to bimodal size distribution, a multimodal distribution model (NICOMP distribution) was found suitable for the vesicle size determination. Liposomes sonicated for 4 min (2 x 2 min) showed a more prominent assembly of vesicles in the targeted size range (Table 5.1).

Table 5.1: Characterization of liposomes containing clotrimazole (n=3)

Time of sonication (min)	Vesicle size				PI*	Entrapment (%)
	Peak 1 (nm)	Intensity (%)	Peak 2 (nm)	Intensity (%)		
1	317 ± 47	56.8	40 ± 5	41.0	0.58	23.2 ± 2.5
2	234 ± 31	53.9	36 ± 5	43.8	0.46	25.0 ± 0.5
2 x 2	111 ± 16	83.1	29 ± 4	16.9	0.46	16.5 ± 4.5

* Polydispersity index

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With increased sonication time, the PI values decreased, as expected. Size reduction by sonication is known to cause a loss of incorporated drug, hence, the exposure to strain by sonication force was kept to a minimum. The effect of sonication on the reduction in clotrimazole entrapment efficiency was detectable for the liposomes sonicated longer than 2 min. Compared to literature reporting liposomal clotrimazole entrapment of over 30 % (Pavelić *et al*., 1999; 2005) and 90 % (Ning *et al*., 2005) the entrapment was lower, however, the literature reports different sonication conditions or lipid compositions used in the compared data. Despite the rather low entrapment efficiency, we decided that the drug load was sufficient to proceed with the selected liposomal size.

The coating of the liposomal surface resulted in an increase in the vesicle size as expected (Karn *et al*., 2011). To explore the influence of polymer concentration on liposomal properties, the coating was performed with 0.1, 0.3 and 0.6 % (w/v) low molecular weight chitosan, respectively. We have selected different concentrations of low molecular weight chitosan as it is known that low molecular weight chitosan exhibits stronger mucoadhesive and anti-microbial properties as compared to chitosan of higher molecular weight (Dash et al., 2011).

Table 5.2: Characterization of non-coated and chitosan-coated liposomes containing clotrimazole (n=3)

Coating (%, w/v)	Vesicle size				PI*	Zeta potential (mV)
	Peak 1 (nm)	Intensity (%)	Peak 2 (nm)	Intensity (%)		
-	107 ± 3	54 ± 3	27 ± 3	46 ± 3	0.34	- 1.6 ± 0.2
0.1	135 ± 21	53 ± 6	42 ± 9	45 ± 5	0.29	25.9 ± 4.0
0.3	141 ± 6	64 ± 6	48 ± 5	35 ± 6	0.27	35.6 ± 1.9
0.6	190 ± 8	58 ± 6	54 ± 2	42 ± 6	0.29	43.8 ± 3.3

* Polydispersity index.

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Results shown in Table 5.2 confirm an increasing vesicle size after chitosan coating. Further, the polymer concentration was shown to influence the vesicle size, with higher chitosan concentration (0.6 %) resulting in larger vesicle size than liposomes coated with lower chitosan concentration. In addition, a reduced PI was seen for the coated liposomes. Chitosan coating is additionally expected to increase the liposomal zeta potential (Berginc *et al*., 2014); a change in the surface charge was detected for the chitosan-coated liposomes with an increase corresponding to the polymer concentrations (Table 5.2).

The coating of clotrimazole-containing liposomes was not performed in the presence of free clotrimazole, hence, no change in drug entrapment was seen after the chitosan coating.

5.1.2 Resveratrol-containing liposomes

To obtain a more uniform size distribution, extrusion was chosen as a size reduction method for liposomes containing resveratrol. The extrusion was performed stepwise and a size of 200 nm was targeted to assure an adequate drug entrapment. Desired vesicle size with a very low polydispersity was attained, indicating a monodisperse size distribution (Table 5.3) and Gaussian distribution gave the best fit for the measurements. Further, a phospholipid assay exhibited a minor loss of lipid in the extruded liposomes of only 5 %, confirming extrusion as a suitable size reduction method for liposomal formulations.

Table 5.3: Characterization of non-coated and chitosan-coated liposomes containing resveratrol (n=3)

Coating (%, w/v)	Resveratrol liposomes			
	Vesicle size (nm)	PI*	Zeta potential (mv)	Entrapment (%)
-	206 ± 10	0.142	-3.17 ± 2.57	80 ± 4
0.1	212 ± 11	0.172	4.15 ± 0.59	77 ± 4
0.3	225 ± 10	0.122	14.77 ± 1.85	74 ± 6

* Polydispersity index.

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Chitosan coating resulted in increased vesicle size and zeta potential for the resveratrol-containing liposomes, yet the change was not as prominent as for the clotrimazole-containing liposomes (Table 5.3). An explanation for this might be the difference in the liposomal size and polydispersity between the two formulations prior to coating. The change in the size and charge was corresponding well to the respective polymer concentration, as expected.

A satisfactory resveratrol entrapment efficiency over 70 % was achieved, in accordance with the literature (Caddeo *et al*., 2008; Kristl *et al*., 2009; Bonechi *et al*., 2012) Polymer coating is known to increase the entrapment efficiency when performed in the presence of free drug (Karn *et al*., 2011). Chitosan coating was performed in the presence of free resveratrol, however, an increased resveratrol entrapment was not seen for the chitosan-coated liposomes. The applied phospholipid assay confirmed that the chitosan coating did not result in any considerable loss of lipids. The data indicate the suitability of the extrusion method as a size reduction method.

5.1.3 PEGylated liposomes containing IFN α-2b

Despite the uncertainty regarding the effect of vesicle size on the mucosal targeting at vaginal site, small particles are expected to facilitate entrapment in the mucus mesh while larger particles are prone to become retained on the mucus surface (Wong *et al*., 2014). However, larger particles (200-500 nm) densely coated with PEG were found to be transported more rapidly through the fresh undiluted human mucus, than the corresponding particles of smaller size (100 nm) (Lai *et al*., 2009). Hence, a vesicle size of 200 nm was the targeted size for the PEGylated liposomes intended for mucus penetration. As for the resveratrol-containing liposomes, the extrusion was used as size reducing method for the PEGylated liposomes containing IFN α-2b, resulting in liposomes with a low polydispersity. A mean vesicle size of 181 nm was obtained (Table 5.4) and measurements were found to fit the Gaussian distribution.

Since phosphatidylcholine (PC) is a neutral lipid, liposomes made of PC are expected to exhibit a neutral or close to neutral zeta potential, as seen for the non-coated liposomes containing clotrimazole and resveratrol (Tables 5.2 and 5.3). However, the PEGylated liposomes exhibited a negative zeta potential (Table 5.4). This is believed to be a result of the selected buffer used in the liposome preparation, namely the Intron A buffer (the composition is presented in paper III).

Table 5.4: Characterization of PEGylated liposomes containing IFN α-2b (n=3)

Pegylated liposomes containing IFN α-2b			
Vesicle size (nm)	**PI***	**Zeta potential (mV)**	**Entrapment (%)**
181 ± 8	**0.129**	**- 13.33 ± 0.81**	**81 ± 10**

* Polydispersity index.

The incorporation of IFN α-2b in liposomes can provide an increase in the IFN α-2b stability. Moreover, an alteration of the IFN α-2b pharmacokinetics is expected (Karau *et al*., 1996; Yang *et al*., 2006). A rather high IFN α-2b entrapment efficiency of 81 % was obtained. This was in accordance with the literature (Takeuchi *et al*., 1999; Vyas *et al*., 2006; Yang *et al*., 2006; Li *et al*., 2011).

5.1.4 Microscopic evaluation of liposomes

Differential interference contrast (DIC) microscopy is an illumination technique applied to gain an increased contrast in unstained, transparent samples (Davidson and Abramowitz, 2002). The method should minimize the pretreatment of the samples such as inclusion of a stain, freeze-fracture, etc. Two mutually coherent polarized light beams (separated approximately 200 nm at the focal plane) are sent through the liposomal samples and, by subsequently recombining these beams, the difference in the optical path length can be detected. The optical path length is the product of the refractive index and the geometric length traveled. Due to a similar geometric length for both beams, this effectively provides an image of the changes in refractive index along the focal plane (Boas *et al*., 2011).

Non-coated, chitosan-coated (0.1 %, w/v) and PEGylated liposomes containing IFN α-2b were examined, both in the original concentration and highly diluted concentration, and images are shown in Figure 5.1. The images of samples with high liposomal concentration appear different from the diluted samples. Additionally, a qualitative difference was detectable in the chitosan-coated liposomes compared to the non-coated and PEGylated liposomes in the concentrated samples (1a-c). This was not visible in the diluted samples (2a-c). Even though the chitosan-coated liposomes seem to be more diffused, measurements of the intensity profile indicated no significant difference in the samples.

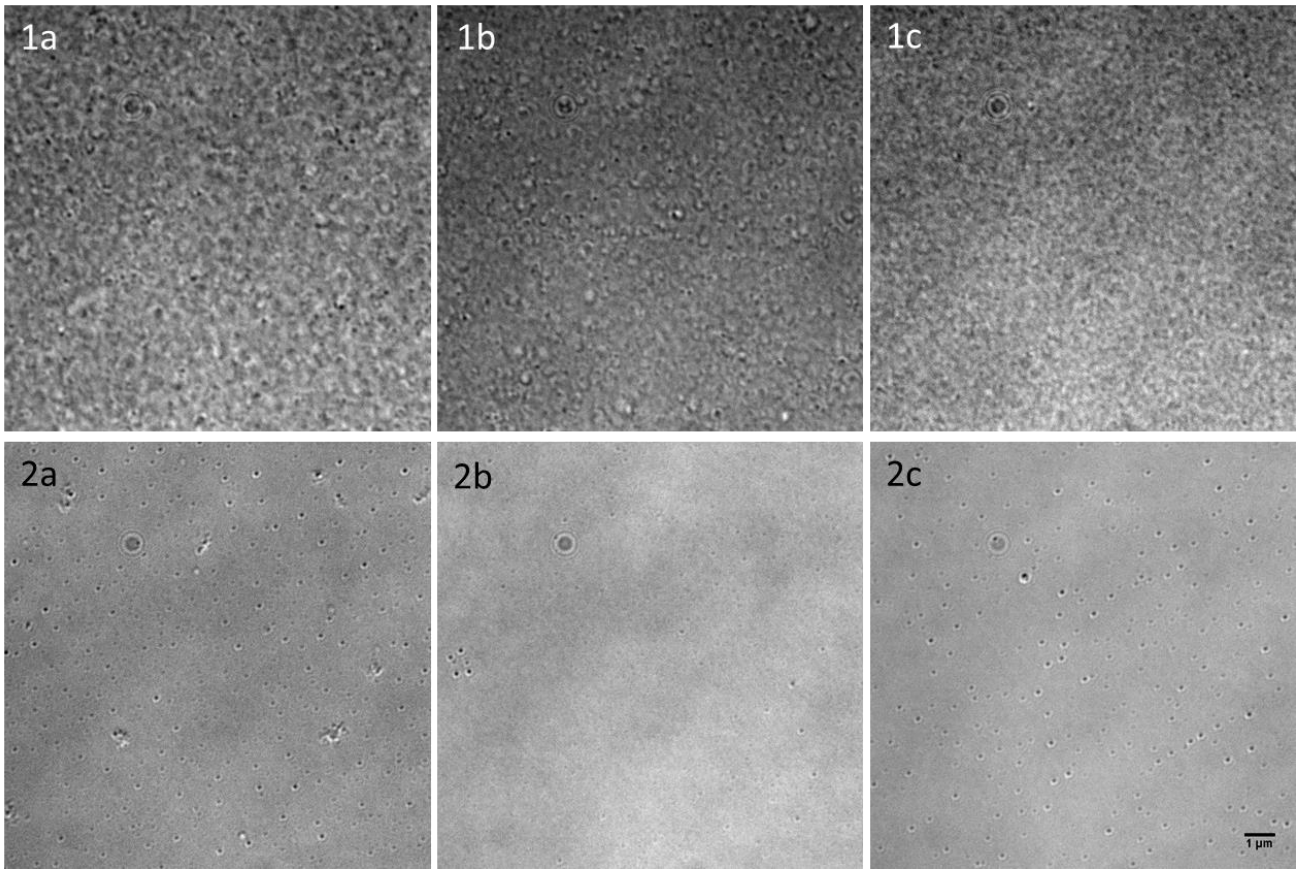

Figure 5.1: Microscopically depicted liposomes. Concentrated suspension; 1a) Plain (non-coated) liposomes 1b) Chitosan-coated liposomes 1c) PEGylated liposomes. Diluted suspension; 2a) Plain (non-coated) liposomes 2b) Chitosan-coated liposomes 2c) PEGylated liposomes.

The concentrated samples were imaged shortly after the preparation of microscope slides and the liposomes were moving in a random Brownian motion. The diluted samples were imaged 48 h after the preparation, and at this time, the samples were static. Hence, one can assume that the initially observed difference in chitosan-coated liposomes is a result of dissimilar movement in this formulation rather than its composition. To our knowledge, no reports on the examination of surface-modified liposomes by this technique have been reported up to now. The preliminary data indicate that the method could provide additional information on the nanosystem in a rather straight-forward manner, if the time is invested in optimization of the experimental set up.

5.2 *In vitro* mucoadhesiveness (Paper I - III)

The natural polymer chitosan is considered biocompatible, biodegradable and mucoadhesive. Its mucoadhesive properties have been studied for various drug delivery systems and chitosan is a preferred polymer for topical drug delivery, including vaginal administration (Valenta, 2005). The cationic character of chitosan is the main property that renders its mucoadhesive properties and also makes it suitable as coating polymer for neutral PC liposomes. During the coating process, it is expected that hydrogen bonds be created between the phospholipid head groups of the liposomes and the cationic amino groups of chitosan (Perugini *et al*., 2000). Both the increase in vesicle size and zeta potential indicate the presence of chitosan on the liposomal surface and the evidence that the coating actually took place (Table 5.2 and 5.3).

5.2.1 Surface-available chitosan

When used as a coating material, the amount of polymer interacting with the liposomes is expected to increase with starting amount of chitosan (Li *et al*., 2009; Perugini *et al*., 2000). To determine the presence of chitosan on the liposomal surface, a colorimetric assay with the anionic reactive dye (Cibacron Brilliant Red) was applied. The assay was performed in the presence of free chitosan, thus the binding efficiency of chitosan to liposomal surface was not directly measured. Nevertheless, we could see a distinguished change in chitosan concentration of the different formulations compared to the starting amount of chitosan (Figure 5.2). For the lower chitosan concentration (0.1 %), 84 % of theoretical amount of chitosan was detected. Further, only 54 % was detected in liposomal suspension coated with higher polymer concentration (0.3 %). The results show that coating with low chitosan concentration (0.1 %) provide more chitosan on liposomal surface. These findings are in accordance with literature (Andersen *et al*., 2015; Li *et al*., 2009) and corresponding to the zeta potential of respective samples.

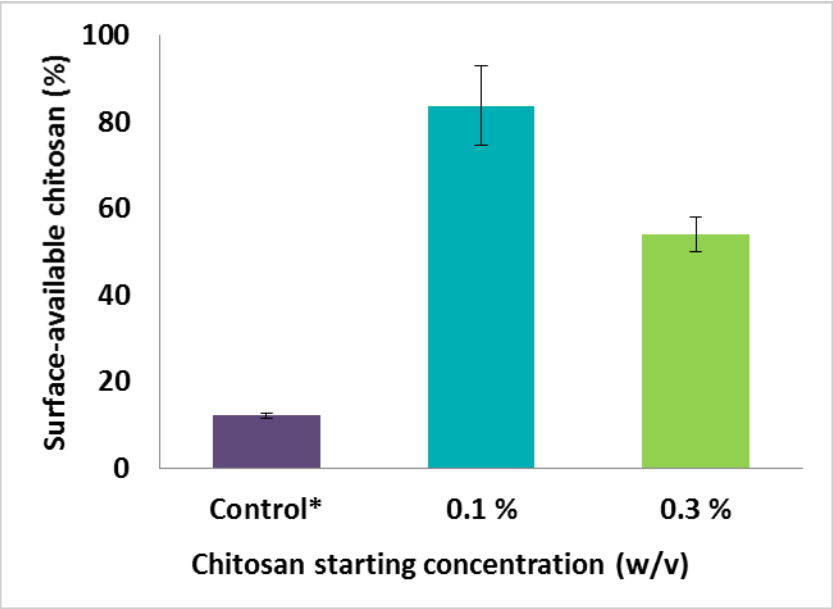

Figure 5.2: Surface-available chitosan (%) on chitosan-coated liposomes (n=3).

* Non-coated liposomes served as a control.

Since no chitosan was removed from the formulations prior to determination, one can discuss if the amount of surface-available chitosan is overexpressed. Yet, an incomplete detection of actual chitosan in the formulation indicates that some of the chitosan is concealed, possibly due to thick layer of polymer. Another explanation might be that chitosan interacts with liposomal surface and adsorbs onto lipid layer. Nevertheless, the liposome coating efficiency has been shown to be superior when using low chitosan concentration; Guo and colleagues found that chitosan coating of liposomes reached a saturation state when exceeded a 0.1 % chitosan concentration (Guo *et al*., 2003).

5.2.2 Mucin-binding

A successful vaginal drug delivery system should assure a uniform distribution of drug onto the mucosal tissue. The interaction between the drug delivery system and mucus can affect the performance of the system and maintaining high drug concentration at vaginal site is challenged by inadequate retention time. To confirm that chitosan coating on the liposomal surface is available for close interactions with mucin and render mucoadhesive properties to the delivery system, we tested the *in vitro* mucin-binding potential of both chitosan-coated and non-coated liposomes. Through ionic interactions, chitosan can acquire strong

adhesion between its cationic groups and the anionic structures of mucus, enabling prolonged retention time in the vaginal cavity. Commercial pig mucin (PM) is commonly used as a mucus substitute due to similarity in the structure and molecular weight of pig mucus and human mucus (Groo and Lagarce, 2014). Due to the variations in vaginal pH, especially when affected by vaginal bacterial infections, the experiment was performed both at pH conditions of a healthy vaginal environment (4.6) and at pH occurring in vaginal bacterial infection (7.4). The changes in the viscosity of mucus occurring during the menstrual cycle or due to vaginal infections were not accounted for, thus, the presented results are preliminary.

The results clearly indicate that liposomes coated with the lowest chitosan concentration (0.1 %) exhibited a superior interaction with mucin compared to all other formulations (Figure 5.3), suggesting prolonged residence time at vaginal site. These results are in agreement with literature (Naderkhani *et al.*, 2014). Additionally, the mucin-binding potential is corresponding to the surface-available chitosan observed for the respective formulations (Figure 5.2). This tendency of reduced amount of surface-available chitosan and reduced mucin-binding when coating is performed with high polymer concentrations is strengthened by the low PM binding expressed by liposomes coated with 0.6 % chitosan.

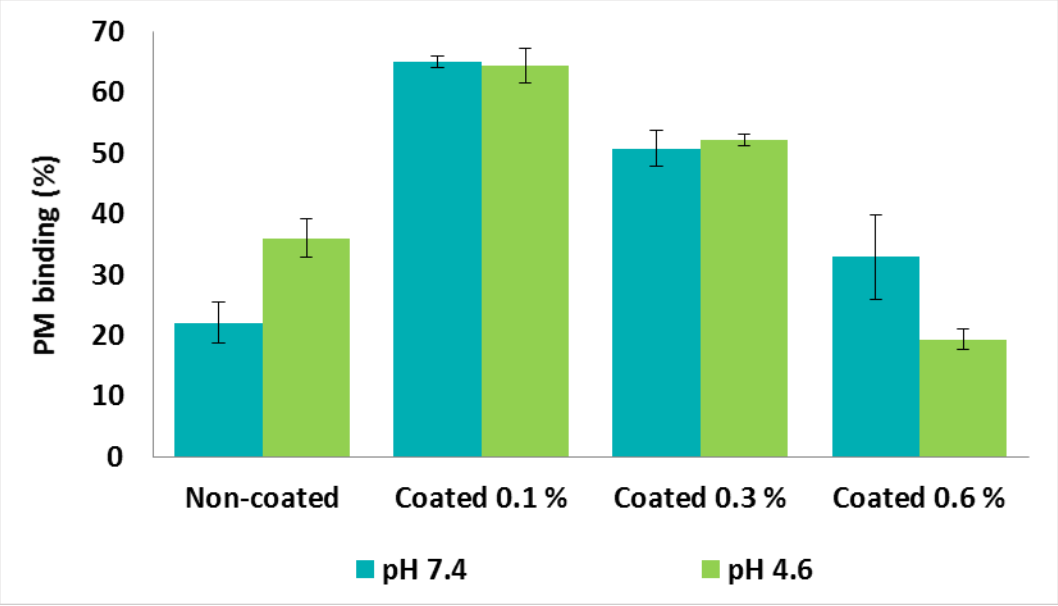

Figure 5.3: Pig mucin (PM) binding (%) of non-coated and chitosan-coated liposomes (n = 3).

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Non-coated liposomes are not expected to exhibit mucin-binding activity, yet a distinct binding potential was seen also for the non-coated formulation. Literature data show similar findings (Andersen *et al*., 2015; Naderkhani *et al*., 2014; Qiang *et al*., 2012). Physical interactions during the centrifugation step is a possible explanation for these results, as actual electrostatic interactions are unlikely to occur between mucin and neutral non-coated liposomes.

An increased mucoadhesiveness was expected in the acidic environment due to the pKa of chitosan (6.3 to 6.6) (Krajewska *et al*., 2011). However, an equal mucin-binding potential was seen for liposomes coated with low chitosan concentration at both pH conditions, indicating that mucoadhesion can be achieved regardless of vaginal pH conditions. This suggests that the mucoadhesive properties of the system should not be compromised due to elevated pH occurring as a result of vaginal infection or due to the presence of semen.

It is expected that mucoadhesion will increase with decreasing vesicle size due to larger surface area and higher availability to mucin interaction. Chitosan-coated nanoparticles in sizes of about 200 nm and 300 nm are shown to exhibit significantly enhanced mucoadhesive properties on porcine tissue, compared to larger particles (900 nm) (Meng *et al*., 2011). Hence, it is reasonable to expect that liposomes in the size range of 200 nm might exhibit even greater mucin-binding potential compared to liposomes in original vesicle size.

The other approach to obtain a uniform distribution and a sufficiently high drug concentration at the site of action, is rapid penetration through the mucus mesh to avoid clearance and reach the epithelial surface. Low molecular weight PEG (20 kDa) used on vesicle surface is found to allow nanoparticles to avoid interactions with mucus and enable penetration of the cervicovaginal mucus. Thus, the mucin-binding potential of PEGylated liposomes was determined to confirm the mucoresistant effects of PEG. Compared to non-coated and chitosan-coated liposomes, the PEGylated liposomes did not exhibit prominent mucin-binding activity (Figure 5.4), as expected.

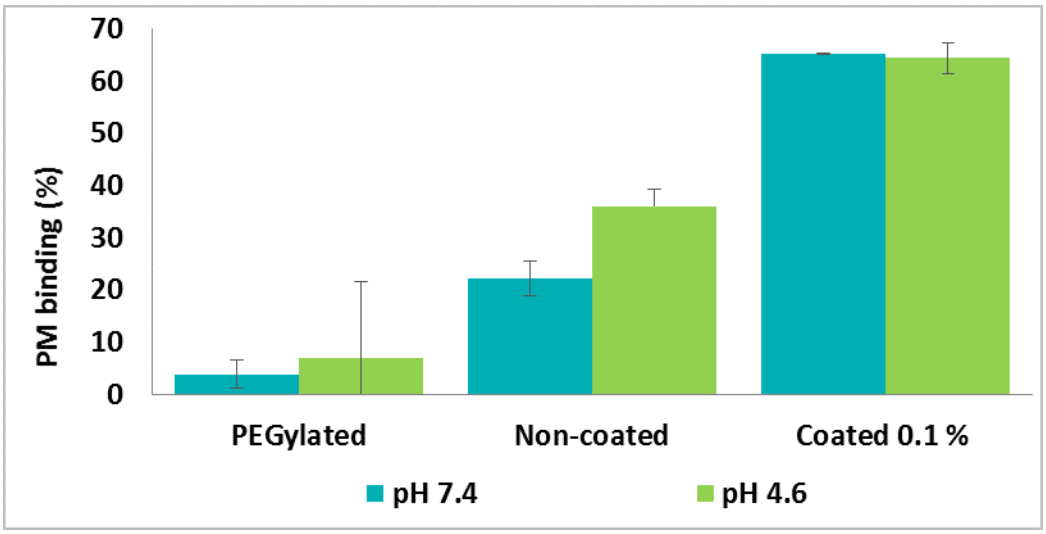

Figure 5.4: Pig mucin (PM) binding (%) of PEGylated, non-coated and chitosan-coated liposomes (n = 3).

These findings indicate reduced mucin interaction when liposomal surface is modified with PEG, enabling penetration through mucus. This allows closer contact with vaginal epithelium and enables improved drug effectiveness.

5.3 *In vitro* drug release (Paper I - III)

A predictable and controlled release is essential in the development of an optimal drug delivery system. Surface modification of liposomes by chitosan coating was confirmed to render mucoadhesive properties to the delivery system, additionally, it may present improved controlled drug delivery and stabilization of the liposomes. To examine the release from both chitosan-coated and non-coated liposomes, an *in vitro* release study was performed. Franz cell diffusion system is generally considered the most appropriate *in vitro* method for evaluating drug release from topical formulations including those for vaginal use (Siewert *et al*., 2003; das Neves and Bahia, 2006), thus was applied in the release studies. The experimental setup was based on human conditions, with an acceptor medium corresponding to the pH of a healthy vaginal environment (4.6) and a temperature of 37 °C. To test the release of clotrimazole a setup of 24 hours was applied based on the mucoadhesive potential of the system, enabling a less frequent application compared to the vaginal dosage forms of clotrimazole that are commercially available.

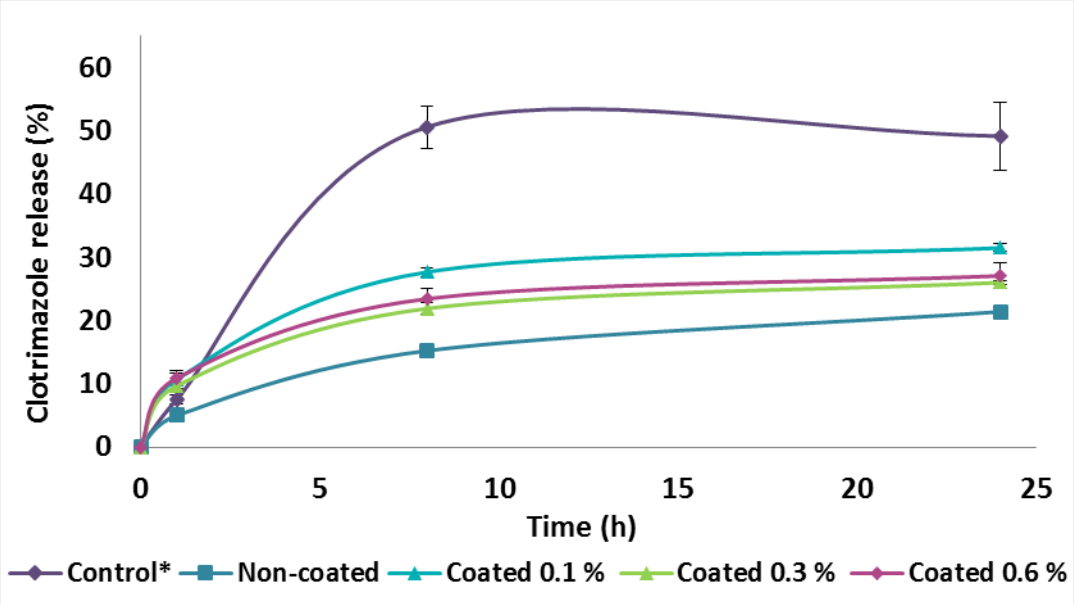

Figure 5.5: *In vitro* clotrimazole release (%) from non-coated and chitosan-coated liposomes (n=3).

* Clotrimazole dissolved in propylene glycol as control.

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A prolonged release was confirmed for the liposomally-associated clotrimazole, compared to clotrimazole solution (propylene glycol) of corresponding concentration (Figure 5.5). A slower clotrimazole release was expected from the chitosan-coated liposomes due to the propensity of liposomes to become leaky when not coated. Interestingly, the non-coated liposomes showed a superior ability to sustaine the clotrimazole release compared to all chitosan-coated formulations. It appears that the clotrimazole release from liposomes is enhanced when chitosan is used as coating material. A possible explanation might be clotrimazoles affinity of the liposomal bilayers rather than the outer aqueous medium, whereas the release from chitosan-coated liposomes is promoted by the precence of chitosan.

A limitation of the Franz diffusion system is the confined volume of the acceptor chamber, which is problematic when analyzing poorly soluble drugs such as clotrimazole. Even after 24 hours, all formulations, including the control, failed to release all incorporated drug. Possibly, this is due to the very limited solubility of clotrimazole in water. Nevertheless, a distinguished difference in the release of liposomally-assosiated clotrimazole and clotrimazole in solution could be detected.

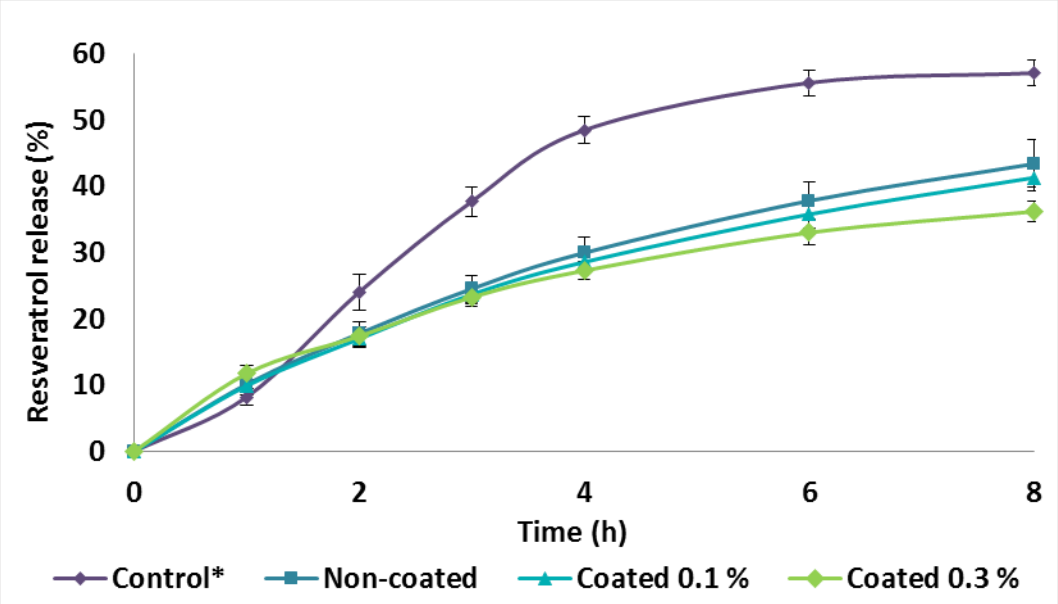

Figure 5.6: *In vitro* resveratrol release (%) from non-coated and chitosan-coated liposomes (n = 3).

* Resveratrol dissolved in propylene glycol as control.

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The same tendency was seen for the *in vitro* release of resveratrol, namely a prolonged release of liposomally-associated resveratrol compared to resveratrol solution (propylene glycol) in corresponding concentration (Figure 5.6). However, chitosan-coated liposomes expressed a more sustained resveratrol release compared to non-coated liposomes, unlike the release of clotrimazole. This agrees with the expectations of a prolonged drug release when liposomes are chitosan-coated. A possible explanation for the unequal effect of coating on the release of the different entrapped substance is that, although lipophilic, resveratrol displays a minor preference to the liposomal bilayer compared to clotrimazole. Due to the poor mucoadhesive properties exhibited by chitosan-coated liposomes with high polymer concentrations, the 0.6 % chitosan-coated liposomes were not included in the *in vitro* release determination of resveratrol.

The release from PEGylated liposomes was compared to a control (corresponding concentration of IFN α-2b in Intron A buffer), and the experiment was done in the presence of vaginal fluid simulant (VFS) to closer mimic the *in vivo* conditions. Both formulations failed to release the incorporated drug, yet a poor release of liposomally-associated IFN α-2b was seen after 6 and 8 hours (Figure 5.7). After 8 hours of

experiment, a considerable amount of IFN α-2b was retained within the artificial membrane. This retention was seen for both formulations (data not shown).

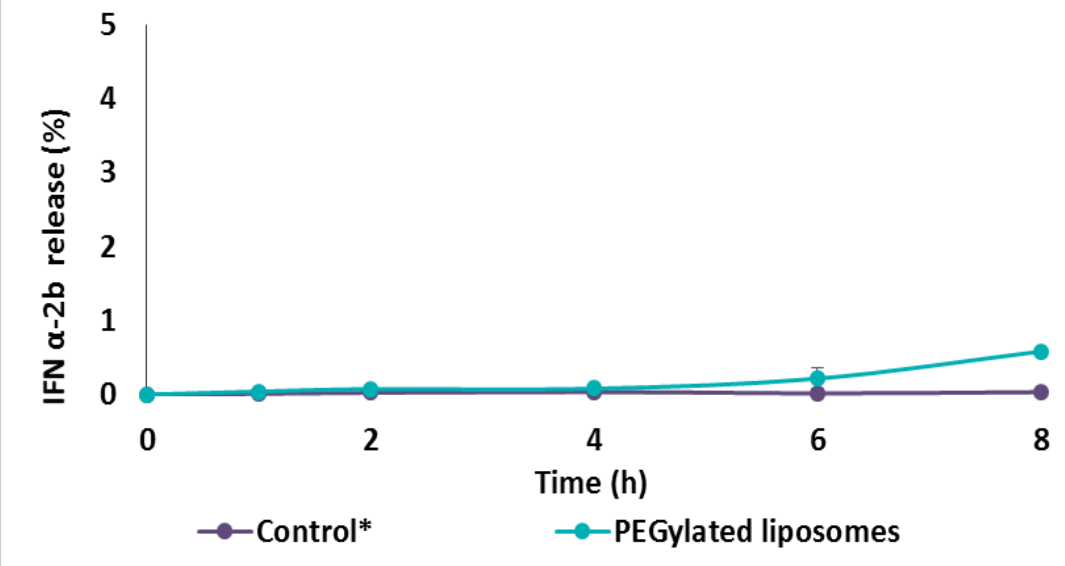

Figure 5.7: *In vitro* IFN α-2b release (%) from PEGylated liposomes (n=3).

* IFN α-2b in Intron A buffer as a control.

A sustained IFN α-2b release from liposomal formulation is in agreement with literature (Qiu *et al*., 2005; King *et al*., 2013).

5.4 *Ex vivo* penetration (Paper I and III)

As for the *in vitro* drug release, the Franz cell diffusion system is found to be suitable in the determination of *ex vivo* tissue penetration (Sandri *et al*., 2004; Bonferoni *et al*., 2008; das Neves *et al*., 2013; Machado *et al*., 2015). When aiming at safe topical drug delivery applied to pregnant patients, a limited drug absorption is desirable, at the same time, an adequate level of drug at the vaginal site should be achieved. To acquire a closer approach to *in vivo* conditions, vaginal tissue from pregnant sheep was applied in the *ex vivo* penetration experiments. Sheep vaginal epithelium is stratified squamous tissue, similar to that of

human, only thinner (Moss *et al*., 2012). Further, no significant differences have been observed in using fresh or snap-frozen tissue samples (Sassi *et al*., 2004).

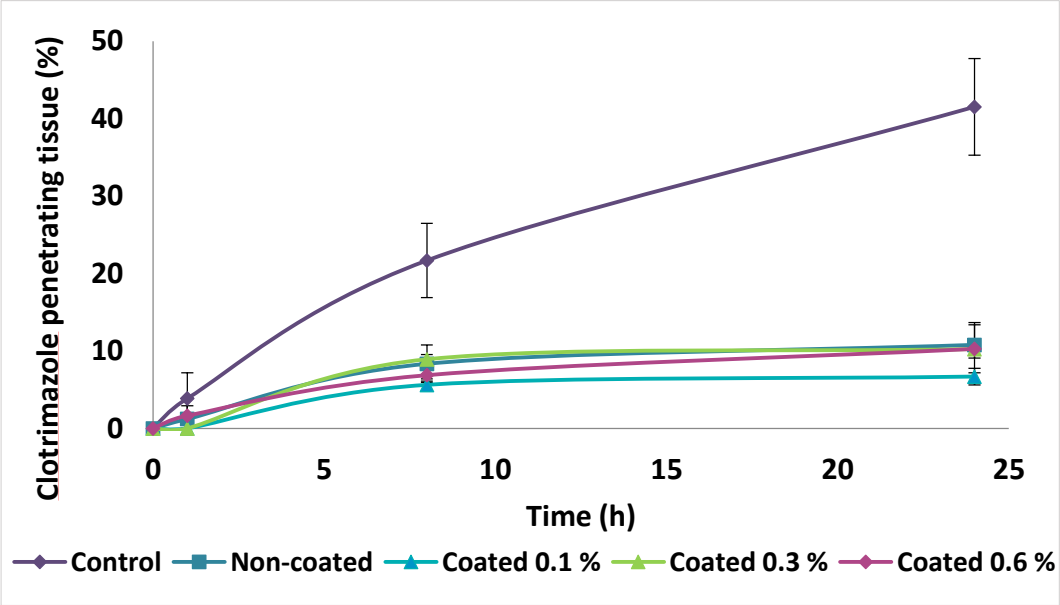

Figure 5.8: Clotrimazole penetration (%) of sheep vaginal tissue *ex vivo* for non-coated and chitosan-coated liposomes (n=3).

* Clotrimazole dissolved in propylene glycol as control.

Modified from Jøraholmen *et al*., 2014 (Paper I) with permission from Elsevier.

Liposomally-associated clotrimazole penetrated significantly less than clotrimazole in solution (propylene glycol). The results showed that noticeable amounts of free clotrimazole penetrated through the vaginal tissue whereas clotrimazole penetrated to lesser extent from all liposomal formulations (Figure 5.8). The data demonstrate that liposomes are able to retain associated clotrimazole on the surface of the vaginal tissue, preventing an undesired drug penetration through the vaginal tissue.

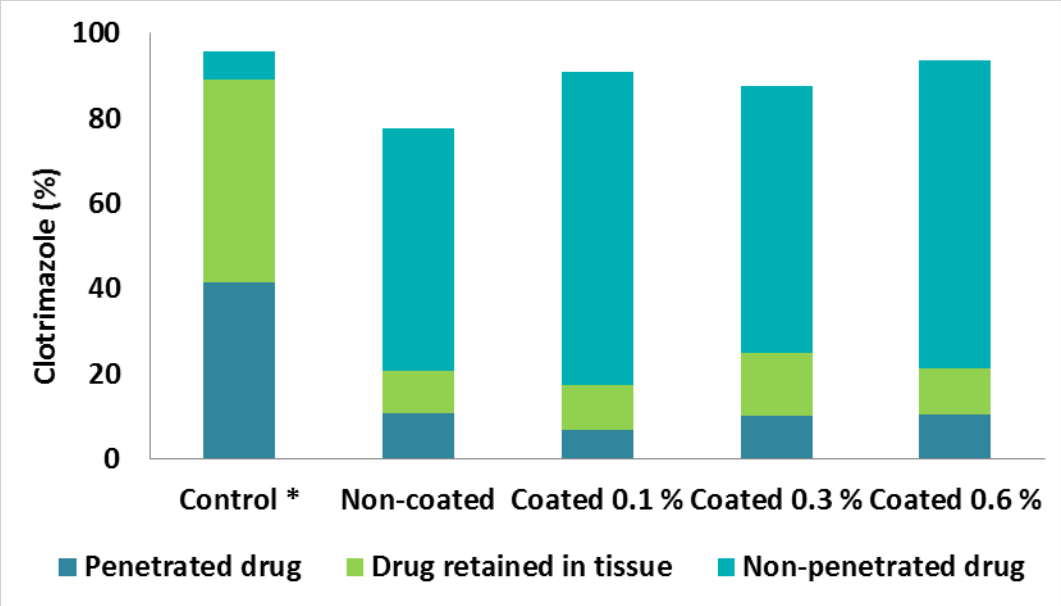

Figure 5.9: Clotrimazole distribution (%) after 24 hours *ex vivo* studies in sheep vaginal tissue (n=3).

* Clotrimazole dissolved in propylene glycol as control.

Modified from Jøraholmen *et al*., 2014 (Paper I) with permission from Elsevier.

After 24 hours, the clotrimazole distribution was measured and the results emphasized the difference in the *ex vivo* behavior of clotrimazole in solution and liposomally-associated clotrimazole (Figure 5.9). Chitosan is known to act as a permeation enhancer by decomposition of the tight junctions (in intestinal epithelium), this is however associated with high molecular weight chitosan (Bernkop-Schnürch and Dünnhaupt, 2012). Our liposomes coated with low molecular weight chitosan were shown to be associated with a lower clotrimazole penetration through the vaginal tissue. The results indicate that liposomally-associated clotrimazole will be retained on the vaginal tissue or within tissue, making it safe for administration during pregnancy.

When aiming for a non-invasive treatment of HPV, reaching into vaginal mucosal tissue and obtain sustained concentrations within affected tissue, while avoiding high systemic levels, is desirable (Foldvari, 2012). Compounds from the vaginal fluid might influence the performance of the delivery system, including the drug release (Owen and Katz, 1999). Thus, to obtain conditions that are closer to those encountered *in vivo*, the experiment was performed in the presence of VFS. The IFN α-2b penetration

through the sheep vaginal tissue was prominent compared to the *in vitro* drug release. Further, a distinct increased IFN α-2b penetration was seen from the PEGylated liposomes compared to control solution (corresponding concentration of IFN α-2b in Intron A buffer) (Figure 5.10).

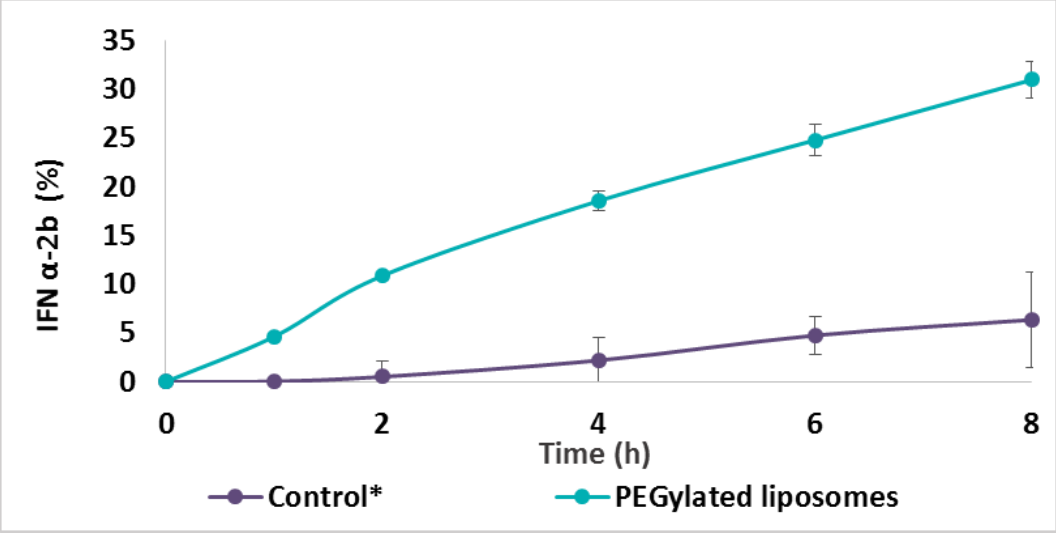

Figure 5.10: IFN α-2b penetration (%) through sheep vaginal tissue from PEGylated liposomes (n = 3).

*IFN α-2b in Intron A buffer as control.

This demonstrates that the PEGylated liposomal formulation enables IFN α-2b to reach into vaginal tissue to a higher extent compared to free IFN α-2b. The findings are in agreement with the liposomal delivery of IFN α-2b to skin; increased skin penetration of IFN α-2b was assured by liposomal formulation (Foldvari et al., 1999).

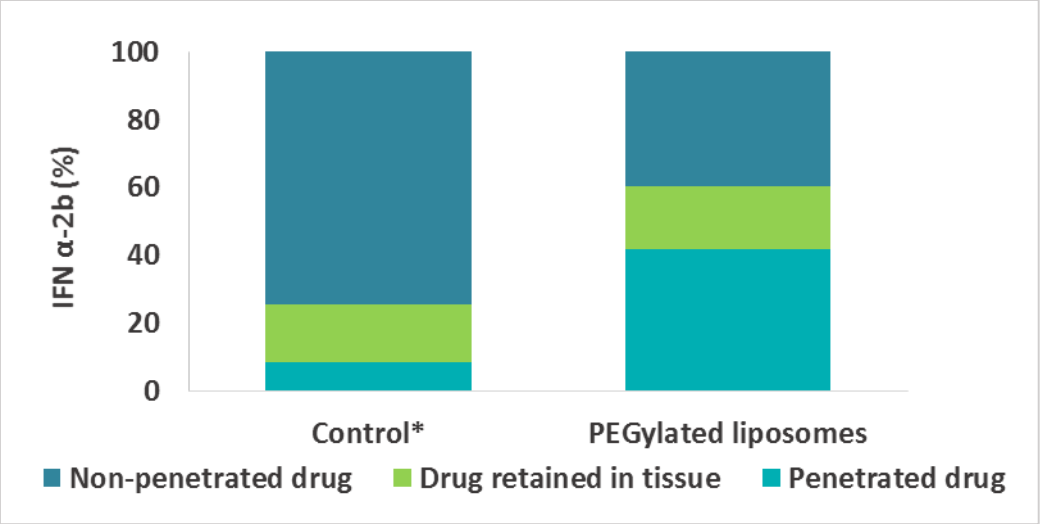

Figure 5.11: IFN α-2b distribution (%) after 8 hours *ex vivo* penetration experiment.

*IFN α-2b in Intron A buffer as control.

After 8 hours, the amount of IFN α-2b retained on top of the vaginal tissue and within tissue was measured. An equal retention of liposomally-associated IFN α-2b and IFN α-2b solution within the tissue was seen (Figure 5.11).

5.5 Liposomal stability

Vesicle size and polydispersity are often parameters for the physical stability of liposomes, and retention of originally entrapped drug indicates the stability of the delivery system. Vesicles have a tendency to form agglomerates, which will behave in a different manner than single vesicles (das Neves *et al.*, 2011a). No significant increase in the vesicle size of the chitosan-coated liposomes was seen after storage at 4 °C for one month (data not shown), indicating that the formation of agglomerates did not occur at this time point.

To obtain a controlled drug release at vaginal site, the delivery system needs to sustain the physical and chemical properties when exposed to the vaginal environment. Local application might affect the stability and performance of the drug delivery system (Owen and Katz, 2005), and the stability of PEGylated

liposomes containing IFN α-2b in the presence of vaginal fluid simulant (VFS) and semen fluid simulant (SFS) was evaluated. The composition, volume, pH and rheological properties of vaginal fluids are affected by age, the menstrual cycle or sexual arousal. Therefore, the exact amount of fluid is not defined, however, studies suggest that an average of 0.5 – 0.75 g of vaginal fluid is generally present in the vagina (Owen and Katz, 1999). The average volume of human ejaculate is found to be 3.4 mL (Owen and Katz, 2005), and the presence of semen is expected to affect the vaginal pH for hours (das Neves *et al*., 2011a). Based on these findings, the PEGylated liposomes were diluted 1:10 (v/v) with VFS and 1:1 (v/v) with SFS to determine the ability of the delivery system to retain entrapped IFN α-2b once exposed to vaginal environment. An incubation time of 2 hours was found appropriate.

A minor leakage of merely 5.1 % of originally entrapped IFN α-2b was detected, indicating the stability of PEGylated liposomes in a simulated vaginal environment and in the presence of SFS.

5.6 Safety and non-irritability *in vivo*

Irritation of the vaginal tissue can lead to increased susceptibility to foreign pathogens and inflammation, hence, delivery systems for the topical application should be safe and non-irritant. Our system was applied *in vivo* to exclude any visual irritation of the vaginal mucosal tissue caused by the system.

Non-coated and chitosan-coated (0.1 and 0.6 %, w/v) liposomes containing clotrimazole, empty non-coated liposomes and clotrimazole in solution (propylene glycol) were applied *in vivo* and photographic documentation of tissue were taken before and after administration. Tissue was studied carefully examining the possible signs of redness or changes in the mucosal tissue after exposure to formulation. No signs of irritation of the mucosal tissue could be visually observed after the local administration of any of the formulations. The absence of irritation of the tissue was confirmed also after the second administration (data not shown).

A change in the normal structure of mucus can be induced by the local administration of nanoparticles and high concentrations of applied nanoparticles might result in disruption of the mucus structure (das Neves *et al*., 2011a). Wang and colleagues demonstrated that surface chemistry might also play a role in whether the mucus barrier is vulnerable to disruption by nanoparticles (Wang *et al*., 2011). They examined the effect of synthetic particles on cervicovaginal mucus and found that high concentrations of mucoadhesive nanoparticles (200 nm) might increase the mucus pore size, allowing foreign particles to penetrate across

mucus to a higher extent. The consequence of continuous application of mucoadhesives on mucus structure should be further evaluated *in vivo*.

5.7 Anti-oxidative and anti-inflammatory activities of resveratrol and liposomal resveratrol (Paper II)

Among several beneficial effects, the strong anti-oxidant and anti-inflammatory effects of resveratrol presents great potential in the treatment of vaginal inflammations and infections. The vaginal administration of resveratrol has shown to reduce HSV replication (Docherty *et al*., 2005). Prior to evaluating the anti-viral potential of liposomes containing resveratrol, we have focused on comparing anti-oxidative and anti-inflammatory activities of resveratrol and liposomally-associated resveratrol.

5.7.1 Radical scavenging activity

Radical scavenging assay was applied to confirm the *in vitro* anti-oxidative activities of resveratrol and liposomal resveratrol compared to other well-known anti-oxidants. The *in vitro* anti-oxidative activities of resveratrol were expressed as the amount of DPPH and $ABTS^{+\bullet}$ radicals scavenged, as the UV absorbance will decrease equivalently to the decreasing number of radicals. We examined the anti-oxidant activity of resveratrol compared to the other well-known anti-oxidants vitamin C and vitamin E. All substances expressed a concentration-dependent radical scavenging activity (Figure 5.12). Resveratrol exhibited a stronger radical scavenging activity for $ABTS^{+\bullet}$ radicals compared to DPPH free radicals. Additionally, a superior $ABTS^{+\bullet}$ scavenging activity was exhibited by lower concentrations of resveratrol compared to respective concentrations of vitamin C and vitamin E. These results are supported by literature (Stojanović *et al*., 2001).

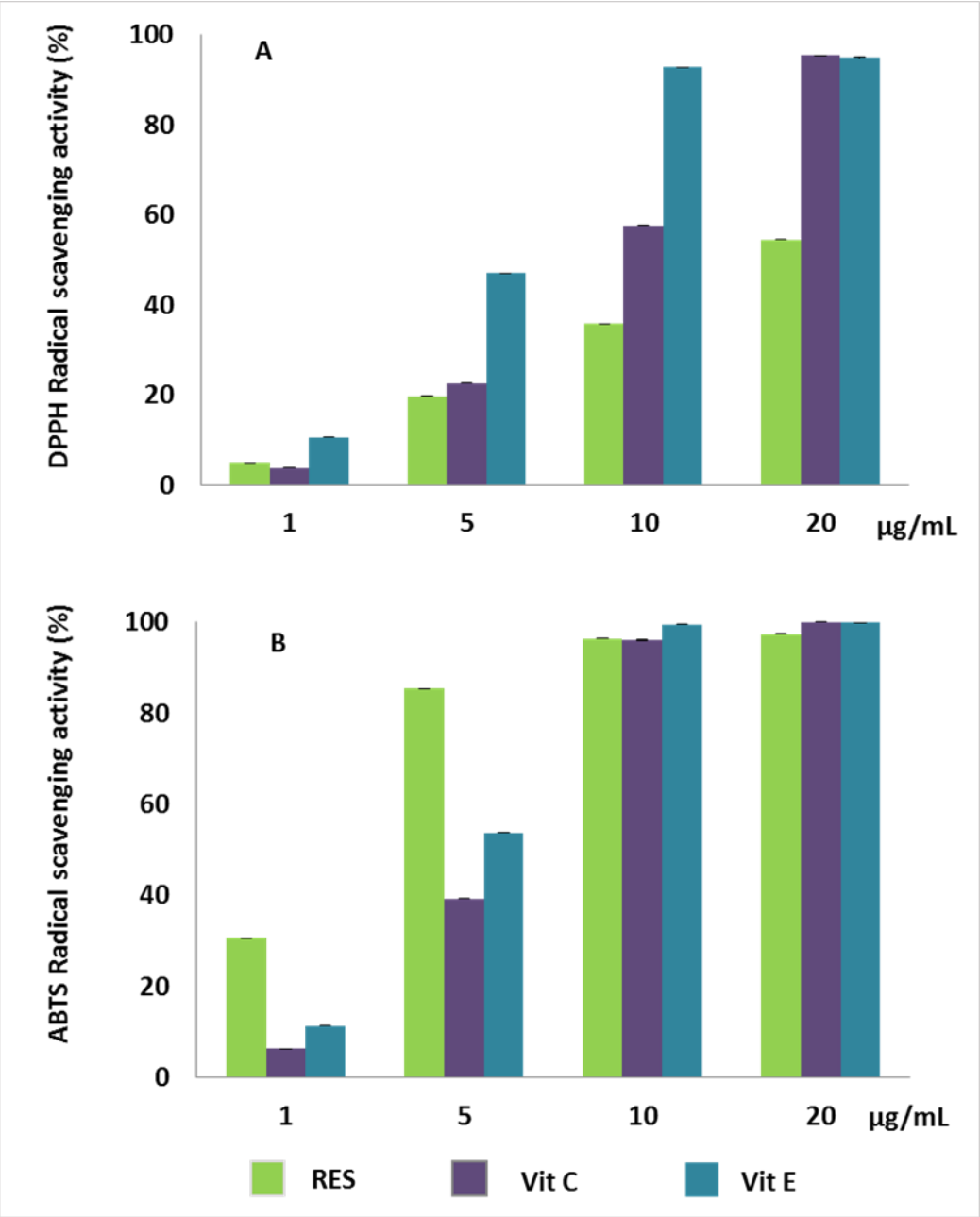

Figure 5.12: Anti-oxidant activities (%) of resveratrol (RES), vitamin C and vitamin E (n=3). A) DPPH radical scavenging activity. B) $ABTS^{+\bullet}$ scavenging activity.

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Polyphenols are a class of compounds which can easily transfer electron and resveratrol is classified under this group. $ABTS^{+\bullet}$ radicals are more reactive with the resveratrol due to the electron transfer process involved, compared to DPPH which involves H atom transfer (Gülçin, 2010), resulting in superior $ABTS^{+\bullet}$ scavenging capacity for resveratrol compared to vitamin C and vitamin E. This was confirmed by a separate experiment, measuring effective concentration causing 50 % decrease of radicals (EC_{50}) (Figure 5.13).

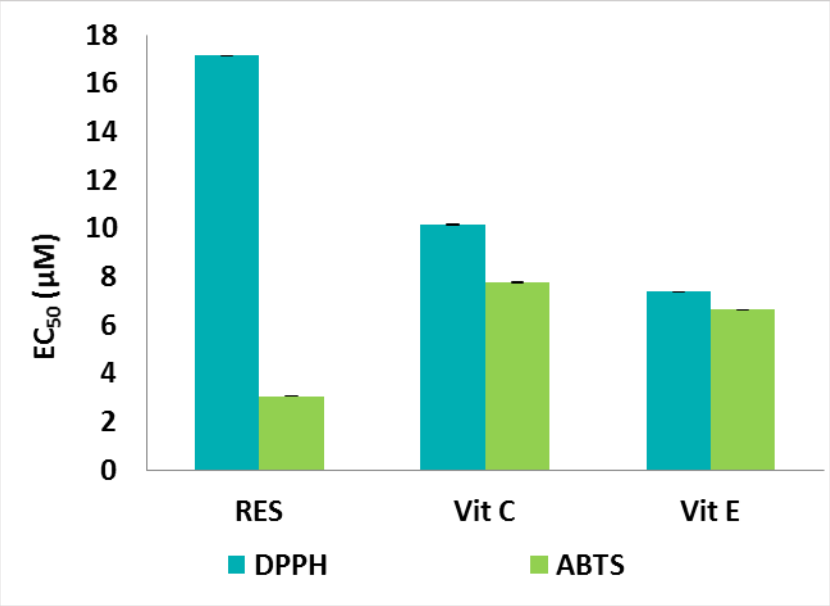

Figure 5.13: Anti-oxidative activity of resveratrol (RES), vitamin C and vitamin E expressed as EC_{50} in µM (n=3).

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Compared to vitamin C and vitamin E, resveratrol exhibited a stronger reactivity towards $ABTS^{+\bullet}$ radicals than DPPH, with an EC_{50} of 17.15 and 3.05 µM against DPPH and $ABTS^{+\bullet}$ radicals, respectively. While vitamin C showed an EC_{50} of 10.25 and 7.77 µM and vitamin E an EC_{50} of 7.38 and 6.64 µM against DPPH and $ABTS^{+\bullet}$ radicals, respectively.

5.7.2 Effect of liposomal resveratrol

The biological activity of resveratrol is limited due to its low solubility and chemical instability, hence these limitations needs to be overcome to assure use of resveratrol as a therapeutic agent. Liposomes have the ability to incorporate poorly soluble substances, and a satisfactory resveratrol entrapment was determined (Table 5.3). In addition to the ability of chitosan-coated liposomes to assure controlled release of resveratrol, we wanted to explore the effect of liposomal formulation on the anti-oxidative and anti-inflammatory activities of resveratrol.

5.7.2.1 Anti-oxidative activity

Normal cellular metabolism produces low concentrations of reactive oxygen species (ROS). When exposed to pathogens, an increased metabolic activity and immune cell reaction is triggered causing an excess production of ROS, which causes oxidative stress. The process of inflammation and infection is accelerated due to the overproduction of free radicals and pro-inflammatory cytokines that causes prominent damage to cells. Anti-oxidants may impair the effect of free radicals by scavenging and inhibit this inflammation process. Further, an increased activity of superoxide dismutase (SOD) may restore the balance of ROS production. The anti-oxidant effect of resveratrol is expressed either directly by donating an electron to free radicals and/or providing hydrogen or indirectly by increasing the SOD activity (Gülçin, 2010; Zheng *et al*., 2010). We compared *in vitro* anti-oxidative activity of resveratrol and liposomal resveratrol by measuring the SOD activity in murine macrophages, J774.1 cells. Cells were cultured by general procedure and the media of the monolayer confluents of cells were replaced by the fresh media or media containing 1µg/mL lipopolysaccharides (LPS) together with different concentration of the samples (resveratrol solution or liposomal resveratrol). The SOD activities of the resveratrol and liposomal resveratrol were expressed in percentage by comparing to corresponding control basal levels.

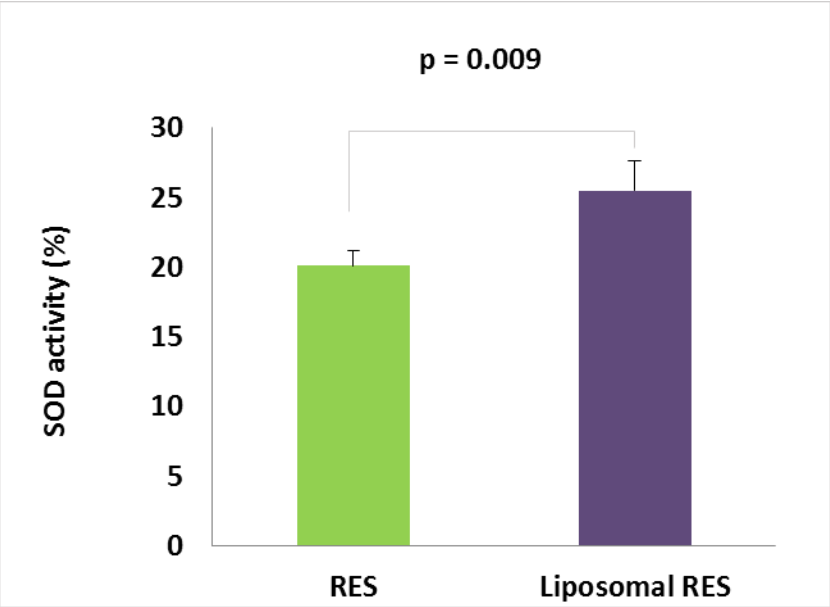

Figure 5.14: The effects of resveratrol (RES) and liposomal resveratrol on enhancement of superoxide dismutase (SOD) activity (%) in LPS-induced macrophages (n=3).

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The SOD activities in lipopolysaccharide (LPS)-induced J774.1 cells were measured to compare the anti-oxidant activity of resveratrol in solution (DMSO) and liposomal resveratrol. Compared to the basal SOD activity of the controls (0.2 % DMSO and empty liposomes, respectively), the activity was increased by 20 % and 26 % for resveratrol solution and liposomally-associated resveratrol, respectively (Figure 5.14). These results show a significantly increased SOD activity for liposomal formulations as compared to solution.

5.7.2.2 Anti-inflammatory activity

Inflammation is one of the first responses of the immune system to infection and is caused by eicosanoids and cytokines, which are released by injured or infected cells. Pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) are involved in the process of pathological infection (Dinarello, 2000). Hence, the anti-inflammatory activities were expressed by the effect of resveratrol on the production of nitric oxide (NO), IL-1β, and TNF-α.

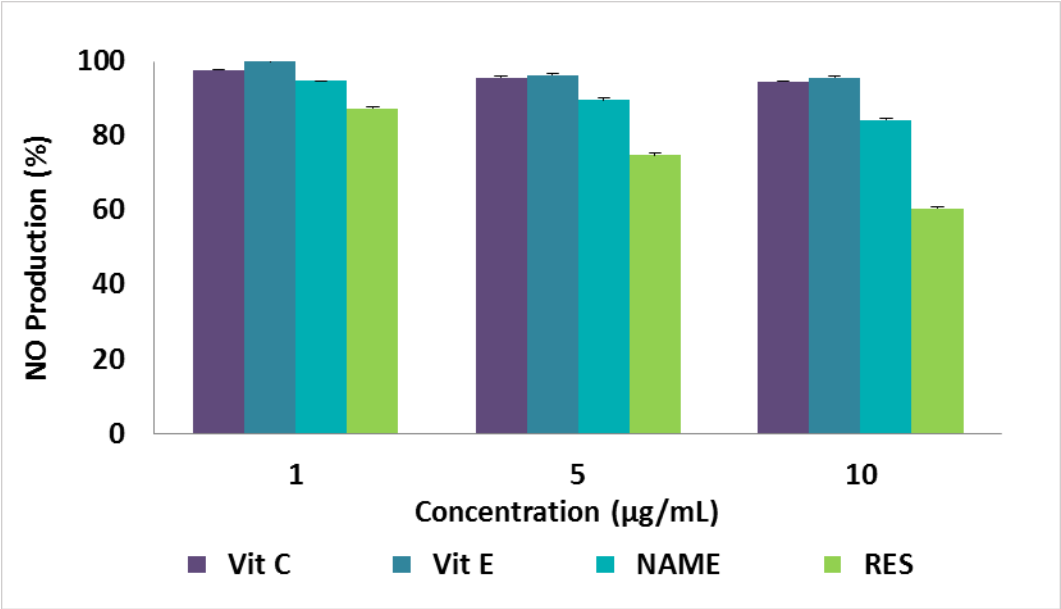

Figure 5.15: The effects of resveratrol (RES), vitamin C, vitamin E and L-nitro-arginine methyl ester (NAME) on nitric oxide (NO) production (%) in LPS-induced macrophages (n=4).

Modified from Jøraholmen *et al*., 2015 (Paper II) with permission from Elsevier.

When treated with LPS, macrophages intensifies the NO production resulting in unstable NO radicals. The activity of resveratrol was expressed as percentage of NO production inhibition. A superior NO production inhibition was seen for resveratrol compared to the activity of vitamin C and vitamin E. Both resveratrol and L-nitro-arginine methyl ester (NAME) expressed a concentration dependent inhibition of NO production, however resveratrol was found to be more potent than NAME (Figure 5.15).

Further, the inhibitory activity of resveratrol was compared to that of liposomal resveratrol. The amount of resveratrol required to show a 50 % inhibition of NO production (IC_{50} µg/mL) in LPS-induced J774.1 cells was reduced by liposomal incorporation (Figure 5.16). Resveratrol and corresponding liposomal formulation showed an IC_{50} of 13.5 and 9.6 µg/ml, respectively. The increased activity of liposomally-associated resveratrol confirm the superior anti-inflammatory effect of resveratrol when in drug delivery system.

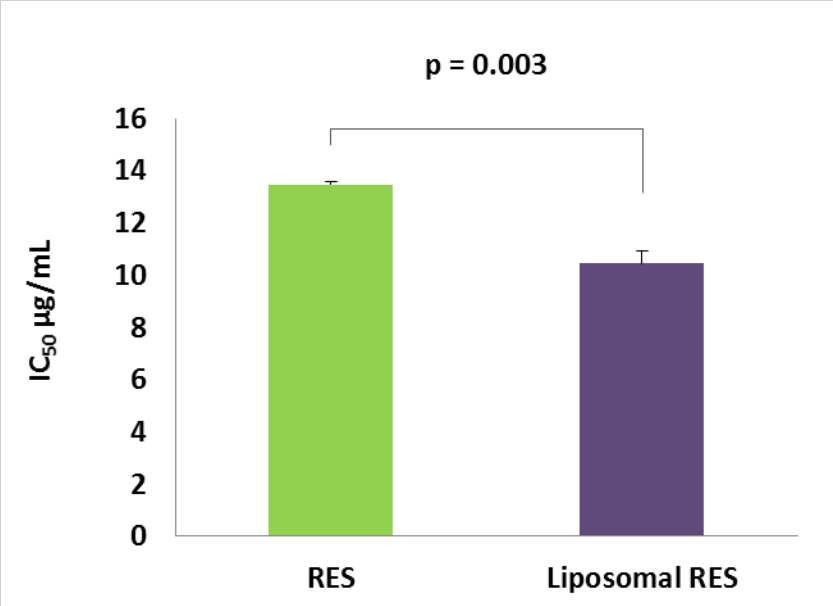

Figure 5.16: The effect of resveratrol (RES) and liposomal resveratrol on NO production in LPS-induced macrophages expressed as IC_{50} in µg/mL (n=4).

Modified from Jøraholmen *et al*., 2015 (Paper II) with permission from Elsevier.

The production of cytokines TNF-α and IL-1β by LPS-induced macrophages in the presence of resveratrol (10 µg/ml) and corresponding liposomal formulation was measured and compared to a control group. A superior TNF-α production inhibition was seen for liposomally-associated resveratrol (Figure 5.17), with a 52 % and 70 % inhibition for resveratrol and liposomal resveratrol, respectively. Further, the IL-1β production was reduced in the presence of resveratrol (Figure 5.17). Compared to the control group, resveratrol (10 µg/ml) and corresponding liposomal formulation inhibited the IL-1β production by 60 % and 62 %, respectively.

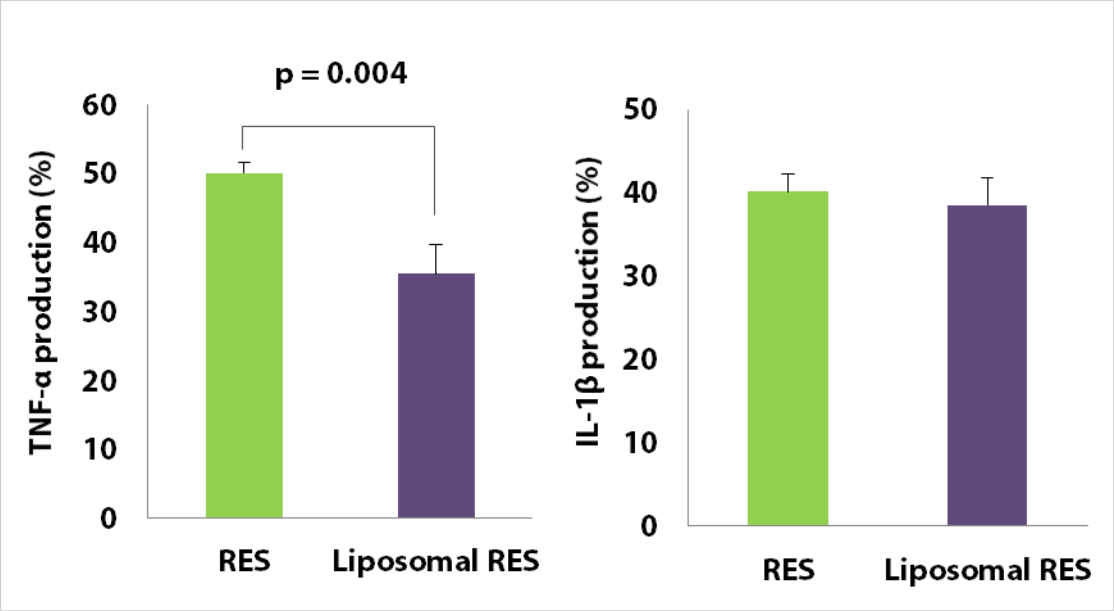

Figure 5.17: The effects of resveratrol (RES) and liposomal resveratrol on TNF-α and IL-1β production (%) in LPS-induced macrophages (n=3).

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Liposomal resveratrol was shown to be superior in both the anti-oxidative and anti-inflammatory studies. The reason for the enhanced inhibitory effect might be an increased cellular uptake and/or maximized effect due to the liposomal entrapment of resveratrol. Additionally, the anti-microbial properties of chitosan may provide an advantage against vaginal pathogens. However, further *in vivo* and clinical studies are needed to obtain the direct evidences.

The findings imply that liposomal resveratrol might be suitable treatment for vaginal inflammation. The mechanism of which resveratrol inhibits HSV production is not known (Docherty *et al*., 2005), however, the enhanced anti-inflammatory effect of liposomal resveratrol suggest an improved anti-HSV activity might be possible by liposomal delivery system.

6 Conclusions

We have developed and optimized liposomal drug delivery systems for vaginal application, which can be either mucoadhesive or mucoresistant depending on the type of polymer used in the formulations. Chitosan coating of liposomes will result in mucoadhesive delivery system able to retain at the administration site for a sufficiently long period of time to assure drug presence at the site, whereas PEGylation of liposomes will results in ability of the system to penetrate deeper within vaginal epithelium and act on deep infections. The systems could be optimized to entrap different active molecules, as we have shown for three compounds of different molecular weights and solubilities. Clotrimazole (conventional therapeutic drug for vulvovaginal candidiasis), resveratrol (a potential candidate for genital HSV) and IFN α-2b (a potential biological drug for genital HPV) were separately studied in their liposomal formulations. An acceptable entrapment of both lipophilic (clotrimazole and resveratrol) and hydrophilic (IFN α-2b) molecules confirmed the system's robustness.

Chitosan-coated liposomes exhibited mucoadhesive properties *in vitro*, with superiority achieved by coating with low polymer concentration, demonstrating the ability to obtain prolonged retention time at vaginal site. Further, chitosan-coated liposomes were shown to enable controlled and prolonged release of associated drug as well as reduced penetration through the vaginal tissue. *In vivo* evaluations confirmed the system to be non-irritant to vaginal mucosal tissue.

PEGylated liposomes were shown to avoid interactions with mucin, indicating mucoresistant properties, enabling mucus-penetration. Further, PEGylated liposomes exhibited enhanced penetration through the vaginal tissue enabling drug release in close proximity to vaginal epithelium.

Resveratrol was confirmed to exhibit strong anti-oxidative and anti-inflammatory activities. These activities were further enhanced by the resveratrol incorporated liposomal formulations making liposomal resveratrol an interesting option in treatment of vaginal inflammation.

7 Perspectives

Further *in vivo* and clinical studies are required to confirm the safety of the system, particularly the continuous application of mucoadhesive system to compromised vaginal epithelium needs to be evaluated.

Chitosan is known to be non-toxic, the toxicity of PEG-modified liposomes should however be evaluated to confirm the safety in topical vaginal application. Additionally, a focus on how the local treatment might influence the natural vaginal flora should be included in the studies to enable the restoring of the healthy vaginal environment and avoid increased post-therapy susceptibility to infection.

An evaluation of the anti-microbial activities of the mucoadhesive delivery system should be performed to explore the possible additional advantages against vaginal pathogens provided by chitosan coating.

As a biological substance, the stability of IFN is limited and the stability when liposomally-associated should be evaluated.

Further, the longtime stability of all liposomal formulations should be tested at different storing conditions.

It would be further interesting to study the effect of PEG chain length when used in the surface modification of liposomes. Recent suggestions that PEG with molecular weight down to 7.5 kDa will enable a more rapid mucus penetration as compared to 20 kDa should be explored and compared to the presented findings.

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Chitosan-coated liposomes for topical vaginal therapy: Assuring localized drug effect

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ABSTRACT

The choice of drug therapy in pregnant patients suffering from vaginal infections is limited by the safety profile of the drug. Assuring the efficient topical therapy to avoid systemic absorption is considered the best therapy option. Chitosan-coated liposomes have been developed and optimized to assure localized therapy of clotrimazole. Chitosan was selected as mucoadhesive polymer both to prolong system's retention at the vaginal site and act on biofilms responsible for high recurrence of infections. Sonicated liposomes were coated with chitosan in three different concentrations, namely 0.1, 0.3 and 0.6% (w/v). Clotrimazole-containing (22 μg/mg lipid) chitosan-coated liposomes were in the size range of 100–200 nm. The *in vitro* release studies confirmed prolonged release of clotrimazole from both non-coated and chitosan-coated liposomes as compared to control. The *ex vivo* penetration experiments performed on the pregnant sheep vaginal tissue showed that coated liposomes assured increased clotrimazole tissue retention and reduced its penetration as compared to the control. Mucin studies revealed that the coating with lower chitosan concentration increased the system's mucoadhesive potential, as compared to coating with higher concentrations. These results provide a good platform for further *in vivo* animal studies on mucoadhesive liposomes destined to localized vaginal therapy.

1. Introduction

Although the occurrence of vaginal infections in pregnancy is common, the choice of drug therapy is rather limited (das Neves et al., 2008). In particular, topical antifungal therapy is preferred due to the systemic toxicity of antifungal drugs (Chang et al., 2002). In pregnant patients, the two main therapy goals can be summarized as (i) assuring the high local drug concentration with concomitant avoidance of systemic absorption and (ii) prevention of infection recurrence (Vanić and Škalko-Basnet, 2013). We propose that coating of liposomal surfaces with chitosan can assure both of the goals. When vagina is the site of drug administration, it is also important that both the drug and corresponding delivery system are safe and non-irritating to the delicate vaginal mucosa (Woodrow et al., 2009). The selection of mucoadhesive polymer will be therefore based on its biodegradability, biocompatibility and confirmed mucoadhesivness. Chitosan fulfils all the above mentioned criteria (Bernkop-Schnürch and Dünnhaupt, 2012; Bhattarai et al., 2010). Moreover, chitosan as mucoadhesive polymer is suited for repeated adhesion, as it does not become inactivated after the first contact with mucus; no reduction in its mucoadhesiveness has been reported (Valenta, 2005). In respect to recurrence, it is now clear that bacterial biofilms play an important role, as the negatively charged polysaccharide matrix coats the bacteria in the biofilm and restricts the penetration of antimicrobial in deeper parts of biofilm. Recently, chitosan was proposed to be able to disrupt bacterial biofilms in vaginal environment more efficiently than other polymers (polycarbophil). Even more importantly, its anti-biofilm effect was found to be pH-independent (Kandimalla et al., 2013).

The mucoadhesiveness of chitosan-based delivery systems has been studied in various routes of drug administration (das Neves et al., 2011a; Gradauer et al., 2012; Sugihara et al., 2012; Takeuchi et al., 2001; Takeuchi et al., 2005; Wang et al., 2011); however, its potential in vaginal drug delivery was comparatively less studied (Valenta, 2005; Bonferoni et al., 2008; Kast et al., 2002; Perioli et al., 2008; Perioli et al., 2009; Berginc et al., 2014). Based on its confirmed mucoadhesiveness, it is reasonable to expect that chitosan-based delivery systems will be superior in vaginal drug

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delivery, as some recent studies on chitosan nanoparticles indicate (Meng et al., 2011).

The success of non-invasive drug delivery via vaginal mucosa will be the result of the interplay between the local vaginal environment, drug and physicochemical properties of drug carrier (Berginc et al., 2014). However, the interaction between drug delivery system and cervicovaginal mucus can affect the performance of drug nanocarrier, as the carrier must migrate through the vaginal or cervical fluid in order to deliver drug to the underlying mucosal surface (das Neves et al., 2012; Vanić and Škalko-Basnet, 2013). Vaginal mucosal tissue has relatively low turnover, which would be beneficial for prolonged residence time (Andrews et al., 2009). Vaginal absorption of drugs occurs in two main steps, namely the drug dissolution in vaginal lumen followed by the membrane penetration (Hussain and Ahsan, 2005).

As a model drug we selected clotrimazole, often prescribed in vulovaginal candidiasis. Its local therapy is recommended to pregnant and breast-feeding patients, as well as to patients not using reliable birth control methods, or planning to become pregnant (das Neves et al., 2008).

2. Materials and methods

2.1. Materials

Lipoid S 100 (PC, soybean lecithin, >94% phosphatidylcholine) was a generous gift from Lipoid GmbH, Ludwigshafen, Germany. Chitosan, low molecular weight (Brookfield viscosity 20,000 cps, degree of deacetylation (DD of 92%), acetonitrile (CHROMASOLV® gradient grade), bovine serum albumin, clotrimazole, glycerol, methanol CROMASOLV®, mucin from porcine stomach (type III, bound sialic acid 0.5–1.5%, partially purified) and sodium chloride were the products of Sigma–Aldrich, Chemie GmbH, Steinheim, Germany. Acetic acid (glacial), anhydrous potassium phosphate and sodium hydrogen phosphate were purchased from Merck KGaA, Darmstadt, Germany. Calcium hydroxide, glucose, lactic acid, potassium hydroxide, propylene glycol, sodium hydroxide and urea were obtained from NMD, Oslo, Norway. Ammonium acetate was the product of BHD Prolab, Leuven, Belgium.

2.2. Preparation of liposomes with clotrimazole

Liposomes were prepared by the method described earlier (Berginc et al., 2014). In brief, clotrimazole (20 mg) and PC (200 mg) were dissolved in methanol in a round bottom flask. The solvent was evaporated using rotoevaporator system (Büchi rotavapor R-124 with vacuum controller B-721, Büchi Vac® V-500, Büchi Labortechnik, Flawil, Switzerland) for at least 1 h at 50 mm Hg and 40 °C. The remaining film was then re-suspended in 10 mL of distilled water. If necessary, ultrasonic bath was used to completely dislodge the film from the flask. Liposomal suspensions were stored in the refrigerator (4–8 °C) overnight prior to further use.

2.3. Vesicle size reduction

Liposomes (4 ml) were transferred to a 10 mL beaker and placed on ice bath. The needle probe tip of probe sonicator was placed in the centre of the beaker containing liposomal suspension. The sonicator (Ultrasonic processor 500 W, Sigma–Aldrich, St. Louis, MO, USA) was set to 40% amplitude and the liposomes were exposed to ultrasonic irradiation for 1, 2 or 2 × 2 min, respectively. The sonicated liposomes were stored in the refrigerator for at least 6 h prior to further use.

2.4. Particle size analysis

The particle size distributions of liposomes were determined by photon correlation spectroscopy (Submicron particle sizer model 370, Nicomp, Santa Barbara, CA, USA). In order to avoid interference from dust particles, the test tubes to be used for the determination were filled with distilled water and sonicated for 10 min in ultrasonic bath, then rinsed with filtered water (using 0.2 μm filter) prior to the experiments. Small aliquots of the samples were diluted with the filtered water to obtain particle intensity of approximately 200–350 kHz (di Cagno et al., 2011). All formulations were prepared in a laminar airflow bench and analyses run in vesicle mode and the intensity–weight distribution at 23 °C. Three parallels were determined (run time 10 min) for each sample measurement.

2.5. Zeta potential determination

Zeta potential measurements were performed on a Malvern Zetasizer Nano Z (Malvern, Oxford, UK). To ensure the validity of the measurements, the instrument was calibrated throughout the measurements using the Malvern zeta potential transfer standard (−50 ± 5 mV). The liposomal suspensions were diluted in 1:40 ratio in filtrated water before measurements to achieve the proper count rate. All measurements were performed at 23 °C and the results were expressed as the average of at least three independent samples.

2.6. Entrapment efficiency determination

To separate free from liposomally entrapped drug, the sonicated liposomes were ultracentrifuged (Beckman model L8-70M preparative ultracentrifuge with SW 60 Ti rotor, Beckman Instruments, Palo Alto, CA, USA) for 30 min, at 10 °C and 85,000 *g*. The pellet (containing unentrapped drug and liposomes larger than 200 nm) was separated from the supernatant (smaller liposomes containing clotrimazole), re-suspended in 500 μL of distilled water and finally diluted to 2 mL with methanol. Drug content in both supernatant and pellet was determined by the HPLC method. A reversed phase column (XTerra® RP18 5 μm, 3.9 × 150 mm column, Waters, Dublin, Ireland) installed in a Waters e2795 separations module coupled with a Waters 2489 UV–vis detector was used in the measurements. The mobile phase consisted of acetonitrile and MilliQ water in a gradient starting at 30% acetonitrile (A), increasing to 90% A over 10 min, then to 100% A after 11 min. The HPLC measurements settings were as follows: flow rate 1 mL/min, column temperature of 25 °C, sample temperature 25 °C, injection volume 20 μL, run time 11 min and the detection wavelength 210 nm. The correlation coefficient was 0.9997 and the minimum detectable amount of clotrimazole 0.5 μg/mL. The entrapment was expressed as the amount of drug present in sonicated vesicles. The measurements were performed in triplicates.

2.7. Phospholipid content

An enzymatic assay was used to determine the amount of lipid present in liposomes in order to calculate the entrapment efficiency. For this purpose a commercial test kit (Phospholipids B; Wako Chemicals USA, Inc., Richmond, VA, USA) was applied in the method described earlier (Basnet et al., 2012). Measurements were performed in triplicates.

2.8. Coating of liposomes

The chitosan solutions (0.1, 0.3 and 0.6%, w/v) used for liposome coating were prepared in 0.1% and 0.5% (v/v) glacial acetic acid,

respectively. The chitosan solution was added drop-wise to an equal volume of liposomes free from unentrapped clotrimazole, under controlled magnetic stirring at room temperature for 1 h, followed by incubation in the refrigerator overnight. The rate of stirring was kept constant for all preparations (Karn et al., 2011).

2.9. In vitro *release study*

Drug release was followed by the method described earlier (Hurler et al., 2012). The Franz cell manual diffusion system (Perme Gear Ink, Diffusion cells and Systems, Hellertown, USA) was properly cleaned with methanol, demineralized water and distilled water, respectively. The heating circulator (Julabo Laboratechnik, F12-ED, Seelback, Germany) was set to 37 °C. The acceptor chamber (12 mL) was filled up with acetate buffer (pH 4.6, 77.1 g of CH_3COONH_4, 70 mL of glacial acetic acid and distilled water up to 1000 mL). Cellophane membrane (Max Bringmann KG, Wendelstein, Germany) was pre-soaked in the same buffer. Liposomal samples (both chitosan-coated and non-coated and sonicated for 2×2 min) or controls (clotrimazole in propylene glycol) were added in the donor chamber and the system was properly sealed. The drug content in all tested samples was determined (HPLC) prior to the study and the volumes of formulations in donor chambers normalized to assure the same drug amount. The samples (500 μl) were collected after 1, 2, 3, 4, 8 and 24 h. The samples were replaced by an equal volume of buffer upon removal of sample from the acceptor chamber. The drug content was determined by the HPLC method. The experiments were performed in triplicates.

2.10. Preparation of vaginal tissue

The sheep vaginal tissue (from pregnant animals) was obtained from the Laboratory Animal Centre, University of Oulu, Finland. The vaginal tissue was carefully removed from the underlying tissue and cleaned with the physiological solution (pH 7.4). Adequate sized pieces were moisten by 0.9% (w/w) NaCl and packed in clinging film, and frozen (−20 °C). They were left to defrost at room temperature for at least 2 h prior to experiments. The thickness of the tissue was determined to be ranging from 900 to 1140 μm. It was earlier confirmed that no significant differences were observed in using fresh or snap-frozen tissue samples (Sassi et al., 2004). We have earlier also compared the barrier properties of the fresh vaginal tissue and frozen (−20 °C) and thawed tissue (both animal skin and vaginal tissue) and have not observed any difference in the properties.

2.11. Ex vivo *penetration study*

The defrosted vaginal tissue was cut to fit the Franz diffusion cells surface (1.77 cm^2). The acceptor chamber was filled up with phosphate buffer (pH 7.4, 8 g/L NaCl, 0.19 g/L KH_2PO_4, and 2.38 g/L Na_2HPO_4) and the vaginal tissue fixed between donor and acceptor chamber. Samples (600 μL) were added into the donor cells and the system was properly sealed. The amount of the drug in each cell was the same, as confirmed by the HPLC analysis. Samples of 500 μL were collected at 1, 2, 3, 4, 8 and 24 h and replaced with an equal amount of buffer. Drug content was determined by the HPLC method. The measurements were performed in triplicates.

2.12. In vitro *mucin-binding test*

The mucoadhesion was measured by determining the binding of liposomes to pig mucin. Liposomes (1 mL) were mixed with equal volume of pig mucin (PM) suspension (400 μg/mL) in 0.05 M PBS (pH 7.4) and incubated at room temperature for 2 h, followed by ultracentrifugation for 1 h, at 10 °C at 216,000 *g* (Optima LE-80; Beckman Instruments, Palo Alto, USA). Aliquots of 200 μL (4 from each sample) of the supernatants (free PM) were transferred to a microtitre plate (Costar® UV 96-well plate with UV transparent flat bottom, Acrylic, Costar®, Corning, NY, USA) and measured spectroscopically at 251 nm (Microtitre plate reader; Spectra Max 190 Microplate, Spectrophotometer Molecular devices, Sunnyvale, CA, USA). The PM binding efficiency was calculated according to Naderkhani et al. (2014).

2.13. Statistical evaluation

The student's *t*-test was used for comparison of two means. A significance level of $p < 0.05$ was considered to be appropriate.

3. Results and discussion

In order to achieve optimal therapeutic outcome, the delivery system is expected to provide a sufficient amount of the active ingredient (in our case clotrimazole) at the desired site of action (vaginal mucosal tissue) for a sufficiently long period of time to enable drug to perform its therapeutic action. Therefore, in the first step in optimization of chitosan-coated liposomes for topical vaginal delivery, we were focusing on the relationship between encapsulation yields *vs* particle size. Smaller particles are expected to provide larger surface area, however those particles carry less clotrimazole load, whereas larger particles will incorporate more of the drug, but will provide smaller contact surface for possible mucoadhesion.

3.1. Liposomal characteristics

Liposomes have been studied as drug delivery systems for almost 5 decades. A number of liposomes-based products are on the market and many more are in pipelines (Allen and Cullis, 2013). We have previously reported on the potential of liposomes to enhance the anti-inflammatory properties of associated curcumin destined for vaginal therapy (Basnet et al., 2012). Guided by those promising results, we selected liposomes as carrier for clotrimazole and used chitosan-coating to (i) assure prolonged and controlled release of clotrimazole and (ii) assure its retention at vaginal site, without significant penetration through the vaginal tissue.

Size and size distribution of liposomes are important characteristics of liposomes destined for topical drug delivery. The effect of liposomal size on the efficacy of liposomal delivery to skin is rather well studied (Cevc, 2004); however, relatively little is known about the effect of vesicle size on the delivery of drugs intended for mucosal targeting at vaginal site. Takeuchi et al. (2001) have found that the amount of liposomes penetrating into the intestinal mucous layer increased when the size of the liposomes was reduced to approximately 100 nm for both non- and chitosan-coated liposomes. In previous experiments (Berginc et al., 2012) we observed that curcumin in smaller vesicles penetrated less into the upper layers of vaginal tissue as compared to curcumin in mulitilammelar vesicles. Both types of liposomes exhibited better tissue retention as compared to curcumin in solution form. Similarly, polymer nanoparticles smaller than 200 nm were reported to successfully deliver small-interfering RNA and provided sustained gene silencing throughout the female reproductive tract for at least 14 days (Woodrow et al., 2009).

The particle size shown in Table 1 indicates that the liposomes sonicated for 2×2 min were in the desired size range. Liposomal dispersions exhibited two distinguished peaks in distributions, indicating bimodal distribution and rather high polydispersity

Table 1
Liposomal characteristics ($n = 3$).

Time of sonication (min)	Vesicle size				PI	Entrapment (%)
	Peak 1 (nm)	Weight intensity (%)	Peak 2 (nm)	Weight intensity (%)		
1	317 ± 47	56.8	40 ± 5	41.0	0.58	23.2 ± 2.5
2	234 ± 31	53.9	36 ± 5	43.8	0.46	25.0 ± 0.5
2 × 2	111 ± 16	83.1	29 ± 4	16.9	0.46	16.5 ± 4.5

Table 2
The effect of chitosan-coating on liposomal size distribution and zeta potential ($n = 3$).

Coating (%, w/v)	Vesicle size				PI	Zeta potential (mV)
	Peak 1 (nm)	Weight intensity (%)	Peak 2 (nm)	Weight intensity (%)		
–	107 ± 3	54 ± 3	27 ± 3	46 ± 3	0.34	−1.6 ± 0.2
0.1	135 ± 21	53 ± 6	42 ± 9	45 ± 5	0.29	25.9 ± 4.0
0.3	141 ± 6	64 ± 6	48 ± 5	35 ± 6	0.27	35.6 ± 1.9
0.6	190 ± 8	58 ± 6	54 ± 2	42 ± 6	0.29	43.8 ± 3.3

(Table 1). With the increase in sonication time, the polydispersity index values decreased, as expected (Table 1).

We tried to minimize the exposure of liposomes to the sonication force, as it is known that extensive sonication can lead to the release of originally incorporated drug and lipid degradation (di Cagno et al., 2011).

Due to highly lipophilic nature of clotrimazole (log P of 3.5), clotrimazole was dissolved in the organic solvent together with lipid during the preparation of liposomes and was expected to incorporate itself within lipid bilayers of liposomes. We observed similar entrapment efficiencies for liposomes sonicated for 1 and 2 min, and the loss of originally entrapped clotrimazole was remarkable only after sonication for 2 × 2 min, resulting in smaller liposomes (Table 1). In comparison to literature data, our entrapment (up to 22 μg clotrimazole/mg lipid) was found to be lower; however, none of the published articles describes the use of the same liposome preparation method and phospholipid composition. Although Ning et al. (2005) reported a very high entrapment of clotrimazole (over 90%) using the film method in preparation of liposomes, they used dialysis to separate unentrapped from liposomal drug. The fact that clotrimazole has aqueous solubility of only 5.5 μmol/L (molecular weight 344) (Bilensoy et al., 2006), and that no data is available on whether or not the sink conditions were assured in the experimental set up of Ning et al. (2005), it might be possible that part of the drug was accumulated in the dialysis-tube in a form of precipitates, falsely contributing to high entrapment values. Liposomes prepared by the proliposome and polyol dilution methods were reported to incorporate more clotrimazole (Pavelić et al., 1999, 2005); however, the methods of preparations differed from the method used in our experiments, and, more importantly, the vesicle size was larger than in our case. Proliposome method is known to yield multilamellar liposomes thus enabling high incorporation of lipophilic drug (Pavelić et al., 1999). An additional difference between the previous and current experiments was the liposomal composition (Pavelić et al., 2005). We choose to prepare liposomes from a simple lipid mixture to be able to follow the effect of chitosan coating in a rather straightforward manner, avoiding the interference of possible ionic interactions between lipid and chitosan and consequently mucin.

The coating of liposomes is expected to result in an increase in their original size (Filipović-Grčić et al., 2001; Karn et al., 2011). Although the entrapment of clotrimazole was lower than we have desired, considering that the obtained vesicle size was in the optimal range for the purpose of development of mucoadhesive liposomal delivery system, we proceeded with coating of those vesicles. Prolonged retention time would be a rationale for lowering the dose needed to induce antifungal effect; therefore lower drug load should not limit the applicability of the system.

3.2. Coating of liposomes

It is well established that the polymer concentration significantly influences the strength of mucoadhesion. Moreover, the optimal polymer concentration depends on the physical state of the delivery system (Andrews et al., 2009) and, in the case of coated liposomes; the liquid nature of the system needs to be taken into consideration. Therefore, we have used three different concentrations in the coating of liposomes, all resulting in liquid formulations. The coating resulted in an increase in particle size (Table 2), in agreement with the results reported by Karn et al. (2011) and Gradauer et al. (2012). The coating of liposomes with chitosan also resulted in an increase of liposome zeta potential (Table 2) in agreement with Berginc et al. (2014). Moreover, the pH of liposomal suspensions changed upon coating; non-coated liposomal suspensions had a pH of 6.0 whereas 0.1, 0.3 and 0.6% coated liposomal suspensions had a pH of 4.12, 4.02 and 4.07, respectively. This would represent additional advantage of chitosan-coated liposomes; it is well known that *C. albicans* adheres to vaginal tissue with higher affinity at pH 6 than at pH 4 and that acidic formulations also restore the physiological acid pH of vagina (Chang et al., 2002).

It was previously reported that the amount of polymer used for coating of nanoparticles and found on vesicle surface was similar regardless of the molecular weight of chitosan used (Llabot et al., 2011). However, nanoparticles coated with low molecular weight chitosan where found to be more bioadhesive than those coated with high molecular weight chitosan. This could be explained by the interpenetration mechanisms with the mucin chains, as it is known that long polymer chains reduce the interpenetration, reducing the bioadhesive strength (Llabot, et al., 2011). This was the reason why we have selected low molecular weight chitosan as a coating material.

Although there is no consensus on the optimal size range of nanocarriers expected to penetrate mucus layer, it was suggested that the nanocarriers in the size range of 200–500 nm are superior to both much smaller and also larger nanosystems (das Neves et al., 2011b). Takeuchi et al. (2005) confirmed the superiority of nanosize chitosan-coated liposomes in prolonging the retention time in the intestinal mucosa as compared to larger vesicles. However, the vaginal mucosa has unique features which make direct translation of the results rather difficult.

Regarding the optimal size, it is also important to consider that some of the particles tend to agglomerate, and that agglomerates behave in a different manner than single particles (das Nevas et al., 2011a). We have tested our liposomes for one month stability (at 4 °C) and have not observed a significant increase in the original size of chitosan-coated vesicles (data not shown). It can be hypothesized that the chitosan-coating is working as a stabilizer by prohibiting agglomeration of the liposomes, even though the chitosan is not bound to the lipid surface either by covalent or ionic bonds. Although liposomes made of phosphatidylcholine have no surface charge, the electrostatically driven binding of chitosan to the lipid membrane is energetically favoured, even for neutral liposomes, leading to further stabilization of the vesicle suspension, as reported recently (Mertins et al., 2010; Mertins and Dimova, 2011).

3.3. In vitro *release of liposomally-associated clotrimazole*

There are several means to achieve the prolonged release of drugs destined for vaginal administration. Most of the approaches rely on the use of polymer in a form of hydrogel, to assure both prolonged release and intimate contact between drug and vaginal mucosa, simultaneously using liposomes as solubilizers for poorly soluble drugs (Pavelić et al., 2005). Other approaches use the complexation of drug with cyclodextrine, followed by incorporation of a complex in a hydrogel, such as for example pluronic thermosensitive gel, exhibiting prolonged release of clotrimazole when the drug was complexed with β-cyclodextrine prior to inclusion into the hydrogels (Bilensoy et al., 2006).

We have followed the release of liposomally-associated clotrimazole (Fig. 1) in comparison to clotrimazole in a free form (propylene glycol as vehicle). All liposomal formulations exhibited prolonged release as compared to free drug (control). All formulations, even the control, also failed to release all of the incorporated clotrimazole, which may be explained by the fact that clotrimazole, a very hydrophobic molecule, has very limited solubility in water. In the case of the control (propylene glycol), an osmotic effect was observed drawing water from the acceptor medium into the sample in donor chamber. As a consequence, clotrimazole started to precipitate (in donor chamber) when a critical amount of drug dissolved in propylene glycol is mixed with water. Therefore, we assume that it would be necessary to add some sort of solubilizing agent into the acceptor medium (acceptor chamber) to secure sink conditions. The limitation of Franz diffusion system is the limited volume which can be used in the acceptor chamber, causing the problems for the poorly soluble drugs. However, we could detect the differences between control (free drug) and the drug released from liposomes. Although we have expected the slowest release from chitosan-coated liposomes, interestingly, the slowest release of the drug was perceived from non-coated (plain) liposomes ($p < 0.05$). Non-coated liposomes act as solubilizer for clotrimazole in the lipid membrane and the drug only diffuses from the lipid membrane as liposomes become leaky, if not coated. It seems that the partitioning of clotrimazole between outer aqueous medium and liposomal bilayers is in favour of liposomal bilayers, whereas in coated liposomes, the release is supported by the presence of chitosan coating. We have also observed the difference in the release from liposomes coated with three different concentrations of chitosan; those coated with higher concentration of chitosan exhibited more pronounced sustained release, however not on a significant level. Chitosan is hydrophilic in nature and makes the surface of liposomes less hydrophobic, as in chitosan-coated liposomes. The thicker coatings (0.3 and 0.6%, w/v, respectively) will cause the diffusion obstacle for the drug released from the surface, resulting in the slower release. Berginc et al. (2014) reported that increasing the amount of chitosan in the liposomal coating had no beneficial effect on the permeability of liposomally-associated curcumin. This is very interesting, and could be relevant for different types of mucoadhesive coatings on the surface of various nanoparticles and requires further studies. This finding is in agreement with report by Fang et al. (2001) who proposed that even the lowest chitosan mole fraction is able to reduce the cooperative unit of the DPPC bilayer and lead to certain degree of membrane bilayer perturbation. Reduction of pH increased the number of protonated amines on the chitosan backbone and caused further disruption on the membrane organization. Although we have used different lipid in our experiments, the interactions between chitosan and polar head of phospholipids are well established (Mertins et al., 2010, 2011). Whether the observation we made for chitosan-coated liposomes incorporating clotrimazole would also apply for liposomally-entrapped hydrophilic drugs and for liposomes of various phospholipid compositions remains to be determined.

3.4. Ex vivo *penetration of liposomally-associated clotrimazole*

The mucoadhesive properties of chitosan are mostly result of its cationic character. Mucoadhesion of chitosan-based delivery systems can therefore be achieved through ionic interactions between the cationic primary amino groups of chitosan and the anionic substructures of the mucus. In addition, the hydrophobic interactions might contribute to its mucoadhesive properties (Bernkop-Schnürch and Dünnhaupt 2012). Although thiolated chitosans have stronger mucoadhesive properties than non-modified chitosan, their compatibility with vaginal environment remains to be proven, and we have therefore opted for non-modified chitosan as a coating material. Chitosans of high degree of deacetylation and of a high molecular mass were reported to cause an increase in the epithelial permeability (Bernkop-Schnürch and Dünnhaupt, 2012), which needs to be taken into consideration when choosing the type of chitosan for the coating of liposomes intended for administration into pregnant patients, and was the reason that we selected low molecular weight chitosan. We are currently evaluating the system in the *in vivo* conditions in pregnant sheep to confirm the safety and non-irritability of the system, as it is well-known that vaginal irritation can lead to increased susceptibility to foreign pathogens and inflammation.

Mucus is a viscous coating on many epithelial surfaces and consists mainly of water (up to 95% weight), inorganic salts, carbohydrates, lipids and glycoproteins, termed mucins. Mucins are hydrosoluble and responsible for the gel-like properties of the

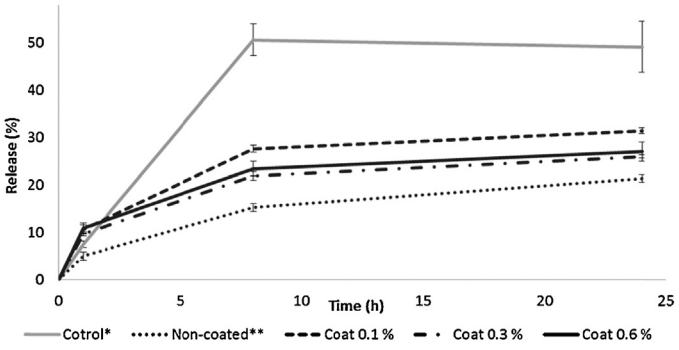

Fig. 1. *In vitro* release of clotrimazole from coated and non-coated liposomes ($n = 3$).
The concentration of clotrimazole in all formulations was the same. Control (*) contained clotrimazole in propylene glycol; non-coated liposomes (**) were diluted to obtain the same concentration of clotrimazole as in coated liposomes.

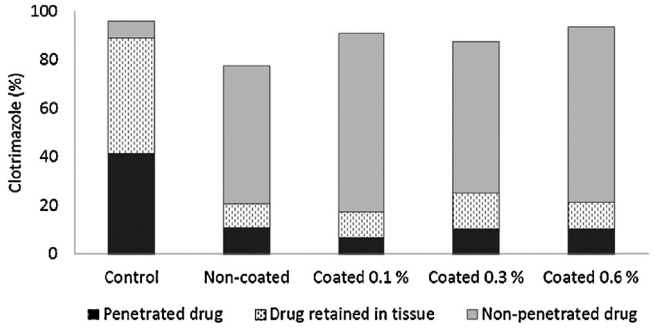

Fig. 2. *Ex vivo* penetration studies (24 h) in vaginal tissue ($n = 3$).
The concentration of clotrimazole was the same in all formulations. Control contained clotrimazole in propylene glycol; non-coated liposomes were diluted to obtain the same concentration of clotrimazole as in coated liposomes. Liposomal formulations assured significantly less ($p < 0.01$) drug penetration as compared to the control.

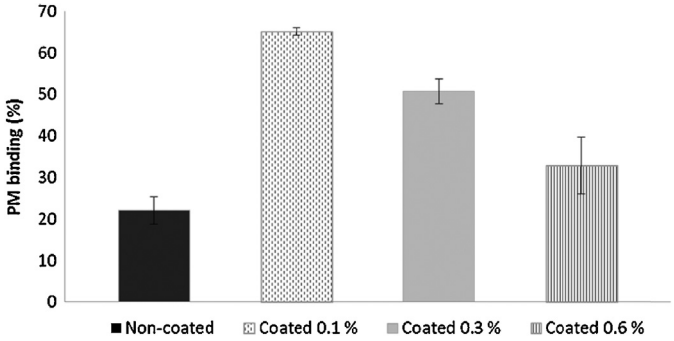

Fig. 3. Mucin-binding (PM) efficacy for non-coated and chitosan-coated liposomes ($n = 3$).

mucus (Serra et al., 2009). In order for mucoadhesion to take place, the wetting and swelling of polymer should enable an intimate contact with the mucosal tissue, followed by interpenetration of the polymer chains and entanglement between the polymer and mucin chains. Chitosan exhibits strong bioadhesive properties through the electrostatic interactions with sialic groups in mucins of the mucosal layer. The high positive charge density of chitosan enhances its mucoadhesiveness (Meng et al., 2011). It is also important to consider the concentration of liposomes applied to vaginal tissue, as this may affect the normal structure of mucus and cause the collapsing of mucin fibres (das Neves et al., 2011b). The concentration of liposomes used in our experiments was low and not expected to cause changes in mucosal structure and was considered to be safe. However, it remains to be evaluated in the *in vivo* studies in suitable animal model.

Although the mucoadhesive behaviour of bulk material such as polymer-based hydrogels made of chitosan for example is well characterized, rather little is known about the behaviour of chitosan at the nanoscale (das Neves et al., 2011b). We have previously confirmed the bioadhesion potential of chitosan-based hydrogels onto the skin (Hurler and Škalko-Basnet, 2012); however, the bioadhesiveness of chitosan-coated liposomes in nanosize range cannot be directly compared to hydrogels.

We have used the vaginal tissues of pregnant sheep to mimic closer the *in vivo* evaluation of our formulation in pregnant animals. The results presented in Fig. 2 indicate that liposomes are able to retain associated clotrimazole on the vaginal tissue and in the tissue, thus preventing undesired penetration through the vaginal tissue. This is of great importance considering clotrimazole therapy in pregnant patients. The free drug, a form of propylene glycol solution, penetrated through vaginal tissue in remarkable manner (almost 40% after 24 h), whereas the liposomally-associated drug penetrated to significantly lower extent ($p < 0.01$). We are aware that propylene glycol is a known skin penetration enhancer and acts as a carrier-solvent for poorly soluble substances such as clotrimazole. Moreover, propylene glycol may induce the osmotic effects which result in the changed barrier properties of the tissue. However, due to a very low solubility of clotrimazole, the choice of a solvent which we could use to prepare clotrimazole solution was very limited. Most of the other solvents are expected to directly damage the barrier properties of the vaginal tissue, resulting in the even higher penetration.

In respect to the drug retention on and within vaginal tissue, it appears that liposomes coated with 0.1% chitosan solution exhibited lowest penetration of clotrimazole and highest amount of clotrimazole retained on top of the tissue (Fig. 2), both in comparison to plain, non-coated liposomes and liposomes coated with different polymer concentrations. One has to consider that the increased retention time on the target site would outweigh more sustained release from non-coated liposomes (Fig. 1), as reported for mucoadhesive liposomes incorporating curcumin (Berginc et al., 2014).

The vulovaginal candidosis is characterized by the infection reaching the deeper epithelial layers (das Nevas et al., 2008); thus prolonged retention time on the vaginal mucus would be beneficial. To confirm that chitosan coating on liposomal surface is available for close interaction with mucin, we tested non-coated and coated liposomes for mucin-binding potential (Fig. 3). The results clearly indicate ($p < 0.01$) that liposomes coated with 0.1% polymer concentration exhibit superior interaction with mucin in comparison to all other formulations. This indicates that they exhibit potential to retain at the vaginal site. This type of liposomes may improve the effectiveness of model drug, yet prevent the undesired systemic absorption.

It is expected that lipophilic substances/drugs are absorbed from vagina as administration site through the transcellular pathway (Sassi et al., 2004). The passive diffusion was found to be the main mechanism of curcumin penetration into vaginal mucosa when liposomally-associated curcumin was tested in the *ex vivo* conditions. Our current findings are in full agreement. Depending on the liposomal size, the concentration of curcumin in different layers of vaginal tissue was found to be significantly higher as compared to concentration of curcumin applied in a form of solution. The permeability from 0.6% chitosan-coated liposomes was found to be similar or even lower than from 0.1% coated liposomes when tested in artificial and isolated bovine mucus. This can be explained by the fact that an increase in the liposomal size, as well as changes in zeta potential, lead to the major part of the polymer being hindered in the deeper layers and unavailable for immediate adhesion. Only when the uppermost polymer sheets of higher polymer concentrations coatings are removed by erosion or by detachment from mucus, more chitosan becomes available for adhesion. This was proposed as an explanation for the superiority of liposomes coated with lower concentration of polymer (Berginc et al., 2014). In addition, liposomal curcumin administered vaginally exhibited negligible potential for systemic absorption, which would greatly support its administration in pregnant patients (Berginc et al., 2012) in agreement with our findings.

Currently, there is a vivid discussion in the field of vaginal drug delivery whether the mucoadhesiveness of delivery system is advantageous for improved drug therapy or rather disadvantageous (das Neves et al., 2011a). The physical properties of mucus are complex and often described as non-Newtonian behaviour with properties between those of a viscous liquid and an elastic solid. In women with bacterial vaginosis, the viscosity of vaginal

fluid is reduced, leading to increased risk of infections and reduced barrier properties of vaginal mucus (Lai et al., 2009). Nevertheless, there is a consensus that the nanocarrier should first be retained at the vaginal site to increase the residence time and avoid vaginal leakage, and subsequently migrate through mucus towards the mucosal surface (das Neves et al., 2012). Antifungal agents used to treat vaginal candidosis need to penetrate deep into the epithelium to reach invasive *Candida* hyphae and exert a local antifungal action (das Neves et al., 2008). One approach to achieve this purpose has been recently suggested by our groups by using deformable propylene glycol liposomes (Vanić et al., 2014). Another approach is based on applying chitosan-based mucoadhesive liposomes developed in this study.

Our findings clearly indicate that liposomes are a suitable drug delivery system in respect to both prolonged release and limited tissue penetration. Liposomes are able to assure sustained release of associated drug either with or without chitosan coating. However, coated liposomes are expected to prolong the residence time in the vaginal cavity in the *in vivo* conditions and are therefore, considered to be superior. The next step involves the determination of bioadhesiveness of the system by the modified method originally developed for skin (Hurler et al., 2012).

We are aware of a need to address current limitations of experimental set up, such as that our experiments were performed on the vaginal tissue in the absence of cervicovaginal fluid. The reason that we did not use cervicovaginal fluid surrogate, is that it was reported that differences between the surrogate and native mucus may be noticeable. We also did not vary the pH of the donor medium, although it is known that the pH is affecting the transport across mucus (das Neves et al., 2012). The effect of semen and the changes in vaginal pH related to age and disease conditions remain to be evaluated.

4. Conclusions

Chitosan-coated liposomes were shown to exhibit prolonged release of associated clotrimazole. The penetration of liposomally-associated clotrimazole through the vaginal tissue was reduced as compared to non-coated liposomes, an important fact regarding system's potential in topical vaginal therapy, especially in pregnant patients. We are currently evaluating the system in the *in vivo* conditions in pregnant sheep to confirm the safety and non-irritability of the system as it is well-known that vaginal irritation can lead to increased susceptibility to foreign pathogens and inflammation.

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Resveratrol-loaded liposomes for topical treatment of the vaginal inflammation and infections

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Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene)
Vitamin C (ascorbic acid)
Vitamin E (α-tocopherol)
2,2′-Azino-bis(3-ethyl benzothiazoline)-6-sulfonic acid diammonium salt (ABTS)
1,1-Diphenyl-2-picrylhydrazyl (DPPH)
Phosphatidylcholine
Chitosan
L-nitro-arginine methyl ester (NAME)

Keywords:
Resveratrol
3,5,4′-Trihydroxy-*trans*-stilbene
Liposomes
Anti-inflammatory
Anti-oxidant
Mucoadhesive
Vaginal infections

ABSTRACT

Resveratrol (RES), chemically known as 3,5,4′-trihydroxy-*trans*-stilbene, is a promising multi-targeted anti-oxidative and anti-inflammatory natural polyphenol. Preclinical studies showed its biological activities against the pathogens of sexually transmitted diseases causing vaginal inflammation and infections. Due to its low solubility and poor bioavailability, the optimal therapeutic uses are limited. Therefore, a clinically acceptable topical vaginal formulation of RES exhibiting optimal therapeutic effects is highly desirable. For this purpose, we prepared and optimized chitosan-coated liposomes with RES. The coated vesicles (mean diameter 200 nm) entrapped up to 77% of RES, a sufficient load to assure required therapeutic outcome. *In vitro* drug release study showed the ability of liposomes to provide sustained release of RES. *In vitro* anti-oxidative activities of RES, namely DPPH and $ABTS^{\bullet+}$ radicals scavenging assays, confirmed RES to be as potent as standard anti-oxidants, vitamins C and E. The anti-oxidative activities of RES and its corresponding liposomal formulation were also compared by measuring enhanced superoxide dismutase (SOD) activities in lipopolysaccharide (LPS)-induced J774A.1 cells. *In vitro* anti-inflammatory activities were compared by measuring nitric oxide (NO), tumor necrosis factor (TNF)-α and interleukin (IL)-1β production in LPS-induced J774A.1 cells. Liposomal RES was found to exhibit stronger anti-oxidative and anti-inflammatory activities than RES solution.

1. Introduction

Resveratrol (RES), a common natural compound produced by several plants in response to pathogenic infection (Houille et al., 2014), is identified chemically as 3,5,4′-trihydroxy-*trans*-stilbene. RES and its derivatives were reported to exhibit fungicidal and anti-microbial activities (Chan, 2002; Baur and Sinclair, 2006; Adrian and Jeandet, 2012; Houille et al., 2014). It also showed anti-viral effect (Docherty et al., 2005). In addition to this, diverse pharmacological activities such as anti-oxidative, anti-inflammatory, neuro-protective, anti-aging, anti-cancer and cardio-protective effects of RES have been reported which can carry potential therapeutic application to humans (Bhat et al., 2001; Smoliga et al., 2011; Vang et al., 2011; Lu et al., 2013). Due to the multi-targeted microbicide activities and significantly low toxic effects, RES might be the potential candidate in safe topical treatment of vaginal inflammation and infection especially in pregnant women. The vaginal environment and structure are highly vulnerable towards the pathogens such as various bacteria, fungi, viruses or protozoa (*Trichomonas*) which cause vaginal inflammation and infection and are often transmitted easily during sexual intercourse. Contamination and rapid growth of these pathogens lead not only to inflammation

Abbreviations: ABTS, 2,2′-azino-bis(3-ethyl benzothiazoline)-6-sulfonic acid diammonium salt; CAT, catalase; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified eagle medium; GPX, glutathione peroxidase; HIV-1, human immunodeficiency virus-1; HPV, human papilloma virus; HSV-2, human simplex virus type 2; IL-1β, interleukin-1β; *i*NOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; L-NAME, L-nitro-arginine methyl ester; MDZ, metronidazole; NO, nitric oxide; PBS, phosphate buffer solution; PM, pig mucin; PC, phosphatidylcholine; PI, polydispersity index; ROS, reactive oxygen species; RES, resveratrol; STD, sexually transmitted diseases; SOD, superoxide dismutase; TH1, T-helper cell type 1; TNF-α, tumor necrosis factor-α.

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and infection but also facilitate human immunodeficiency virus (HIV-1), human simplex virus type 2 (HSV-2) and human papilloma virus (HPV) infections (Nikolic and Piguet, 2010). Therefore the understanding on the cross-links between various diseases and microbicides together with the link between HPV and cervical cancer was recognized by the award of the 2008 Physiology and Medicine Nobel Prize (Abbott and Brumfiel, 2008). Particularly, it is interesting that RES uses different mechanisms to induce cell death in cervical cancer cell lines (Garcia-Zepeda et al., 2013). In addition, pregnant women are vulnerable to vaginal infection because of the reduced T-helper cell type 1 (TH1) activities due to the development of protective mode towards the growing fetus. If they are not treated in time, the pregnancy might result in impaired fetal growth and development or even termination. Although anti-microbial agents are commonly used in the treatment of pathogenic vaginal infection, contemporary normal course of anti-microbial therapy cannot be applied during pregnancy. Moreover, the problem of anti-microbial resistance such as the one linked to metronidazole (MDZ) and other 5-nitroimidazoles (tinidazole, ornidazole, and secnidazole) used against trichomoniasis needs to be taken into consideration (van de Wijgert and Shattock, 2007).

Regarding the pathogen resistance and serious side effects linked to current anti-microbial options in vaginal therapy, especially in pregnant patients, a multi-targeted, less toxic and potential candidate, such as RES could be an ideal molecule. However, due to its low solubility and poor bioavailability, the possible clinical uses against vaginal inflammation and infection remain limited. Clinically applicable and safe formulation of RES assuring its optimal therapeutic value in the treatment of vaginal inflammation and infection in pregnancy is needed. By applying the chitosan-coated liposomal carrier for RES, we aimed to utilize the ability of chitosan not only as microbicide target but also to disrupt bacterial biofilms, which is of great importance in the treatment of vaginal bacterial inflammation and infections (Kandimalla et al., 2013). This paper describes the nanomedicine-based topical formulation of liposomal RES targeted to vaginal inflammation and infection. *In vitro* anti-oxidative and anti-inflammatory effects of free RES were compared with that of the corresponding liposomal formulation.

2. Materials and methods

2.1. Materials

Lipoid S 100 (PC, >94% phosphatidylcholine) was a gift from Lipoid GmbH, Ludwigshafen, Germany. Vitamin C (ascorbic acid), chitosan [low MW, Brookfield viscosity 20.000 cps, degree of deacetylation (DD) of 92], 1,1-diphenyl-2-picrylhydrazyl (DPPH), mucin from porcine stomach (type III, bound sialic acid 0.5%–1.5%, partially purified), phosphorus standard solution (0.65 mM), sodium chloride, resveratrol (RES: 3,5,4′-trihydroxy-*trans*-stilbene, purity ≥ 99%), vitamin E, 2,2′-azinobis(3-ethyl benzothiazoline)-6-sulfonic acid diammonium salt (ABTS) and potassium peroxodisulphate were the products of Sigma-Aldrich, Chemie GmbH, Steinheim, Germany. Acetic acid (glacial), anhydrous potassium phosphate, and sodium hydrogen phosphate were purchased from Merck KGaA, Darmstadt, Germany. Ammonium acetate was obtained from BHD Prolab, Leuven, Belgium. Cibacron brilliant red 3B-A was purchased from Santa Cruz Biotech, Dallas, TX, USA. Glycine hydrochloride Plusone® was obtained from Pharmacia Biotec, Uppsala, Sweden. Dulbecco's modified eagle medium (DMEM), trypsin–ethylenediaminetetraacetic acid, lipopolysaccharide (LPS; *Escherichia coli*, 055:B5), L-nitro-arginine methyl ester (L-NAME), sulfanilamide, naphthylethylenediamine dihydrochloride, and phosphoric acid were purchased from Sigma Life Science (Sigma–Aldrich Norway AS, Oslo). Assay kit for SOD activity measurement was from Abnova GmbH EMBLEM, Heidelberg, Germany, and TNF-α, and IL-1β measured spectrophotometrically with the assay kits were from Cell Biolabs, Inc., San Diego, CA, USA. All chemicals and solvents used were of analytical grade.

2.2. Cell culture

Murine macrophage, J774A.1 (ATCC® TIB67™) cells were purchased from ATCC and used in the *in vitro* anti-oxidative and anti-inflammatory studies. Cells (1×10^5 cells/ml) were cultured in 24-well plates with DMEM medium containing glutamine and 10% calf serum by incubating at 37 °C in 5% CO_2 for 24 h to stabilize and adhere on the plate. After 24 h the cell medium was replaced with the LPS (1 μg/ml) and/or test samples (RES/corresponding liposomal formulations) containing medium. The effects of the test samples on the LPS-induced pro-inflammatory cytokines (TNF-α, IL-1β) and NO expressed in the medium and SOD activity were measured after 24 h according to the instruction provided in the commercial kits.

2.3. Preparation of stock solutions and application to cells

Stock solutions (10 mg/ml) of RES and vitamin E were prepared by dissolving with dimethyl sulfoxide (DMSO). They were diluted with DMEM medium to the desired concentration before the treatment with the cells. In case of DMSO solution (for vitamin E and RES), maximum concentrations of DMSO were not more than 0.2% (*v*/*v*). All other samples were prepared and with the medium and applied directly into the cells.

2.4. Anti-oxidative assays

2.4.1. Measurement of DPPH radical scavenging activity

Effect of RES on DPPH free radical was determined by the similar method as reported previously (Basnet et al., 2012). In brief, DPPH solution (60 μM, 0.3 ml) in ethanol was mixed with an equal volume of each sample solution (1, 5, 10 or 20 μg/ml as the final concentrations). The reaction mixture was thoroughly mixed and kept in the dark for 30 min at room temperature. The anti-oxidative activity of RES was expressed by measuring the decreased absorbance intensity at 519 nm with UV spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) using following formula: Radical scavenging activity (%) $= 100 \times (A - B) / A$, where A is the control (absorbance of DPPH radicals without sample) and B is the absorbance of radicals after reacting with the sample. The anti-oxidative activity of RES was also compared to that of vitamin C and vitamin E under the same experimental conditions. The results are expressed as mean ± S.D. of three experiments.

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

$ABTS^{\bullet+}$ radicals were generated by mixing equal volumes (3 ml) from each of the stock solutions of ABTS (7.4 μM) and potassium peroxodisulphate (2.6 μM) in distilled water. The reaction mixture was allowed to stabilize for 3 h at room temperature and then diluted with ethanol to 100 ml as the $ABTS^{\bullet+}$ radicals working solution. The green colour $ABTS^{\bullet+}$ radicals working solution (0.3 ml) was mixed with an equal volume of sample solutions at the 1, 5, 10 and 20 μg/ml concentration. After mixing, it was kept in the dark at room temperature. After 30 min, optical density was measured with UV spectrophotometer at 731 nm. As the number of $ABTS^{\bullet+}$ radicals decreases, the intensity of green colour reduces. Results were expressed as described for DPPH radical assay. The anti-oxidative activity of RES was compared to that of the vitamin C and vitamin E under the same experimental conditions.

2.5. Preparation of liposomes

Liposomes were prepared by the film hydration method as described earlier (Jøraholmen et al., 2014). Briefly, RES (10 or 20 mg) was dissolved in methanol and mixed with phosphatidylcoline (PC, 200 or 400 mg) in methanol and solvents were evaporated on Büchi rotavapor R-124 (with vacuum controller B-721, Büchi Vac® V-500, Büchi Labortechnik, Flawil, Switzerland) for at least 3 h at 50 mm Hg

and 50 °C. The remaining film was then re-suspended in distilled water (10 ml). Throughout the preparation RES solution was kept protected from light. Empty liposomes were prepared by similar method. Liposomal suspensions were stored in refrigerator (4–8 °C) for at least 12 h prior to further use.

Liposomal size was reduced through the extrusion through polycarbonate membranes with defined pore sizes (Nuclepore Track-Etch Membran, Whatman House, Maidstone, UK). The extrusion was performed stepwise through the 0.8, 0.4 and 0.2 μm pore size filters and 5 extrusions were executed for each step.

2.6. Coating of liposomes

Coating of liposomes was performed in the presence of unentrapped RES. In brief, chitosan solutions (0.1, 0.3 and 0.6%, *w*/*v*) were prepared in 0.1% and 0.5% (*v*/*v*) glacial acetic acid, respectively. The chitosan solution (2 ml) was added drop wise to an equal volume of liposomal dispersion under controlled magnetic stirring at room temperature for 1 h, followed by the incubation in the refrigerator (4–8 °C) overnight. The rate of stirring was kept constant for all preparations (Jøraholmen et al., 2014).

2.7. Characterization of liposomes

2.7.1. Size

The liposomal size distributions were determined by photon correlation spectroscopy (Submicron particle sizer model 370, Nicomp, Santa Barbara, CA, USA) as reported previously (Jøraholmen et al., 2014). The particle intensity was approximately 250–350 kHz the analyses run in vesicle mode and intensity-weight distribution. Three parallels (with a run time of 10 min for each parallel) were determined for each sample measurement.

2.7.2. Zeta potential measurements

Zeta potential measurements were performed on a Malvern Zetasizer Nano ZS (Malvern, Oxford, UK) according to Jøraholmen et al. (2014). To assure accuracy, the instrument was calibrated throughout the measurements using the Malvern Zeta Potential Transfer Standard (-50 ± 5 mV). Measurement cell (DTS1060) was cleaned with ethanol and filtrated water (0.2 μm), respectively, prior to loading of sample. The liposomal suspensions were diluted with filtrated water to appropriate concentrations (typically 1:20) before the measurements, to achieve the proper count rate. All measurements were performed at 25 °C and three parallels were measured.

2.7.3. Entrapment efficiency determination

RES liposomes (2 ml) were dialyzed (Mw cutoff: 12–14,000 Da, Medicell International Ltd., London, UK) against distilled water (500 ml) for 4 h at room temperature. The volume of medium was adjusted to assure the solubility of RES. Aliquots of sample and medium were diluted in methanol, and the amount of liposome-associated RES was determined by UV spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) at 306 nm. The correlation coefficient for standard curve was 0.9958 and the minimum detectable amount for RES was 1 μg/ml.

2.7.4. Phospholipid assay

The content of PC was measured using the modified Bartlett method as reported previously (Andersen et al., 2015). Phospholipid content was measured by UV spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) at 830 nm. The phosphorous standard solution was used to prepare standard curve in concentration range of 1–10 μg/ml.

2.8. Chitosan determination

The surface-available chitosan was determined by a modified colorimetric method (Andersen et al., 2015). In brief, glycine buffer (pH 3.2) was prepared by dissolving glycine (1.87 g) and NaCl (1.46 g) in 250 ml of distilled water; an aliquot of 81 ml was further diluted with 0.1 M HCl to a final volume of 100 ml. Cibacron Brilliant Red 3B-A (150 mg) was dissolved in 100 ml of distilled water. The dye solution (5 ml) was further diluted to 100 ml with the glycine buffer. Vesicle suspensions were diluted with distilled water to desirable concentration (1:6, *v*/*v*) before 3 ml of the final dye solution was added. UV–vis absorbance was measured at 575 nm with a spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The percentage of surface-available chitosan was calculated using the following equation:

$$\text{Percentage of surface available chitosan} = \frac{Cs}{Cc} \times 100$$

where, C_s is the concentration of chitosan in the sample and C_c is the concentration of chitosan used to prepare the liposomal formulations.

A chitosan standard solution (0.05 % *w*/*v*) was prepared in 0.05 % (*v*/*v*) glacial acetic acid. The standard solution was diluted in glycine buffer to desired concentrations. The correlation coefficient for the standard curve was 0.9997 and the minimum detectable amount of chitosan was 2.27 μg/ml.

2.9. In vitro mucoadhesive properties

The mucoadhesive properties were determined by measuring liposomes binding to the pig mucin (PM) as described earlier (Jøraholmen et al., 2014). Briefly, non-coated and chitosan-coated liposomes (1 ml) in original vesicle size were mixed with equal volume of PM suspension (400 μg/ml) in 0.05 M phosphate buffer saline and incubated at room temperature for 2 h, followed by ultracentrifugation (216,000 ×*g*) for 1 h at 10 °C (Optima LE-80; Beckman Instruments, Palo Alto, CA, USA). Aliquots of 200 μl (4 from each sample) of the supernatants were transferred to a microtitre plate (Costar® UV 96-well plate with UV transparent flat bottom, Acrylic, Costar®, Corning, NY, USA) and free PM was measured spectrophotometrically at 251 nm (Microtitre plate reader; Spectra Max 190 Microplate, Spectrophotometer Molecular devices, Sunnyvale, CA, USA). The PM binding efficiency was calculated according to Naderkhani *et al.* (2014).

2.10. In vitro RES release

The *in vitro* RES release experiment was performed by the method described earlier (Jøraholmen et al., 2014). The Franz cell manual diffusion system (Perme Gear Ink, Diffusion Cells and Systems, Hellertown, PA, USA) and the heating circulator (Julabo Laboratechnik, F12-ED, Seelback, Germany) was set to 37 °C. The acceptor chamber (12 ml) was completely filled with acetate buffer (pH 4.6) containing CH_3COONH_4 (77.1 g) and glacial acetic acid (70 ml) and distilled water up to 1000 ml. Cellophane membrane, cut to appropriate size, was pre-soaked in the same buffer prior to fixation. Liposomal samples and controls (RES in propylene glycol) (600 μl) were added to the donor chambers and the system was properly sealed. The RES content in all tested samples was determined spectrophotometrically prior to the experiment to assure the same concentration gradient in the samples and controls, and to assure sink conditions. Samples (500 μl) were withdrawn from acceptor chamber after 1, 2, 3, 4, 6 and 8 h. The collected samples were replaced by an equal volume of buffer in the acceptor chamber. All collected samples, the remaining suspension on and retained in the cellophane membrane were dissolved in methanol and measured spectrophotometrically (Agilent Technologies, Santa Clara, CA, USA) at 306 nm.

2.11. Measurement of SOD activity

The effects of RES and liposomal RES on SOD activity were measured in LPS-induced J774A.1 cells by the similar method as reported previously

(Basnet et al., 2012). Cells were cultured by the method as described above. The controls for RES and liposomal formulations were medium containing 0.2% DMSO and empty liposomes, respectively. In both controls, SOD activity induced with LPS (1 μg/ml) after 24 h, were expressed as basal level. The SOD activities (%) exhibited by the LPS-induced macrophages in the presence of test samples (RES, and liposomal RES) at 10 μg/ml were assayed according to the protocol for colorimetric assay kit. The measurements were performed in triplicates.

2.12. Measurement of NO production

The effects of RES and liposomal RES on production of NO in the LPS-induced J774A.1 cells were measured as reported previously (Basnet et al., 2012). The cells were treated with LPS (1 μg/ml) and/or samples at various concentrations. After 24 h, the effects of the samples (RES, and liposomal RES) on the production of NO released in the medium were measured in terms of nitrite formation by Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% H_3PO_4) at 550 nm using $NaNO_2$ as the standard. The measurements were performed in quadruplets. The effects of the RES on the production of NO were also compared to some well-known anti-oxidants (vitamin C and vitamin E) and *i*NOS inhibitor (L-NAME). The controls for RES and liposomal RES were 0.2% DMSO and empty liposomes, respectively. The inhibitory activities of RES and liposomal-RES on NO production were expressed as IC50 (μg/ml).

2.13. Effect on IL-1β and TNF-α production

The effects of RES and liposomal-RES on the production of TNF-α and IL-1β in the LPS-induced J774A.1 cells were measured as reported previously (Basnet et al., 2012). The cell medium was replaced with the medium containing LPS (1 μg/ml) and/or samples (10 μg/ml). After 24 h, the medium (500 μL) was removed and stored at −70 °C until cytokine assays were carried out. Controls for RES and liposomal RES were 0.2% DMSO and empty liposomes, respectively. The production of TNF-α or IL-1β in the medium by the LPS-induced cells were measured and expressed as 100%. Both pro-inflammatory cytokines (TNF-α and IL-1β) were measured in the same set of experiments by the assay kits as described in the manufacturer's protocols. Measurements were performed in triplicates.

2.14. Statistical analyses

Data were analyzed using the SPSS 19.0 software (SPSS Inc. Chicago, IL, USA). Continuous variables are expressed as mean and categorical variables are reported as percentage. Statistical significance of difference between the control and test groups or corresponding groups was determined by one-way ANOVA, and $P < 0.05$ was considered statically significant. Results are expressed as mean ± S.D., where $n = 3$ or 4.

3. Results

3.1. The effect on DPPH/$ABTS^{+\bullet}$ radicals

Although RES, vitamin C and vitamin E are structurally different (Fig. 1) and differ significantly in their physical properties, they show similar chemical reaction towards free radicals and anti-oxidative potentials.

Anti-oxidative activity is expressed as DPPH or $ABTS^{+\bullet}$ radicals scavenging capacity of RES. We examined the radical scavenging activity of RES at 1, 5, 10 and 20 μg/ml concentrations for DPPH or $ABTS^{+\bullet}$ radicals. The anti-oxidative activities of RES were compared to those of vitamin C and vitamin E under the similar conditions. All samples showed concentration-dependent radical scavenging activity and the results are shown in Fig. 2(A and B).

In a separate experiment, the anti-oxidative activities of RES were compared with that of vitamin C or vitamin E by measuring their effective concentrations required for the 50% decrease of radicals (EC50) and the results are shown in Fig. 3. EC50 for RES were 17.15 and 3.05 μM against DPPH and $ABTS^{+\bullet}$ radicals, respectively. While vitamin C and vitamin E showed 10.25 and 7.38 μM against DPPH radicals and 7.77 and 6.64 μM against $ABTS^{+\bullet}$ radicals, respectively. Comparing to vitamin C and vitamin E, RES showed stronger reaction towards $ABTS^{+\bullet}$ radicals rather than DPPH (Fig. 3).

3.2. Liposomal characteristics

The vesicle sizes, polydispersity index (PI), zeta potential and entrapment efficiency of coated and non-coated liposomes are presented in Table 1. Considering the optimal vesicle size for topical vaginal administration (Vanic and Skalko-Basnet, 2013) and to assure the

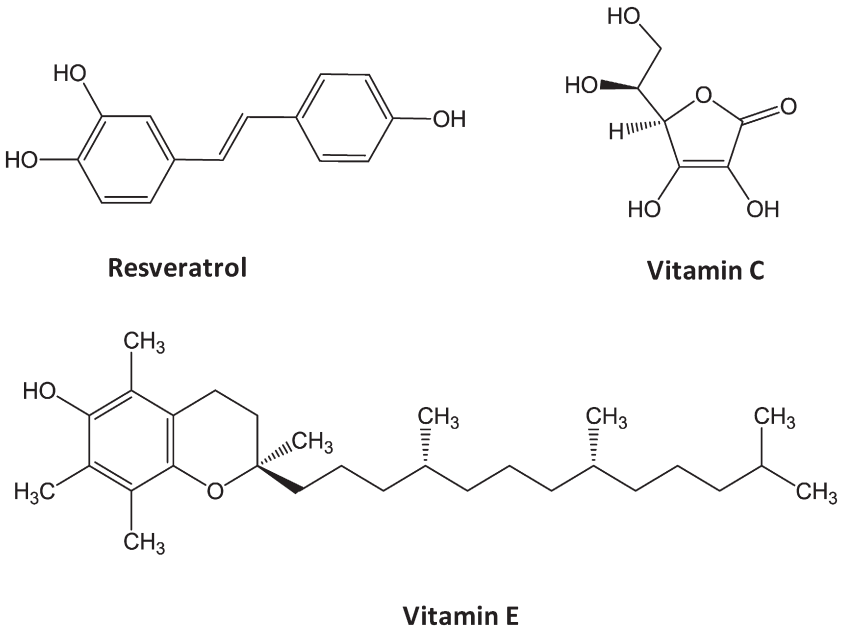

Fig. 1. Chemical structures of resveratrol (*trans*-3,5,4′-trihydroxystilbene), vitamin C (ascorbic acid) and vitamin E (α-tocopherol).

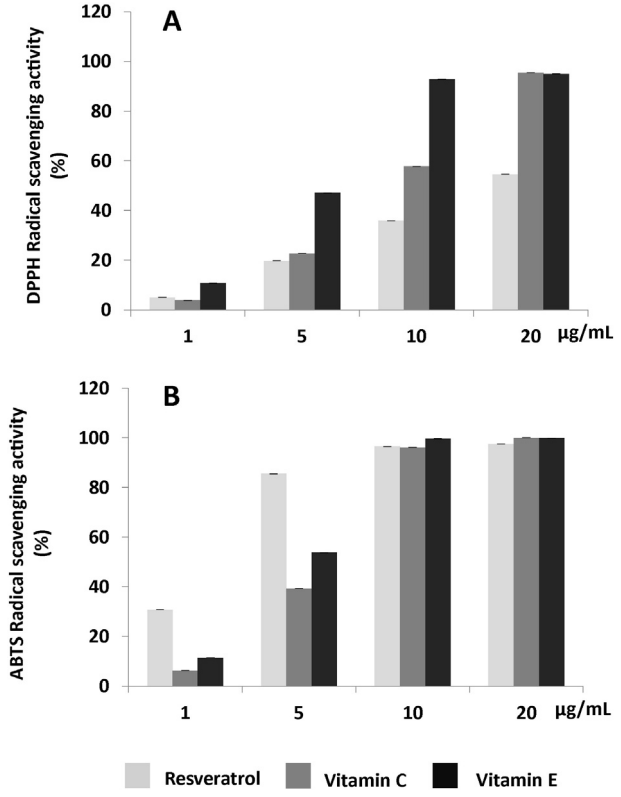

Fig. 2. Anti-oxidative activities of resveratrol, vitamin C and vitamin E. A) DPPH radical scavenging activity B) ABTS + • radical scavenging activity. Results are expressed as percentage mean ± SD ($n = 3$).

sufficient entrapment efficiency, the liposomal sizes with the mean diameter of 200 nm were targeted. The coating resulted in an increase in the mean liposomal size; the increase in the vesicle size corresponded to the increasing polymer concentration, as expected. All liposomal dispersions exhibited low PI, indicating a rather monodisperse size distribution. The low PI confirmed that the extrusion could be a suitable method to obtain liposomes of desired vesicle size with limited polydispersity.

The differences in zeta potential on liposomal surface between non-coated and chitosan-coated liposomes also confirmed that liposomes were indeed coated (Table 1). We also observed the differences between liposomes coated with 0.1 and 0.3% (*w/v*) chitosan, respectively. Moreover, an entrapment efficiency of over 70% of the starting amount of RES was obtained which is sufficient to assure the required RES concentrations for therapeutic effects (Table 1). Although the chitosan-coating was performed in the presence of unentrapped RES, no significant increase in RES incorporation was seen for the chitosan-coated liposomes. A phospholipid assay was applied to assure that the vesicle size reduction via extrusion and the chitosan-coating did not result in a loss of lipids. The loss of lipid was found to be less than 5% and accepted as satisfactory.

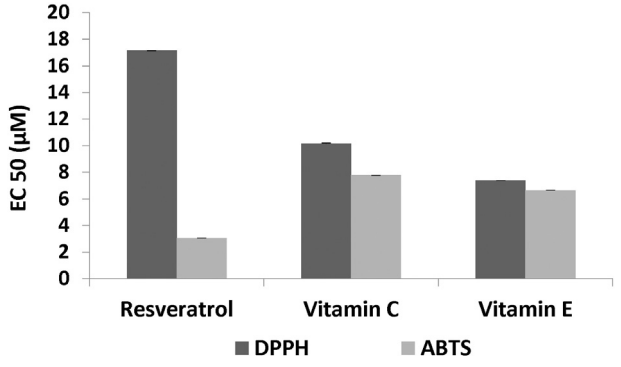

Fig. 3. Anti-oxidative activity of resveratrol, vitamin C and vitamin E expressed as EC50 in µM. Results are expressed as mean ± SD ($n = 3$).

3.3. Mucoadhesive properties of liposomal formulation

The increased concentration of polymer is expected to lead to more efficient coating of liposomes (Li et al., 2009). A colorimetric assay with the anionic reactive dye (Cibacron Brilliant Red) was performed in the presence of free chitosan, thus the binding efficiency of chitosan to liposomal surface could not directly be measured. However, for the lower chitosan concentration (0.1%, *w/v*), 84% of chitosan was found to be surface available, whereas 54% of chitosan was detected on liposomes coated with higher polymer-concentration (0.3%, *w/v*). The results are in agreement with the findings in the literature (Li et al., 2009; Andersen et al., 2015).

We tested the *in vitro* mucin-binding potential for both coated and non-coated liposomes to confirm the mucoadhesive properties of chitosan-coated liposomal delivery system (Fig. 4). Since the vaginal environment varies in pH, the experiments were performed at pH corresponding to healthy vaginal conditions (4.6) and vaginal bacterial infections conditions (7.4). The results indicate that PM-binding properties are significantly ($P < 0.001$) improved for chitosan-coated liposomes compared to non-coated liposomes which are in accordance with the literature (Jøraholmen et al., 2014; Naderkhani et al., 2014). The superior mucin-binding potential of liposomes coated with low chitosan concentration (0.1%, *w/v*) was confirmed (Jøraholmen et al., 2014), compared to all other formulations. Importantly, the superiority was confirmed at both pH, suggesting that mucoadhesiveness will be assured regardless of the vaginal pH conditions. Non-coated liposomes are not expected to exhibit mucin-binding activity, however a noticeable binding efficiency was also observed for non-coated liposomes. One possible explanation for observed PM-binding to plain liposomes can be by physical interactions occurring during the ultracentrifugation and not an actual electrostatic interaction between liposomes and mucin. However, the binding was significantly lower than PM-binding of chitosan coated liposomes (0.1 and 0.3%, *w/v*).

3.4. In vitro release of RES from coated and non-coated liposomes

The Franz diffusion system was employed to assess the RES release from liposomal formulations comparing the release to free RES in propylene glycol solution serving as a control. Liposomes coated with the 0.6% of chitosan showed poor mucoadhesive properties (Fig. 4), therefore were not included in the RES release study. An apparent sustained release from all liposomal formulations was determined (Fig. 5) and the *in vitro* release studies confirmed prolonged release of RES from both the non-coated and chitosan-coated liposomes as compared to the control. Further, the release of RES was slower from the chitosan-coated liposomes as compared to the non-coated liposomes. This indicates that chitosan-coated liposomes have the ability to prolong the release of RES to a greater extent than non-coated liposomes.

Table 1
Liposomal size, zeta potential and entrapment efficiency of non-coated and chitosan-coated liposomal resveratrol.

	Resveratrol-loaded liposomes			
Chitosan (%)	Vesicle size (nm)	PI[a]	Zeta potential (mv)	Entrapment (%)
–	206 ± 10	0.142	−3.17 ± 2.57	80 ± 4
0.1	212 ± 11	0.172	4.15 ± 0.59	77 ± 4
0.3	225 ± 10	0.122	14.77 ± 1.85	74 ± 6

Results are expressed as mean ± S.D. ($n = 3$). Data for chitosan solution (0.1 and 0.3 %, *w/v*) are presented.
[a] PI: polydispersity index.

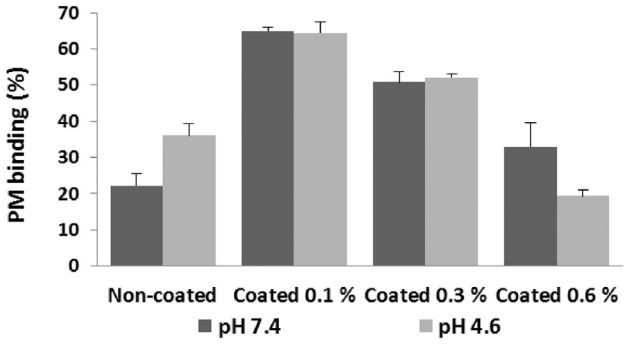

Fig. 4. Pig mucin (PM) binding (%) of non-coated and chitosan-coated liposomes. The values represent the percentage mean ± SD ($n = 3$).

3.5. Effect of RES and liposomal RES on SOD activity

The effects of RES and liposomal RES on SOD activity in LPS-induced J774A.1 cells were evaluated and results are shown in Fig. 6. Comparing to the basal SOD activity of the controls, RES increased the SOD activity by 20%. Under a similar condition, liposomal formulation of RES increased the SOD activity by 26%. Liposomal formulations significantly increased ($P = 0.009$) SOD activity as compared to RES solution.

3.6. Effect of RES and liposomal RES on NO production

LPS treatment induces macrophages to increased amount of NO production by the conversion of L-arginine to L-citrulline. Thus produced unstable NO radical, is rapidly converted to NO_2^- or NO_3^-. Therefore the amount of NO_2^- measured quantitatively by the Griess reagent can be directly correlated to the NO production by the macrophages. We measured the NO_2^- produced by LPS and RES (1, 5 and 10 μg/ml) treated cells and expressed the activity as percentage of NO production and results are shown in Fig. 7. RES showed a concentration-dependent NO production inhibition. The activities of RES were also compared to that of vitamin C, vitamin E and L-NAME under similar conditions. Vitamin C and vitamin E showed only a weak inhibitory activity at higher concentrations (10 μg/ml).

L-NAME (*i*NOS inhibitor) showed, as expected, concentration-dependent activity at 1, 5, and 10 μg/ml. Under similar conditions, RES was found to be more potent than L-NAME (Fig. 7). In another similar experiment, the inhibitory activity of RES and corresponding liposomal formulations against NO production was measured at 1, 2, 5, 10, 15, 20, 25, and 30 μg/ml RES concentrations. The results were expressed in 50% inhibitory concentration (IC50) as μg/ml (Fig. 8). RES and corresponding liposomal formulation showed IC50 as 13.5 and 9.6 μg/ml, respectively.

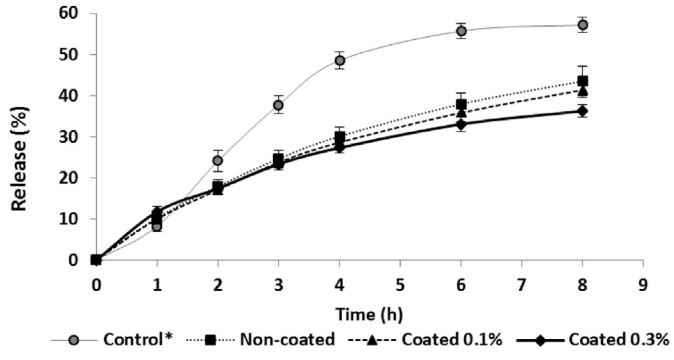

Fig. 5. *In vitro* resveratrol release from non-coated and chitosan-coated liposomes. *As a control, resveratrol in the same concentration as in liposomes was dissolved in propylene glycol. Results are expressed as percentage mean ± SD ($n = 3$).

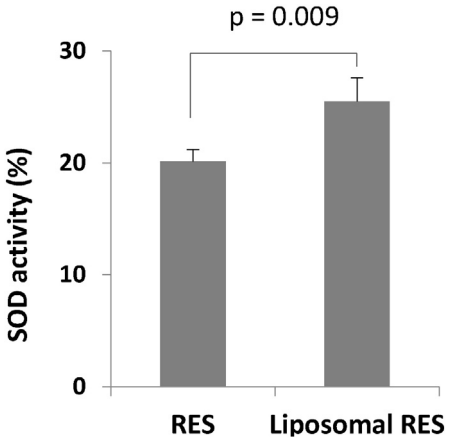

Fig. 6. The effects of resveratrol (RES) and liposomal RES on SOD activity in LPS-induced macrophages. Stock solution of RES in DMSO was diluted with the cell culture medium euaivalent of RES present in liposomal RES and applied same volume containing 10 μg/ml as the final concentration to cells. Results are expressed as mean ± SD ($n = 3$). Control for RES and liposomal RES were 0.2% DMSO and empty liposomes, respectively.

Liposomal RES was found to be more potent than the corresponding RES solution ($P = 0.003$), confirming the need for delivery system (Fig. 8).

3.7. Effect of RES and liposomal RES on TNF-α and IL-1β production

We measured the TNF-α and IL-1β production by the LPS-induced macrophages in the presence of RES or liposomal RES. As compared to the control group, RES at a concentration of 10 μg/ml inhibited 52% of TNF-α production. Under the similar condition, liposomal RES inhibited 70% of TNF-α production which were significantly different ($P = 0.004$). We also measured the effects of RES and liposomal RES on the production of IL-1β. Compared to the control group, RES and liposomal RES at a concentration of 10 μg/ml inhibited 60% and 62% of IL-1β production, respectively (Fig. 9).

4. Discussion

RES acts as a potent defensive anti-oxidant by inhibiting reactive oxygen species (ROS) mainly by activating AMPK (Pangeni et al., 2014). It exhibits stronger anti-radical activity than α –tocopherol, catechin, myricetin and naringenin. RES, vitamin C and vitamin E used in our experiments were supplied commercially (99.0% pure). Their structures are given in Fig. 1. Vitamin C is a hydrophilic and vitamin E a lipophilic anti-oxidant with well-defined bioavailability. However, RES remains to be a biopharmaceutical challenge with regard to its solubility limitations (Das et al., 2008). Taken orally RES is relatively well absorbed, rapidly metabolized and generally well tolerated, although limited long-term toxicity studies have also been performed (Cottart et al., 2010). Extensive intestinal and hepatic metabolism is the rate limiting step for the systemic bioavailability resulting in a half-life of only 8–14 min (Das et al., 2008). With log*P* > 3.1 it is classified as a class-II compound in the Biopharmaceutical Classification System. *Trans*-RES is a pharmacologically active isomer. RES is a photosensitive compound and becomes converted into *cis*-RES after exposure to light for just 1 h in solution (Singh and Pai, 2014). This is an additional reason to incorporate RES in liposomal delivery system enabling the protection against light (Coimbra et al., 2011).

Our interests were in anti-microbial potential of RES, as increasing number of *in vitro* and *in vivo* studies suggest that RES exhibits anti-parasitic (*Trihomonas vaginalis*) (Mallo et al., 2013), anti-fungal (*Candida* species) (Houille et al., 2014), anti-viral (Docherty et al., 2005) and anti-bacterial (Nawrocki et al., 2013) activities. Prior to evaluating the anti-viral potential of liposomal RES, we have focused on the anti-oxidative and anti-inflammatory activities of RES and corresponding formulation.

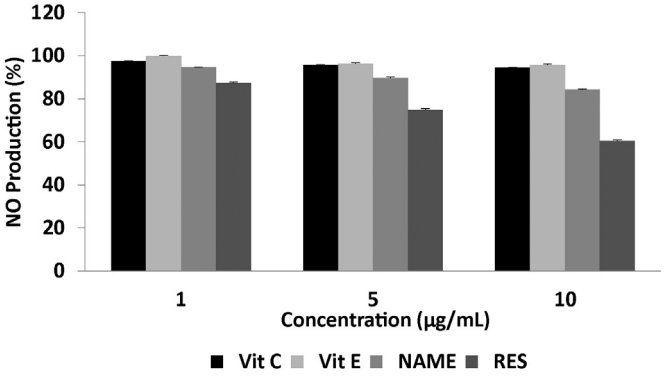

Fig. 7. The effects of resveratrol, vitamin C, vitamin E and NAME on NO production in LPS-induced macrophages. RES: resveratrol; NAME: L-nitro-arginine methyl ester. Results are expressed as mean ± SD ($n = 4$).

Inflammation is one of the first responses of the immune system to infection. The symptoms of inflammation include redness and swelling, which are caused by increased blood flow into the tissue. Inflammation is caused by eicosanoids and cytokines, which are released by injured or infected cells. There is abundant evidence that certain pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α are involved in the process of pathological infection (Dinarello, 2000). Therefore, to express the anti-inflammatory activity, we focused on effect of RES on the production of nitric oxide, IL-1β, and TNF-α.

4.1. Liposomal RES

In the past ten years, an increasing number of publications dealing with RES in nanosystems have been reported (Amri et al., 2012). Moreover, an extensive list of patents on the use of RES in diverse formulations indicates increasing interest of academia and industry for its commercialization (Singh and Pai, 2014). The limitation of the solubility of RES can be overcome by incorporating RES into delivery system which acts as a solubilizer and improve the delivery of the drug candidate. Several novel delivery systems were proposed in recent years as means to improve its bioavailability, including polyethylene glycol-polylactic acid polymeric nanoparticles (Jung et al., 2014), solid lipid nanoparticles (Teskac and Kristl, 2010; Gokce et al., 2012), polymeric micelles (Lu et al., 2009), RES-loaded poly(*N*-vinylpyrrolidone)-ь-poly(ε-caprolactone) nanoparticles (Lu et al., 2013). RES self-emulsifying system

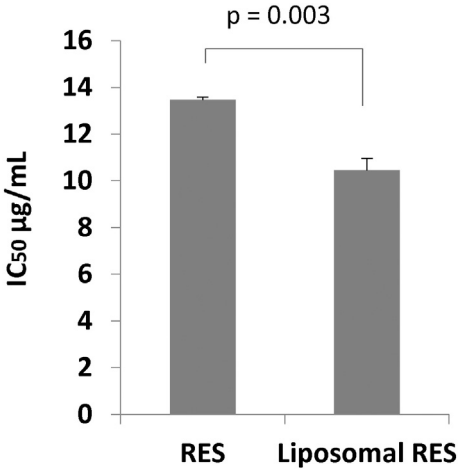

Fig. 8. The effect of resveratrol (RES) and liposomal RES on NO production in LPS-induced macrophages. Results are mean ± SD ($n = 4$) showing the amount RES required to show 50% inhibition of NO production (IC50 µg/ml) in J774A.1 cells induced by LPS (1 µg/ml).

was shown to increase the uptake by endothelial cells and improve protection against oxidative stress-mediated death (Amri et al., 2014). Phospholipids offer means to improve the poor solubility and consequently low bioavailability of various active compounds (Fricker et al., 2010). In addition, liposomes improved the chemical instability of RES by preventing its inactivation through *cis–trans* isomerization (Coimbra et al., 2011; Scognamiglio et al., 2013). Kristl and coworkers confirmed that liposome-mediated uptake of RES improved the cell-stress response in comparison to free RES (Kristl et al., 2009). The same group (Caddeo et al., 2008) proved the enhanced efficacy of RES incorporated in liposomes on proliferation and UV-B protection of cells. Elastic liposomes incorporating RES and quercetin injected subcutaneously offered a new strategy for reducing the subcutaneous fat (Cadena et al., 2013). In spite of improving delivery and solubility of RES, its wider therapeutic application is still limited because of the required dose needed to assure therapeutic outcome (Augustin et al., 2013).

Considering localized vaginal therapy, relatively little is known about the effect of vesicle size on the delivery of drugs intended for vaginal mucosal targeting. However, it was suggested that the nanocarriers in the size range of 200–500 nm are superior to both much smaller and also larger nanosystems (das Neves et al., 2011a,2011b). Additionally, it was reported that the number of liposomes penetrating the mucous layer increased when the size of the liposomes was reduced to approximately 100 nm for both non- and chitosan-coated liposomes (Takeuchi et al., 2001). There is usually a correlation between the particle size and drug entrapment efficiency; small vesicles are expected to offer greater surface area to interact with the mucus, but bearing less drug load, whereas larger vesicles enable increased drug loading, nevertheless, reduced mucoadhesion due to less surface contact (Vanic and Skalko-Basnet, 2013).

Liposomes are well-established delivery systems able to incorporate poorly soluble drugs and enable their aqueous medium-based vaginal administration (Pavelic et al., 1999; 2005a, 2005b). Liposomes as carriers for RES were also studied by several groups targeting various routes of administration. For example, transferosomes and ethanol-containing vesicles were used to deliver RES through porcine skin (Scognamiglio et al., 2013).

Chitosan is one of the mucoadhesive polymers recommended for the vaginal delivery (Valenta, 2005). Several other potential mucoadhesive polymers were reported as vaginal delivery systems, such as for example Carbopol (Pavelic et al., 2005a,2005b), however the known antimicrobial activities were the main reason to focus on chitosan in the present work. Moreover, chitosan is a natural polymer and considered to be biocompatible, biodegradable and bioadhesive. The cationic properties of chitosan contribute to its mucoadhessiveness. When chitosan is used as a coating material for neutral PC liposomes, it is expected that hydrogen bonds be created between the phospholipid head groups and the cationic polysaccharide. In addition to required mucoadhesive properties, chitosan-coating of liposomes may present improved controlled drug delivery and stabilization of liposomes (Jøraholmen et al., 2014). Moreover, chitosan also exhibits anti-microbial activity against vaginal pathogens (Kim et al., 2003) and its ability to disrupt vaginal bacterial biofilms makes chitosan superior polymer in localized vaginal therapy (Kandimalla et al., 2013). Interactions between the cationic chitosan and the negatively charged mucosal surface are shown to render excellent mucoadhesive properties, making this polymer a well suited coating material for drug delivery systems intended for mucosal tissues (Meng et al., 2011). Our results are in agreement with the findings of Meng *et al.* who reported that chitosan-coated nanoparticles in sizes of about 200 and 300 nm exhibited significantly enhanced mucoadhesive properties on porcine tissue (Meng et al., 2011).

The only other report, to the best of our knowledge, on chitosan-coated liposomes containing RES is by Park *et al.* who tested potential of this system to provide transdermal delivery of RES as delaying skin-aging substance (Park et al., 2014). They also observed that liposomes

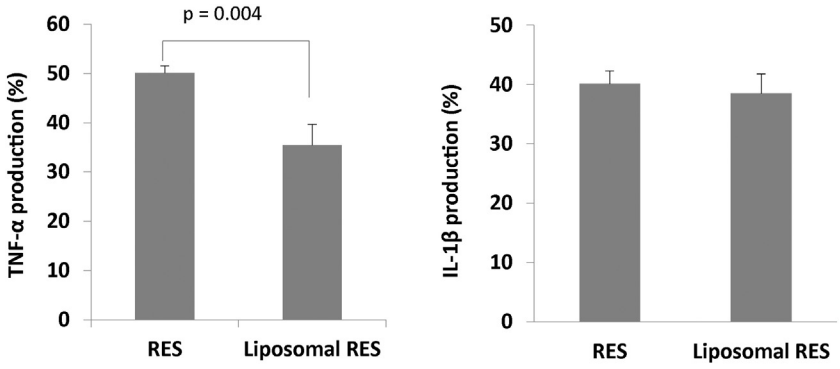

Fig. 9. The effects of resveratrol (RES) and liposomal RES on IL-1β and TNF-α production in LPS-induced macrophages. Results are expressed as percentage mean ± SD ($n = 3$).

coated with lower concentration of polymer maintained their size integrity better than those coated with higher concentrations of polymer (Park et al., 2014).

During the process of chitosan-coating of liposomes, the amount of polymer interacting with the liposomes is expected to increase with an increase in the starting concentration of chitosan (Li et al., 2009). Our findings that lower chitosan concentrations result in more surface-available chitosan are in agreement with Guo *et al.* who reported that chitosan-coating of liposomes reached a saturation state when exceeded 0.1% concentration (Guo et al., 2003).

The effectiveness of drug delivery at the mucosal site is dependent on a series of interconnected actions including i) distribution and retention of nanosystems on the mucosal surface, ii) their penetration into/through the mucus mesh, and iii) release profile of the drug (Vanic and Skalko-Basnet, 2013). The RES release (Fig. 5) indicates that chitosan-coated liposomes enabled sustained release. Chitosan-coated liposomes were superior to non-coated liposomes regarding the ability to sustained RES release, confirming our aim that chitosan-coating assures not only the mucoadhesivness but also the prolonged RES release. This is of great importance considering the vaginal administration, as reduced frequency in need for repeated administration correlates with better patient compliance.

4.2. Anti-oxidative activity

Invading pathogens create oxidative stress because of over production of ROS during increased metabolic activities and immune cell reaction. A number of cellular processes including aerobic metabolism can easily supply one electron to cytoplasmic oxygen to generate $O_2^{\bullet-}$ which is also an essential step for the cellular communication and signaling (Afanas'ev, 2007). Normally when the concentration of $O_2^{\bullet-}$ increases the activity of superoxide dismutase (SOD) and catalase (CAT) increase to dismutate the toxic amount of $O_2^{\bullet-}$ to non-toxic molecules such as water and oxygen. However, as the production of $O_2^{\bullet-}$ and dismutation process are not balanced, there will be the over production of $O_2^{\bullet-}$ or H_2O_2 which can either easily be transformed into the $ONOO^-$ by reacting with $NO^{\bullet}$, the $OH^{\bullet}$ by Fenton reaction (catalyzed by Fe^{+++}/Fe^{++}) or the $R^{\bullet}$ by increased peroxidase activity. The free radicals-ROS, especially $ONOO^-$, $OH^{\bullet}$, and $R^{\bullet}$ are very powerful oxidants as compared to $O_2^{\bullet-}$ and H_2O_2. The pathogen-induced ROS and/or free radicals generated *in vivo* together with the pro-inflammatory cytokines can cause significant damage to cells hasten the inflammation and infection. The anti-oxidants can attenuate the direct effect of radicals by deleting or scavenging which may inhibit the inflammatory processes.

RES was exhibited strong reaction to free radicals comparable with those of vitamin C and vitamin E. In addition, its effects on enhancing the SOD activity would make it a stronger candidate as an anti-oxidant. RES showed stronger radical scavenging activity for $ABTS^{+\bullet}$ radicals, comparing to DPPH free radicals (Figs. 2 and 3). The superior activity of RES (EC50 of 3.05 μM) compared to the other anti-oxidants in case of $ABTS^{+\bullet}$ radicals, is in agreement with literature (Stojanovic et al., 2001). The reaction with $ABTS^{+\bullet}$ radicals involves an electron transfer process while H-atom transfer mechanism involves in DPPH radical reaction therefore, $ABTS^{+\bullet}$ radicals are more reactive with the RES type polyphenols (Gülçin, 2010). Vanaja and colleagues reported on the enhanced anti-oxidative activities of liposomal RES as compared to free RES based on the inhibition of 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH)-induced luminol enhanced chemiluminescence assay (Vanaja et al., 2013). Only one report found so far describes DPPH radical assay directly used for liposomal suspensions measuring with the UV–vis spectrophotometer (Caddeo et al., 2013). It might be possible that the alcoholic solution of DPPH radical solubilizes the liposomal suspension by the destructing of liposomal vesicles to release free RES. We do not see any advantage of measuring direct radical scavenging activity for anti-oxidants-associated liposomal suspension unless used lipid is itself anti-oxidants. RES also showed pro-oxidant properties at higher concentration like other common anti-oxidants since it is a redox-chemistry and clinical application depends on the drug concentration (de la Lastra and Villegas, 2007).

RES is reported to exhibit anti-oxidative effect directly either by donating an electron to free radicals and/or providing hydrogen or indirectly by enhancing the SOD activity (Gülçin, 2010; Zheng et al., 2010). We also compared the *in vitro* SOD activities of RES and the corresponding liposomal formulation. RES and corresponding liposomal delivery system significantly enhanced the *in vitro* SOD activities comparing to controls. Moreover, liposomal RES, under similar conditions, was found superior to RES (Fig. 6).

4.3. Anti-inflammatory activity

Vaginal inflammation and infection are very common and every woman experiences them at least once in her life. They can be caused by various pathogens such as virus, bacteria, fungi, or parasite (*Trichimonas*). Contamination of such pathogens initiates inflammation and their rapid growth lead to infection. Inflammation is the root cause of severe metabolic dysfunction including loss of cell integrity, enzyme function, genomic stability etc. (Hanahan and Weinberg, 2000). Nitric oxide is known not only as a free radical and vasodilator; it also plays a very important role in the pathways of inflammation and as an immunomodulator (Coleman, 2001). Some free radicals mainly such as $O_2^{\bullet-}$, $OH^{\bullet}$, and $NO^{\bullet}$ radicals along with non-free radical species such as H_2O_2 and HNO_2 are responsible for mediating the inflammation (Khan et al., 2008). In addition, cytokines such as TNF-α and IL-1β are playing important roles in chronic inflammation processes and persistent inflammatory tissue damage leading to each stage of infection. Moreover, pro-inflammatory molecules such as NF-κB and non-steroidal anti-inflammatory gene-1 dominating over the effect of SOD, CAT and glutathione peroxidase (GPX), together with a non-enzymatic system such as glutathione and vitamins (A, C, and E) constitute the defense to overreaction of free radicals (Finkel and

Holbrook, 2000). RES showed anti-inflammatory activities by the inhibition of NF-κB activity via multiple mechanisms (Surh and Na, 2008). RES inhibited HSV replication by suppressing NF-κB activity (Faith et al., 2006). NF-κB is a host nuclear transcription factor, activated by multiple stimuli, including inflammatory cytokines, growth factors and bacterial or viral infections (Santoro et al., 2003). RES also showed anti-inflammatory activity by interfering both transcription (Subbaramaiah et al., 1998) and catalytic (Jang et al., 1997) activities of the COX2 enzyme. Therefore, the anti-inflammatory activities of RES can be suggested through ostensibly independent effects on NF-κB, cyclooxygenase and IL-1β (Baur and Sinclair, 2006).

In our experiments, RES was found to be more potent than vitamin C or vitamin E in scavenging $ABTS^{\bullet+}$ radicals (Fig. 3). RES was also found to be a stronger inhibitor of NO production as compared to *i*NOS inhibitor L-NAME, which strengthens its potential as an anti-inflammatory agent (Fig. 7). In addition, liposomal RES showed superior inhibitory properties on NO production (Fig. 8). RES significantly inhibited the production of cytokines TNF-α and IL-1β in the LPS-activated macrophages. In all those set of experiments, liposomal RES was found to be more potent as compared with the corresponding RES especially for TNF-α (Fig. 9). The increased inhibitory activity of liposomal RES might be the consequence of enhanced cellular uptake and/or maximized effect of RES. It requires further investigation at cellular and sub-cellular levels. If the finding reported here can be directly correlated to the normal epithelial cells in human, liposomal formulation of RES for topical anti-inflammatory treatment would be an optimal formulation.

Moreover, trichomoniasis is the most common non-viral STD in the worlds. Currently, metronidazole (MDZ) and other 5-nitroimidazoles (tinidazole, ornidazole, and secnidazole), the potent drugs against infections caused by anaerobic or microaerophilic microorganisms, are the only recommended drugs for standard treatment of *T. vaginalis* infection. However, resistance of *T. vaginalis* to MDZ, allergic reactions, and failure to remedy the infection with two consecutive courses of treatment have been reported (Cudmore et al., 2004; Das et al., 2005; Harp and Chowdhury, 2011; Muzny et al., 2012). Studies have shown that at least 5% of clinical cases of trichomoniasis are caused by parasites that are resistant to the above-mentioned drugs. Because of the lack of approved alternative treatments, the only option for patients with resistant infections is to use higher and sometimes toxic doses of MDZ, which leads to an increase in the occurrence of side effects (Cudmore et al., 2004). On the other hand, RES showed *in vitro* anti-parasitic effect against *T. vaginalis* by altering hydrogenosomal dysfunction (Mallo et al., 2013). Therefore, regarding to the potent anti-oxidative and anti-inflammatory activities and multi-targeted mechanisms, RES could be a potential therapeutic candidate especially for pregnant women against common inflammations and infections caused by diverse type pathogens. Although we have not tested the newly developed systems against *T. vaginalis* or particular pathogens, it is to be expected that the developed system can assure the necessary concentration of RES at vaginal site enabling sufficient interaction time and consequent antimicrobial action.

5. Conclusions

Our findings further confirmed RES as a strong anti-oxidant as well as anti-inflammatory compound. The liposomal formulation solubilized RES and enhanced its anti-oxidative and anti-inflammatory properties. With a combination of liposomal carrier as a solubilizer for RES and the mucoadhesive properties of chitosan in chitosan-coated liposomes, development of an optimal vaginal drug delivery system with specific, prolonged and controlled drug release properties might be possible to enable a controlled delivery as well as provide chemical stability for RES. Moreover, anti-microbial properties of chitosan will provide additional advantage against vaginal pathogens. However, further *in vivo* and clinical studies are needed to obtain the direct evidences.

Conflict of interest

Authors do not have any conflict of interests.

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PEGylated liposomes for topical vaginal therapy: Mucus-penetration improves delivery of interferon alpha

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Abstract

In recent years, a great deal of attention has been given to the surface modification of liposomes as means to prolong the residence time in body or body cavities. In respect to mucosal sites, it has been agreed that mucoadhesive polymers prolong the residence time. Recent findings, however, indicated that nanocarrier systems based on the surface-available polyethylene glycol (PEG) and able to penetrate mucus, assure closer contact with the epithelium, and improved drug delivery to vaginal mucosa. To elaborate on the proposed hypothesis, we developed the mucus-penetrating, PEGylated liposomes, containing Interferon alpha-2b (IFN α-2b) destined to provide localized therapy in the vaginal tissue. IFN α-2b was chosen as a relevant biological with potential to treat human papilloma virus (HPV) infections of vagina. The PEGylated vesicles had a size of 181 ± 8 nm, a negative zeta potential of – 13 mV and an entrapment efficiency of 81 ± 10 %. *In vitro* release experiments on model membrane showed a nearly non-existent IFN α-2b release from both the control and liposomally-associated IFN α-2b. However, the *ex vivo* penetration studies performed on vaginal tissue from pregnant sheep, showed a clear elevated IFN α-2b penetration from PEGylated liposomes compared to the control. Furthermore, mucin studies confirmed the absence of

interaction between the PEG modified liposomes and mucin indicating that the liposomes have the ability to penetrate mucus and reach the deeper epithelium. The system seems to have a promise in topical delivery of IFN α-2b and provide enhanced efficacy in local anti-viral therapy.

Key words: PEGylated liposomes, IFN α-2b, vaginal therapy, HPV, mucus-penetrating

1. Introduction

It is estimated that 80 % of all sexually active women will acquire vaginal human papilloma virus (HPV) infection by the age of 50 (Viera *et al*., 2010). Genital papilloma virus is transmitted via mucosal surfaces and is responsible for genital warts. Current therapy fails to eradicate warts and eliminate the virus and the recurrence rate is up to 90 % (Stockfleth and Meyer, 2012). For visible lesions, intralesional interferon alpha-2b (IFN α-2b) appears to be applicable treatment; however, this treatment option involves pain and severe side effects due to systemic exposure. Moreover, this is not suitable treatment for latent or subclinical infections and a sophisticated non-invasive approach is desirable. The potential of topical treatment of genital warts was one of the first studies reporting vaginal applications of liposomal drugs. In preliminary clinical testing, topical treatment with liposomal IFN α-2b achieved complete resolution of cervical lesions in female patient at the end of therapy (Foldvari and Moreland, 1997). Liposomal formulations can provide a prolonged half-life of IFN α-2b and a shift of distribution towards infected tissues due to increased capillary permeability, thereby improving efficacy and reducing toxicity (Wang *et al*., 2002). Additionally, incorporating IFN α-2b in liposomal formulations may increase its stability and alter its pharmacokinetics, two issues which often limit the success of IFN therapy (Karau *et al*., 1996; Yang *et al*., 2006).

To achieve a successful local delivery to mucosal tissue, the penetration into/through the mucus mesh, uniform distribution of drug into the underlying tissue and maintained sufficiently high drug concentration are required. Vaginal mucosal surface is protected by mucus, a physical barrier in the form of an adhesive gel that stick to most particles, preventing all foreign particles to penetrate to the epithelium surface. Nevertheless, viruses have the ability to overcome this barrier and cause mucosal infection (Ensign *et al*., 2012). For drug delivery systems to be able to successfully reach mucosal tissue, biomimicking of the virus properties might be a promising approach.

Polyethylene glycol (PEG) is an uncharged hydrophilic polymer widely applied in pharmaceutical formulations, including those for topical vaginal therapy. When used as a coating material, PEG enables nanoparticles to diffuse through vaginal mucus by eliminating the adhesive interactions between the nanoparticles and mucus (Ensign *et al*., 2012; Lai *et al*., 2007), achieving a closer contact to the vaginal epithelium, and improved drug effectiveness is possible. With the combination of the carrier properties of liposomes as a protective carrier for sensitive biologicals and the mucoresistant properties of PEG available on liposomal surface, development of an optimal vaginal drug delivery system with controlled drug release in close proximity to the vaginal epithelium is possible.

Following this hypothesis, we developed liposomal carriers containing IFN α-2b with surface-available low molecular weight PEG (Mw of 2000), aiming to obtain a mucus-penetrating delivery system able to distribute IFN α-2b to vaginal mucosa enabling improved localized therapy.

2. Materials and Methods

2.1. Materials

Lipoid S 100 (PC, soybean lecithin, > 94 % phosphatidylcholine) was a generous gift from Lipoid GmbH, Ludwigshafen, Germany; methoxy poly (ethylene glycol)-modified lipids (mPEG 2000) was from the same manufacturer. IntronA® 50 million IU/mL injection fluid in multiple dose pen was the product of MSD AS, Drammen, Norway. Acetic acid, bovine serum albumin, calcium hydroxide, cholesterol, fructose, glycerol, mucin from porcine stomach (type III, bound sialic acid 0.5 % - 1.5 %, partially purified), potassium phosphate monobasic, Sephadex® G-50, Triton® X-100 and zinc chloride were all purchased from Sigma-Aldrich Chemie GMbH, Steinheim, Germany. Di-sodium hydrogen phosphate, sodium dihydrogen phosphate monohydrate, potassium chloride and titriplex (ethylenedinitrilotetraacetic acid disodium salt dihydrate) were obtained from Merck KGaA, Darmstadt, Germany. Glucose, lactic acid, polysorbatum, potassium hydroxide, sodium citrate dihydrate and urea were the products of NMD, Oslo, Norway. Ammonium acetate, magnesium chloride and potassium chloride was the product of VWR International BHD Prolab, Leuven, Belgium. ELISA kit were purchased from Bio-Techne, Abingdon, UK.

2.2. Preparation of liposomes

Liposomes were prepared by the film hydration method as described earlier (Jøraholmen *et al*., 2014). Briefly, cholesterol (10 mg), mPEG 2000 (36.3 mg) and PC (200 mg) were dissolved in methanol and chloroform (1:1, v/v) in a round bottom flask. Solvents were removed through evaporation (Büchi rotavapor R-124 with vacuum controller B-721, Büchi Vac® V-500, Büchi Labortechnik, Flawil, Switzerland) for 2 hours at 50 mm Hg and 50 °C. The remaining film was flushed with nitrogen to assure no residual solvents. The lipid film was then re-suspended in 5 mL of IFN α-2b solution (2 mIU/mL) from IntronA® 50 million IU/mL injection fluid and Intron A buffer (pH 7.4, 7.5 g/L NaCl, 1.8 g/L NaH_2PO_4, 1.3 g/L Na_2HPO_4, 0.1 g/L EDTA and 0.1 g/L Polysorbate 80). Empty liposomes were prepared in a similar way, merely the film was re-suspended in Intron A buffer. Liposomal suspensions were stored in refrigerator (4 - 8˚C) for at least 12 hours prior to further use.

2.3. Vesicle size reduction

Liposomal size was reduced by the extrusion through polycarbonate membranes (Nuclepore Track-Etch Membran, Whatman House, Maidstone, UK) (Jøraholmen *et al*., 2015). The extrusion was performed stepwise through 0.8, 0.4 and 0.2 µm pore size filters (for each step, 3 extrusions were executed). Extruded liposomes were stored in refrigerator for at least 6 hours prior to further use.

2.4. Particle size analysis

The particle size distribution of liposomal samples was determined by photon correlation spectroscopy (Submicron particle sizer model 370, Nicomp, Santa Barbara, California, USA). To avoid interference by dust particles, test tubes used for the determination were filled with distilled water and sonicated for 10 min in ultrasonic bath, then rinsed with filtered, distilled water (using 0.2 µm filter) prior to the measurements. Small aliquots of the samples were diluted with the filtered, distilled water to obtain a particle intensity of approximately 200-350 kHz (Jøraholmen *et al*., 2014). All samples preparations were performed in a laminar airflow bench. Particle size analyses were run in vesicle mode and intensity distribution and three parallels (each with a run time of 10 min) were determined for each sample measurement.

2.5. Zeta potential measurements

Zeta potential measurements were performed on a Malvern Zetasizer Nano ZS (Malvern, Oxford, UK). Prior to measurement, the measurement cells were properly cleaned with ethanol and filtrated water, respectively. The liposomal suspensions were diluted in filtrated water to appropriate concentrations (typically 1:20) before loading the sample into the cells (Jøraholmen *et al*., 2015). Three parallels were determined for each sample measurement.

2.6. IFN α-2b entrapment

Liposomally entrapped IFN α-2b and free drug were separated by size-exclusion gel chromatography. Sephadex® G-50 in Intron A buffer (75 mg/mL) was left to swell overnight (at 4-8 °C). The gel was packed in a column (50 mL) and flushed with Intron A buffer. The stationary phase measured 65 cm^3. Liposomal sample containing IFN α-2b (1.2 mL) was applied on top of the column and 100 fractions of 1 mL was collected. Eluate time was 1.8 mL/min. The column was properly rinsed with Intron A buffer (150 ml) before and after each sample.

An enzyme-linked immunoassay kit (VeriKine™ Human IFN α-2b Multi-Subtype ELISA kit) was used for the quantification of IFN α-2b. Aliquots of the samples were diluted in 0.1 % Triton (Triton X-100 in Intron A buffer) to disintegrate liposomes and further diluted to suitable concentrations with Intron A buffer. The procedure was performed according to enclosed instructions in the ELISA kit. In brief, standards and diluted samples were added to microplate coated with IFN α-2b antibodies. Diluted antibody solution, diluted horseradish peroxidase (HPR) and tetramethyl-benzidine (TMB) substrate were added step-wise after 1 hour incubation. Finally, the reaction-terminating solution was added after 15 min incubation. Amount of liposomally-associated IFN α-2b was determined by UV spectrophotometry at 450 nm (Microtitre plate reader; Spectra Max 190 Microplate, Spectrophotometer Molecular devices, Sunnyvale, California, USA).

2.7. *In vitro* release

Vaginal fluid simulant (3.51 g/L NaCl, 1.40 g/L KOH, 0.222 g/L $Ca(OH)_2$, 0.018 g/L bovine serum albumin, 2 g/L lactic acid, 1 g/L acetic acid, 0.16 g/L glycerol, 0.4 g/L urea, 5 g/L glucose) was prepared according to Owen and Katz (1999). Measured pH was 4.6.

The *in vitro* IFN α-2b release experiment was performed on the Franz cell manual diffusion system (Perme Gear Ink, Diffusion cells and Systems, Hellertown, USA) as described earlier (Jøraholmen *et al*., 2014). The system and accessories were properly cleaned with methanol, demineralized water and distilled water, respectively, prior to experiment. The heating circulator (Julabo Laboratechnik, F12-ED, Seelback, Germany) was set to 37 °C. The acceptor chambers (12.1 mL) were completely filled with acetate buffer (pH 4.6; 77.1 g CH_3COONH_4, 70 mL glacial acetic acid and distilled water up to 1000 mL). Polyamide membrane (Sartorius polyamide membrane, 0.2 µm pore size, Sartorius AG, Gröttingen, Germany) was cut to appropriate size (diffusion area of 1.77 cm^2) and fixed between donor and acceptor chambers. The IFN α-2b content in all tested samples was determined (ELISA) prior to the experiment to prepare a control solution (IFN α-2b in Intron A buffer) containing similar drug concentration. Liposomal samples and controls (each in volume of 550 µL) as well as vaginal fluid simulant (50 µL) were added in the donor chambers and the system was properly sealed. Sampling from acceptor chamber (500 µL) was done after 1, 2, 4, 6 and 8 hours and all the samples withdrawn from the acceptor chamber were replaced by an equal volume of acetate buffer. Concentrations of IFN α-2b in the withdrawn samples, remaining in donor chamber and retained in the polyamide membrane were determined with ELISA kit and UV spectrophotometry as described above. The experiments were performed in triplicates.

2.8. Preparation of tissue

Vaginal tissue from the pregnant sheep was obtained from the Laboratory Animal Centre, University of Oulu, Finland. The experiments were performed according to the guidelines of the National Animal Experiment Board in Finland. The vaginal tissue was carefully removed from the underlying tissue, cleaned and moisten with physiological solution (pH 7.4), packed in clinging film, and then frozen (-20 °C). Prior to the experiments, the tissue was left to defrost in phosphate buffer (pH 7.4) at room temperature for at least 1 hour.

2.9. *Ex vivo* penetration

The experiment was performed on the Franz cell manual diffusion system like described above. Briefly, the acceptor chamber was filled up with phosphate buffer (pH 7.4, 8 g/L NaCl, 0.19 g/L KH_2PO_4, and 2.38 g/L Na_2HPO_4). Sheep vaginal tissue was defrosted, cut to appropriate size (1.77 cm^2) and fixed between donor and acceptor chamber. Samples and controls (550 µL), with similar amount of IFN α-2b, as well as vaginal fluid simulant (50 µL) were added in the donor chambers and the system was properly sealed. Aliquots (500 µL) were collected after 1, 2, 4, 6 and 8 hours and replaced by an equal volume of phosphate buffer. Amount of IFN α-2b in the collected samples, remaining in donor chamber and retained in the vaginal tissue were determined with ElISA kit and UV spectrophotometry as described above. The experiments were performed in triplicates.

2.10. *In vitro* mucin-binding

The binding of PEGylated liposomes to pig mucin (PM) was determined to confirm the lack of mucoadhesiveness. The test was performed as described earlier (Jøraholmen *et al*., 2015). Briefly, empty liposomes (1 mL) with original vesicle size was added to an equal volume of PM suspension (400 µg/mL) in phosphate buffer (pH 7.4) and acetate buffer (pH 4.6), respectively. Incubation at room temperature for 2 hours was followed by ultracentrifugation at 216 000 *g* for 1 hour, at 10 °C (Optima LE-80; Beckman Instruments, Palo Alto, California, USA). From the supernatant, 4 aliquots of 200 µL were transferred to a microtitre plate (Costar® UV 96-well plate with UV transparent flat bottom, Acrylic, Costar®, Corning, New York, USA) and free PM was measured spectroscopically at 251 nm (Microtitre plate reader; Spectra Max 190 Microplate, Spectrophotometer Molecular devices, Sunnyvale, California, USA). PM binding efficiency was calculated according to Naderkhani *et al*. (2014). The experiments were performed in triplicates.

2.11. Stability of PEGylated liposomes in the presence of biological fluids

Human semen simulant was prepared according to Owen and Katz (2005). Briefly, solution 2 (101 mg $CaCl_2 \cdot 2H_2O$, 15.13 mL H_2O), the solution 3 (92 mg $MgCl_2 \cdot 6H_2O$, 15.13 mL) and further solution 4 (34.4 mg $ZnCl_2$, 15.13 mL H2O) were added to solution 1 (5.6 mL 0.123 M $NaH_2PO_4 \cdot H_2O$, 49.14 mL 0.123 M Na_2HPO_4, 813 mg sodium citrate dehydrate, 90.8 mg KCl, 88.1 mg KOH, 272 mg fructose, 102 mg glucose anhydrase, 62

mg lactic acid, 45 mg urea, 5.04 mg bovine serum albumin). The solutions were mixed and pH was adjusted to 7.7 with sodium hydroxide.

Aliquots (500 μL) of liposomal suspensions containing IFN α-2b (free of unentrapped IFN) were added to human semen simulant (500 μL) and vaginal fluid simulant (50 μL), and incubated at room temperature for 2 hours. Potentially leaked (free) IFN α-2b was separated from liposomally-associated IFN α-2b by size-exclusion gel chromatography (according to the method described earlier). Quantification of IFN α-2b in fractions was done with ELISA kit and UV spectrophotometry as described above.

2.12. Statistical analyses

For comparison of two means, statistical significance was determined by the student's *t*-test. A significance level of $p < 0.05$ was considered to be acceptable.

3. Results and Discussion

3.1. Liposomal characteristics

The effectiveness of local drug delivery at the vaginal site is determined by the physicochemical characteristics of the delivery system. The vesicle size affects the ability to fit within the mucin pores, while the particles surface charge and properties establish if there will be attraction or repulsion to mucus. Thus, the main focus in the development of a system aiming for local therapy at the mucosal site is controlling the surface properties, particle size and surface charge (Vanić and Škalko-Basnet, 2013). Penetration into mucus allows closer contact with the vaginal epithelium and enables improved drug effectiveness. The protective barrier function of mucus against all foreign particles complicates this approach, however, virus have the ability to penetrate mucus and cause mucosal infection. Development of delivery systems that are capable of imitating this property is an interesting strategy in the treatment of the same infections. Polyethylene glycol (PEG) available on vesicle surface is shown to effectively minimize the adhesive interactions between vesicles and mucus, creating mucus-penetrating particles that can efficiently penetrate the human mucus (Tang *et al*., 2009; Lai *et al*., 2007). The surface density and molecular weight of PEG affect the particle transport, and literature indicates that dense coating with low molecular weight

PEG increases the transport rate through mucus (Lai *et al*., 2009a). By modifying liposomal surface with low molecular weight PEG we aimed to develop an advanced carrier for IFN α-2b for the local therapy at mucosal site.

Although there is no consensus regarding the effect of vesicle size on mucosal targeting at vaginal site, it is known that small particles will easily be trapped in the mucus mesh while larger particles will be retained on the mucus surface. Lai *et al*. (2009a) showed that larger particles (200-500 nm) densely coated with PEG are more rapidly transported through fresh undiluted human mucus, than the corresponding particles of smaller size (100 nm). Viruses with diameters between 30 and 200 nm are able to reach and infect the vaginal mucosa successfully (Wong *et al*., 2014). Therefore, we aimed for a vesicle size of 200 nm. The vesicle size of our PEGylated liposomes was close to the targeted size range (Table 1). We reported earlier (Jøraholmen *et al*., 2015) that the extrusion as a size reduction method can be applied to obtain vesicle size in desired size range with favourable polydispersity. Our liposomes exhibited rather monodisperse size distribution (Table 1).

Table 1: Characteristics of PEGylated liposomes containing IFN α-2b (n = 3).

	Vesicle size (nm)	PI*	Zeta potential (mV)	Entrapment (%)
PEGylated liposomes containing IFN α-2b	**181 ± 8**	**0.129**	**- 13.33 ± 0.81**	**81 ± 10**

*Polydispersity index

Considering optimal mucus-penetration, a nearly neutral surface charge is desirable to assure mucoresistant properties (Lai *et al*., 2009a). However, it is also known that a negatively charged component present in the liposomal bilayer is beneficial for a stable association and will improve IFN α-2b entrapment and prevent drug leakage from the liposomes (Karau *et al*., 1996). Our PEGylated liposomes had a slightly negative zeta potential (Table 1). However, as seen in mucoadhesiveness testing, the charge did not contribute to mucoadhesiveness.

Even if the system exhibits desired mucoresistant properties, it is very important that the drug load is sufficient to achieve desired therapeutic response. We were able to achieve rather high entrapment efficiency of 81 % (Table 1). This is in accordance with literature data (Yang *et al*., 2006; Vyas *et al*., 2006; Takeuchi *et al*., 1999; Li *et al*. 2011;). By incorporating IFN α-2b in liposomal formulations, an increase in

IFN α-2b stability and positive alteration of the pharmacokinetics can be obtained (Karau *et al*., 1996; Yang *et al*., 2006).

3.2. Mucoresistant properties

A great deal of older literature suggested that PEG owns mucoadhesive properties that are expressed by the interpenetration effects between PEG chains and mucus (Huang *et al*., 2000; Serra *et al*., 2006; Yoncheva *et al*., 2005). Very recent studies, however, have demonstrated that PEG-modified nanoparticles minimize mucoadhesion and effectively penetrate the mucus enabling closer contact to the underlying epithelium (Tang *et al*., 2009; Lai *et al*., 2007; Ensign *et al*., 2012). It is suggested that the molecular weight of PEG is the determining factor whether PEG will exhibit mucoadhesive or mucoresistant characteristics and recent findings indicate that when densely coated with low molecular weight PEG, nanoparticles avoid adhesive interactions with mucus (Ensign *et al*., 2012; Lai *et al*., 2007; Wong *et al*., 2014).

To confirm that PEG on liposomal surface has a mucoresistant effect and contributes to a reduced association between liposomes and mucus, the binding between mucin and liposomal preparations was determined. Commercial pig mucin (PM) is commonly used as a mucus substitute due to similarity in the structure and molecular weight of PM and human mucus (Groo and Lagarce, 2014). Based on the facts that the vaginal pH varies in healthy and infected, as well as pre- and post-menopausal women, the experiment was performed at conditions of both a healthy vaginal environment (pH 4.6) and at conditions expected to occur during the vaginal bacterial infection and post menopause (pH 7.4). As expected, the PEGylated liposomes did not exhibit mucin-binding activity (Figure 1). The results indicate a significantly reduced binding efficiency within the different pH conditions for the PEGylated liposomes compared to both conventional non-coated liposomes and the 0.1 % (w/v) chitosan-coated liposomes ($p < 0.001$).

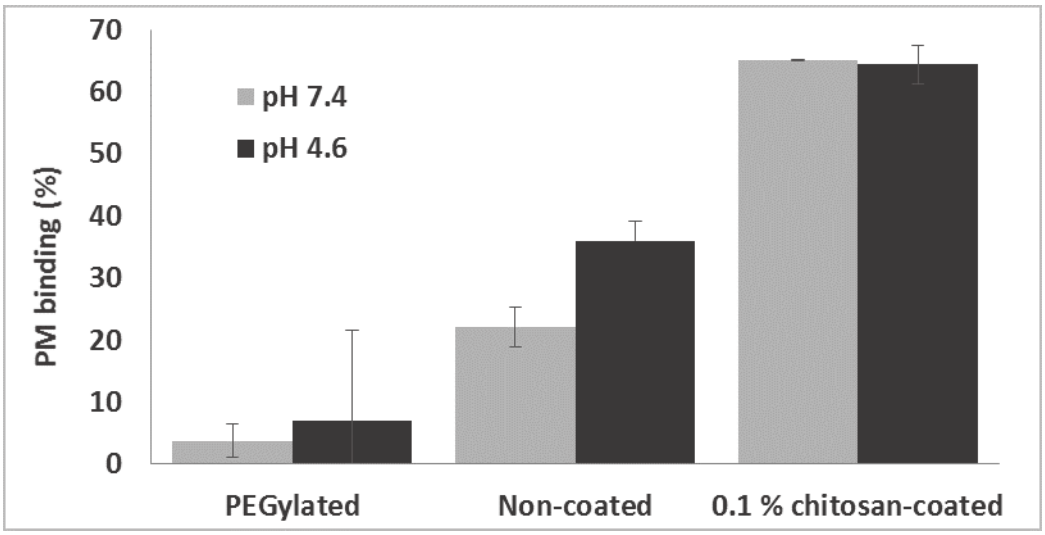

Figure 1: Mucin-binding of PEGylated, non-coated and chitosan-coated liposomes. Results are expressed as percentage mean ± SD (n = 3).

The modest mucin-binding observed for the PEGylated liposomes is likely to be the result of physical interactions due to ultracentrifugation and not the electrostatic interactions. Liposomes coated with the low chitosan concentration (0.1 %, w/v) exhibited a superior interaction with mucin compared to non-coated liposomes (Jøraholmen *et al*., 2015).

The absence of mucin-binding activity coincide with the expectation of mucoresistant properties of PEG-modified liposomes. The results indicate that the PEGylated liposomes might be able to penetrate the mucus and assure closer contact with the epithelium, thus, enhance mucosal delivery of incorporated drug and improve its localized therapeutic outcome. However, one has to consider that the retention time at vaginal site might be insufficient to assure that an adequate amount can actually penetrate mucus due to lack of mucoadhesion and rapid clearance by vaginal discharge. To confirm that mucoresistant delivery system has sufficient time to stay in a closer contact with the vaginal epithelium and assure penetration in deeper layers, *in vivo* testing in infected animals would be required.

Mucin fibres in the healthy human vaginal mucus are negatively charged and found to have a diameter of approximately 340 nm (Lai *et al*., 2007). Mucus properties are affected by the environmental changes, such as changes in pH due to vaginal infections and the viscosity of vaginal fluid. When exposed to bacterial vaginosis, a reduction in the viscosity of vaginal fluid causes reduced barrier properties and increased risk of infection (Lai *et al*., 2009b). It is further suggested that mucoadhesive nanoparticles may disrupt the protective microstructure of mucus by increasing the average pore size of mucin fibers. This can provide

easier access for foreign particles to vaginal mucosa, including the pathogens and other potentially toxic nanomaterials, due to the impaired mucus barrier. This phenomena needs to be further evaluated in *in vivo* testing. However, the limited literature data indicate that the mucoresistant particles did not exhibit similar effect on the cervicovaginal mucus (Wang *et al*., 2011).

3.3. *In vitro* IFN α-2b release

When evaluating *in vitro* drug release from topical formulations, including those intended for vaginal use, the Franz cell diffusion system is generally considered one of the most appropriate methods (Brown *et al*., 2011). In addition to the ability to incorporate both lipophilic and hydrophilic substances, liposomes can provide controlled release of incorporated drug (Jøraholmen *et al*., 2014), and we followed the release of liposomally-associated IFN α-2b over 8 hours compared to the IFN α-2b release from a control solution. The experimental setup was designed mimicking the human conditions; the acceptor medium had the pH of healthy human vaginal environment (4.6) and a temperature of 37 °C. Further, the experiment was performed in the presence of simulated vaginal fluid, since it is known that the flow, retention, drug delivery kinetics, and bioactivity of vaginal formulations are influenced by the compounds from the vaginal fluid (Owen and Katz, 1999). Use of the vaginal fluid simulant has been proven to assist in *in vitro* evaluations of contraceptive and prophylactic drug delivery systems (Sassi *et al*., 2008).

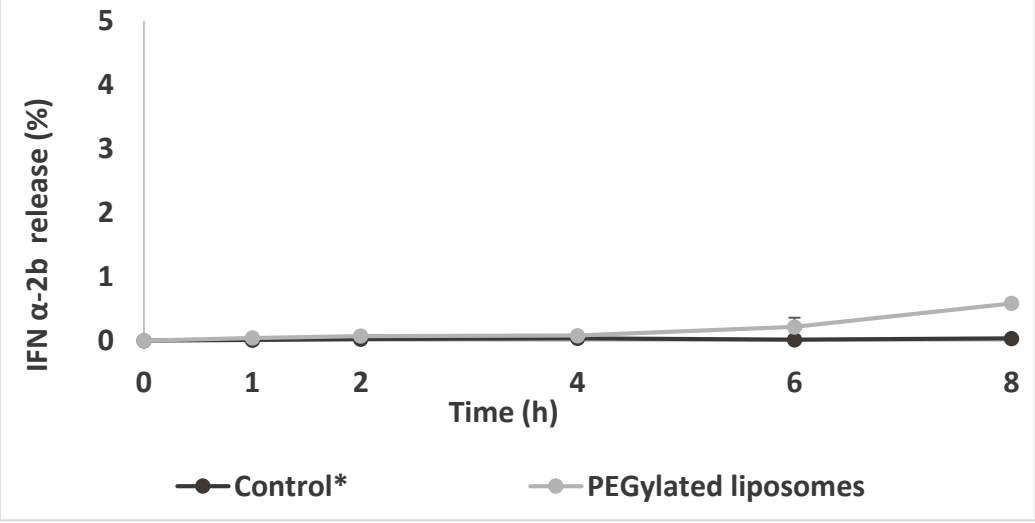

Figure 2: *In vitro* IFN α-2b release. Results are expressed as percentage mean ± SD (n = 3).

*IFN α-2b in Intron A buffer.

Initially, both PEGylated liposomes and control solution showed a strongly retained *in vitro* release of IFN α-2b (Figure 2). Even though the release of liposomally-associated IFN α-2b increased significantly ($p < 0.001$) compared to the control solution after 8 hours, the release was very limited. A considerable amount of IFN α-2b from the control solution was retained within the artificial membrane (Figure 3). Although the pore size of the membrane should be sufficient to allow the passage of IFN molecules through, it seems that IFN α-2b was mostly retained in the membrane, or stayed in the liposomes on the membrane surface (Figure 3).

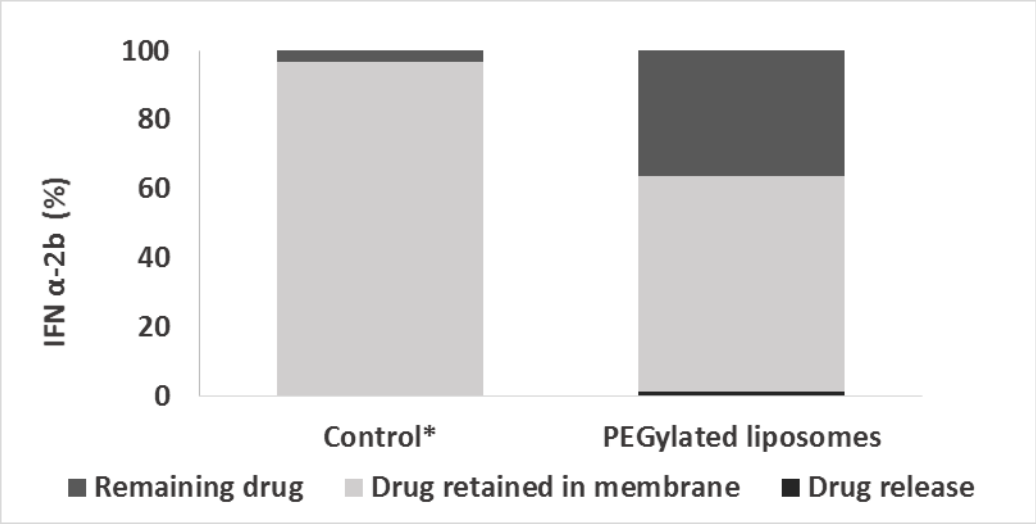

Figure 3: IFN α-2b distribution after 8 hours of *in vitro* release.

*IFN α-2b in Intron A buffer.

3.4. *Ex vivo* penetration

Franz cell diffusion system is shown suitable as a method for determination of *ex vivo* tissue penetration (Machado *et al*., 2015; Sandri *et al*., 2004; Bonferoni *et al*., 2008; das Neves *et al*., 2013). Sheep vaginal epithelium is stratified squamous tissue, similar to that of human, only thinner (Moss *et al*., 2012). The tissue used in this experiment was from pregnant animals and the thickness was measured to be 750 µm. The experiment was performed in the presence of vaginal fluid simulant to closer mimic the conditions the delivery system is expected to face when administered vaginally.

In contrary to the strongly sustained release through the artificial membrane, penetration through the sheep vaginal tissue was prominent, and a distinct increase in IFN α-2b release from the PEGylated liposomes was seen (Figure 4). This demonstrates that the PEGylated liposomal formulation enables IFN α-2b to penetrate the vaginal mucus to a higher extent compared to the solution formulation used as a control.

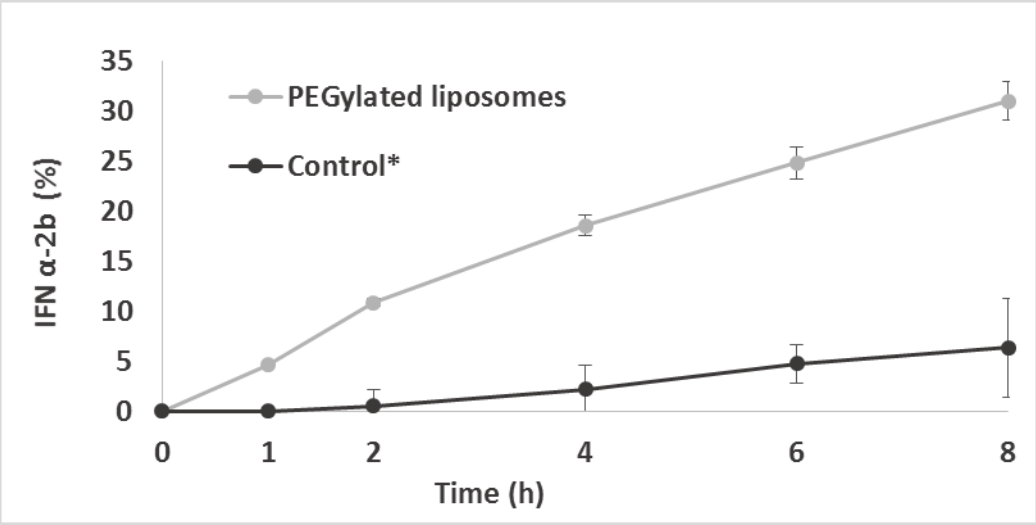

Figure 4: *Ex vivo* IFN α-2b penetration. Results are expressed as percentage mean ± SD (n = 3).

*IFN α-2b in Intron A buffer

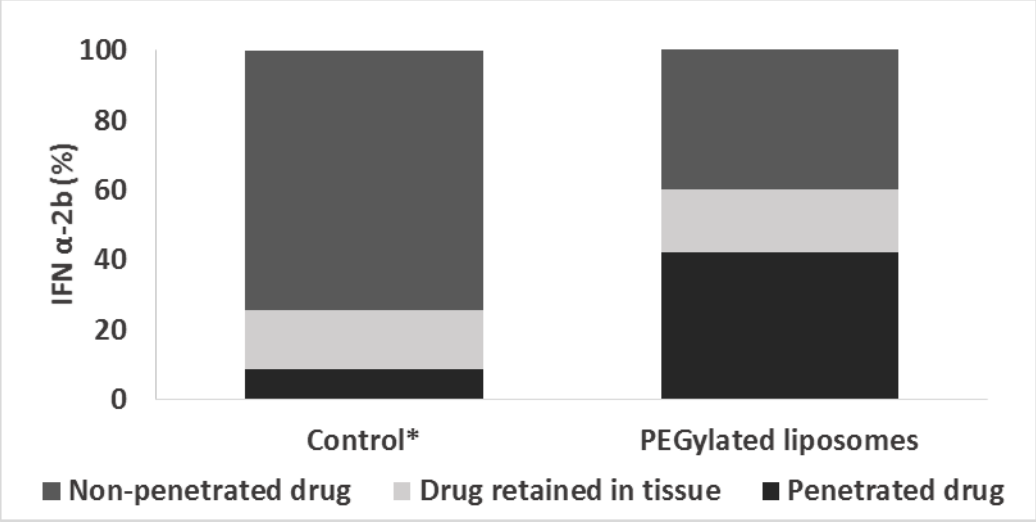

Figure 5: IFN α-2b distribution after 8 hours *ex vivo* penetration experiment.

*IFN α-2b in Intron A buffer

Majority of IFN α-2b from control solution appeared to be retained in or on top of tissue and only a minor amount of drug was able to penetrate through the tissue after 8 hours (Figure 5).

3.5. Stability

The stability of liposomal IFN α-2b has been confirmed for at least 1 month (Eppstein and Stewart, 1981). The physical and chemical properties of the environment might affect the stability and performance of the drug delivery system (Owen and Katz, 2005). The composition, volume, pH and rheological properties of vaginal fluids are affected by age, the menstrual cycle or sexual arousal. Some studies suggest that on average 0.5 – 0.75 g of vaginal fluid is contemporary present in the vagina (Owen and Katz, 1999). Vaginal pH in healthy, pre-menopausal women will vary from 4.0 – 5.0, however, the presence of semen will increase the pH to levels closer to neutral range. The average volume of human ejaculate is found to be 3.4 mL (Owen and Katz, 2005), and expected to affect the vaginal pH for hours (das Neves *et al*., 2011).

Based on the cited literature, PEGylated liposomes were diluted 1:10 (v/v) with vaginal fluid simulant and 1:1 (v/v) with semen fluid simulant to determine possible IFN α-2b leakage from the delivery system once exposed to the vaginal environment. An incubation time of 2 hours was selected as an appropriate challenge. A minor leakage of merely 5.1 % IFN α-2b was detected after 2 hours, indicating the stability of PEGylated liposomes in a simulated vaginal environment and in the presence of semen fluid simulant.

4. Conclusions

Our findings suggest lack of interactions between mucin and PEGylated liposomes, confirming the mucoresistant properties provided when PEG is used as a coating material. The ability of the liposomally-associated IFN α-2b to penetrate through the vaginal tissue was distinctively increased for the PEGylated liposomes as compared to IFN α-2b in solution. This indicates that PEGylation represents an approach to obtain drug delivery in the close proximity to the vaginal epithelium. Furthermore, the PEGylated liposomes were shown to be stable in the vaginal environment simulated by the presence of vaginal fluid simulant and semen fluid simulant.

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