



MASTER THESIS FOR THE DEGREE MASTER OF PHARMACY

DEVELOPMENT OF CHITOSAN-COATED LIPOSOMES FOR IMPROVED
THERAPY OF VAGINAL INFECTIONS: CLOTRIMAZOLE AS MODEL
DRUG

BY

MAY WENCHE BAKKE JØRAHOLMEN

2012

Supervisors

Professor Nataša Škalko-Basnet

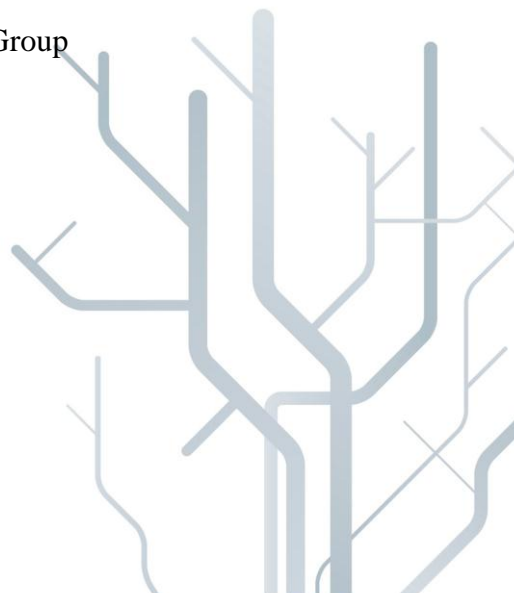
Professor Ingunn Tho

Drug Transport and Delivery Research Group

Department of Pharmacy

Faculty of Health Sciences

University of Tromsø



ACKNOWLEDGEMENTS

This study was conducted in the Drug Transport and Delivery Research Group, Department of Pharmacy, University of Tromsø.

First of all I would like to thank my supervisor Professor Nataša Škalko-Basnet for continuous encouragement and for shearing your endless knowledge. Your patient guidance and support cannot be appreciated highly enough. It has been a honor and pleasure to work with you, and I look forward to continue the work on my Ph.D.

The support of Professor Ingunn Tho is also highly appreciated.

I would also like to thank Merete Skar for always finding time to help and for all technical advice in the lab.

Many thanks to PhD student Julia Hurler for her help, especially with the Franz diffusion cells.

I also want to thank everyone else associated with the *Drug Transport and Delivery Research Group*, for making me feel so welcome and giving me a helping hand whenever needed.

I wish to thank Mydland for providing tissue used in this study.

Special thanks to all my friends here in Tromsø for all the fun times. In particular Hanne; these last 5 years would not have been the same without you.

Last, but not least, I would like to thank my family for your continuous support and encouragement through my years of study. And to my little sister; no one can cheer me up like you.

Tromsø, May 2012

May Wenche Jøraholmen

ABSTRACT (Norwegian)

For mange legemidler er vaginal administrasjon fordelaktig, spesielt gjelder dette for behandling av gynekologiske sykdommer som underlivsinfeksjoner. Det er utviklet mange ulike farmasøytiske formuleringer for vaginal administrasjon, likevel er effektiviteten av de tilgjengelige legemiddelformene begrenset på grunn deres korte retensjonstid på behandlingssted. Den selvrensende prosessen i vagina og fysiologiske forandringer i vaginale slimhinner er fortsatt en utfordring ved utvikling av formuleringer.

Målet med denne studien var å utvikle og karakterisere mucoadhesive (slimhinneklebende) liposomer som kan forbedre lokal, vaginal behandling med klotrimazol.

Klotrimazol ble inkorporert i liposomer ved hjelp av den såkalte “mechanical dispersion” metoden, og den liposomale suspensjonen ble sonikert for å oppnå ønsket vesikkelstørrelse. Størrelse, polydispersitet og grad av legemiddelinkorporering for liposomene ble karakterisert. Kitosan ble valgt som polymermateriale for å skape en hinne på overflaten av liposomene for å forbedre de mucoadhesive egenskapene. Kitosan-overtrekk (0.1 % w/v og 0.6 % w/v) ble påført liposomer etter at fritt legemiddel var separert fra. *In vitro* frigjøring av legemiddel fra kitosanovertrekte liposomer ble sammenlignet med frigjøring fra liposomer uten chitosan og fritt legemiddel. Kitosan-overtrekte liposomer viste forsinket legemiddelfrigjøring i større grad enn liposomer uten kitosan. Vaginalt vev fra ku ble brukt for å studere formuleringens adhesjon til slimhinnen og penetrasjon av vevet. Foreløpige data indikerer at klotrimazol heller forblir i vaginalt vev enn å penetrere gjennom vevet. Eksperimentene viste at kitosan-overtrekte fosfolipide vesikler har et potensial i lokal behandling av vaginale sykdommer.

Nøkkelord: Klotrimazol, mucoadhesive liposomer, kitosan, vaginal drug delivery.

ABSTRACT

For many drugs the vaginal route of administration is favorable, particularly for local therapy of specific gynecological diseases such as vaginal infections. A large variety of pharmaceutical preparations have been developed for vaginal delivery, nevertheless, the efficiency of currently available dosage forms is often limited by their poor retention time at the vaginal site. Physiological changes and the self-cleansing action of the vaginal tract remain a challenge in formulation development.

The aim of this study was development and characterization of mucoadhesive liposomes capable to improve vaginal delivery of clotrimazole. Clotrimazole was incorporated in liposomes by the mechanical dispersion method and the liposomal suspension sonicated to desired vesicle size. Liposomes were characterized for their size, polydispersity and clotrimazole entrapment. Polymer-coating was used to improve the mucoadhesive properties, and chitosan was chosen as a coating material. Chitosan coating (0.1 and 0.6 %, w/v) was performed on liposomes free from untrapped clotrimazole. *In vitro* drug release from chitosan-coated liposomes was compared to release from non-coated liposomes and free clotrimazole. Chitosan-coated vesicles were able to prolong the release of entrapped clotrimazole to greater extent than non-coated liposomes. Cow vaginal mucosa was used as model mucosa in both penetration study and mucoadhesion testing and the preliminary data indicate that clotrimazole stays in vaginal tissue, rather than penetrating through the tissue. The experiments confirmed potential of chitosan-coated phospholipid vesicles in treatment of local vaginal diseases.

Key words: Clotrimazole, mucoadhesive liposomes, chitosan, vaginal drug delivery.

CONTENTS

1. GENERAL INTRODUCTION	1
2. INTRODUCTION.....	3
2.1 Vagina	3
2.2 Vaginal drug delivery.....	4
2.2.1 Vaginal dosage forms and delivery systems	6
2.3 Mucoadhesion	7
2.3.1 Theories of mucoadhesion	8
2.3.2 Factors affecting mucoadhesion.....	9
2.3.3 Mucoadhesive polymers.....	10
2.3.4 Mucoadhesive dosage forms and systems for vaginal administration	11
2.4 Hydrogels	11
2.4.1 Chitosan.....	12
2.5 Liposomes as drug delivery system	13
2.5.1 Introduction	13
2.5.2 Constituents and basic properties	14
2.5.3 Preparation of liposomes	15
2.5.4 Potential of liposomes in drug delivery.....	16
2.5.5 Mucoadhesive liposomes	16
2.6 Vaginal infections	17
2.6.1 Treatment of candidiasis	19
2.7 Clotrimazole	20
2.7.1 General	20
2.7.2 Mechanism of action	20
3. AIMS OF THE STUDY.....	23
4. MATERIALS AND METHODS	25
4.1 Materials.....	25
4.2. Tissues.....	25
4.3 Instruments	26
4.4 Computer programs.....	26
4.5 Methods.....	26
4.5.1 Preparation of liposomes with clotrimazole.....	26
4.5.2 Vesicle size reduction.....	27
4.5.3 Particle size analysis.....	27

4.5.4 Entrapment efficiency determination	27
4.5.5 Calibration curve	28
4.5.6 Coating of liposomes.....	28
4.5.7 Preparation of acetate buffer	28
4.5.8 In vitro drug release study	28
4.5.9 Preparation of vaginal tissue	29
4.5.10 Preparation of phosphate buffer	29
4.5.11 Ex vivo penetration study.....	29
4.5.12 Preparation of vaginal fluid simulant	30
4.5.13 Mucoadhesion testing.....	30
5. RESULTS AND DISCUSSION	31
5.1 Liposome characterization	31
5.1.1 Particle size analysis of uncoated vesicles	31
5.1.2 Clotrimazole entrapment efficiency	33
5.2 Coating of liposomes.....	36
5.2.1 Size characteristics	36
5.2.2 Clotrimazole entrapment in coated liposomes	38
5.3 In vitro clotrimazole release	38
5.4 Ex vivo penetration	41
5.5 Preliminary mucoadhesion testing	42
6. CONCLUSION	45
7. PERSPECTIVES.....	47
8. REFERENCES.....	49

LIST OF FIGURES

Figure 1: Schematic drawing of the vaginal mucosa. 1: capillary vessels; 2: artery; 3: vein (das Neves and Bahia, 2006)

Figure 2: Contact and consolidation stages of mucosal adhesion (Khutoryanskiy, 2011)

Figure 3: Structure of chitosan (Pharmaceutical Excipients)

Figure 4: Liposomal structure (<http://morgengrewreise.de/dubai/liposome-bilayer>)

Figure 5: Structure of phosphatidylcholine

Figure 6: Structure of clotrimazole (Ph Eur)

Figure 7: Mechanism of action of azoles (fluconazole)

(<http://depts.washington.edu/hiv aids/oral/case4/discussion.html>)

Figure 8: Mean diameter and standard deviation of sonicated liposomes

Figure 9: Calibration curve of clotrimazole in methanol

Figure 10: Clotrimazole entrapment efficiency

Figure 11: *In vitro* clotrimazole release

Figure 12: *In vitro* clotrimazole release from coated (0.6 %, w/v) liposomes

LIST OF TABLES

Table 1: Representative particle size distribution – sonicated samples

Table 2: Particle size distribution – before and after ultracentrifugation

Table 3: The effect of chitosan coating on particle size distribution

1. GENERAL INTRODUCTION

Vaginal delivery as a route of drug administration is currently of a great interest to scientists and pharmaceutical industry (Hussain and Ahsan, 2005; Friend, 2011). The main advantages of vaginal drug delivery compared to conventional routes of administrations are the avoidance of the hepatic first-pass metabolism and relatively high permeability for many drugs. In addition to other important factors, such as large surface area, rich blood supply and reduced side effects, the vagina provides a potential as a site for both local and systemic therapy (Vermani and Garg, 2000; Acartürk, 2009). However, conventional vaginal dosage forms, such as creams, foams, pessaries and jellies, are considered to reside at the targeted site for a relatively short period of time due to the self-cleansing action of the vaginal tract resulting in reduced therapeutic outcome (Pavelić *et al.*, 1999). Hence, there is a need to develop effective drug delivery systems that should prolong the contact of the drug with mucosal surface and enable sustained release of incorporated drug, leading to improved drug therapy (Pavelić *et al.*, 2005).

Extended residence at the targeted administration site is of interest for both local treatment, as well as for systemic drug bioavailability when applicable (Lee *et al.*, 2000). The role and potential of mucoadhesion in drug delivery as mean for prolonged residence time for many drug formulations, has been of interest in pharmaceutical technology since the early 1980's (Boddupalli *et al.*, 2010). Mucoadhesion is commonly defined as the adhesion between two materials, of which at least one is a mucosal surface, in this case vaginal mucosa (Shaikh *et al.*, 2011).

Several factors may influence mucoadhesion, and in the case of polymers used in delivery system development, molecular weight, pH, charge, hydrogen bonding capacity and concentration of polymer were reported to be dominant (Boddupalli *et al.*, 2010; Shaikh *et al.*, 2011).

The use of mucoadhesive polymers in drug delivery represents several advantages. Main advantages regarding vaginal administration are the possibility of prolonged *in situ* residence and intimate contact with mucus, hence enhanced and prolonged drug delivery (das Neves *et al.*, 2011). In order to ensure controlled drug delivery, drug carrier system with potential to

control release of associated drug, represent an additional advantage in improved drug therapy.

Liposomes have been widely used as drug carriers in topical treatments of local diseases, especially in dermatology. They are capable of incorporating a variety of both hydrophilic and hydrophobic drugs, and, due to their ability to perform sustained and/or controlled release of entrapped drug, are considered to be suitable for vaginal application as well (Pavelić *et al.*, 2005). However, a major limitation when using liposomes topically is their liquid form, resulting in limited retention time at the administration site. This can be overcome by their incorporation in an adequate vehicle in which the original structure of vesicles is preserved (Pavelić *et al.*, 2001). Another approach to prolong residence time of liposomal formulation would be to modify liposomal surface through coating with mucoadhesive polymers. The choice of mucoadhesive polymer used as coating material will depend on the desired polymer properties (Karn *et al.*, 2011). One of the most suitable polymers, in respect to safety and mucoadhesiveness, is chitosan. Chitosan, a well-known natural-origin mucoadhesive polymer, is recommended for the vaginal administration of drugs as a stable and suitable vehicle (Baloglu *et al.*, 2009). We have used this polymer in current research as selected coating material in an attempt to optimize mucoadhesive delivery system.

For years, antifungal and antibacterial agents have been administered vaginally to treat yeast and bacterial infections, respectively (Friend, 2011). Especially interesting is *Candida*, a common cause of vaginitis. When properly diagnosed, uncomplicated candidiasis may be treated reliably with a number of azole anticandidal drugs, including short-course regimens (Eschenbach, 2004). Among them, clotrimazole is known to be very effective locally without major side effects (Kast *et al.*, 2002). Clotrimazole ($C_{22}H_{17}ClN_2$) is an imidazole derivative generally used for the treatment of vaginal yeast infections (Kast *et al.*, 2002). Although clotrimazole-containing products are available on the market, the therapeutic outcome for those products is very often limited and patient compliance reduced, leading to repeated treatments. Clotrimazole is a lipophilic molecule with limited solubility; therefore, incorporation of clotrimazole in liposomes would result in its solubilization and potentially improved anticandidal action. Chitosan coating on liposomal surface is expected to provide prolonged residence time of the system at vaginal site.

2. INTRODUCTION

2.1 Vagina

The human vagina is a fibro muscular tube, with a length between 6 and 10 cm, extending from the cervix of the uterus to the exterior of the body. The vagina is slightly S-shaped and the tissue morphology varies with respect to the anatomical region (Hussain and Ahsan, 2005; Mallipeddi and Rohan, 2010). Histologically, the vaginal wall consists of three layers (Figure 1); the epithelial layer (stratified squamous epithelium resting on a *lamina propria*), the muscular coat and the *tunica adventia* (Justin-Temu *et al.*, 2004; Valenta, 2005).

The surface area of vagina is enlarged due to numerous folds in the mucosal layer, often referred to as rugae (das Neves and Bahia, 2006). In addition, rugae provide distensibility and support of the vaginal wall (Alexander *et al.*, 2004).

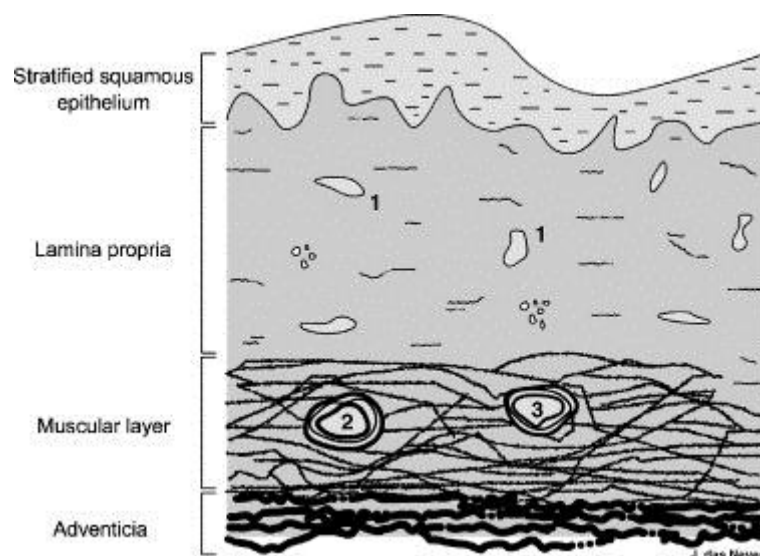


Figure 1: Schematic drawing of the vaginal mucosa. 1: capillary vessels; 2: artery; 3: vein (das Neves and Bahia, 2006)

The thickness of the vaginal epithelium is age-dependent. With hormonal activity the thickness increases, hence the epithelium is relatively thin before puberty, increasing after puberty and in post-menopause returning to a state similar to pre-puberty (Justin-Temu *et al.*, 2004; Valenta, 2005).

Arteries, blood vessels and lymphatic vessels are abundant in the vaginal walls. The blood leaving the vagina enters the peripheral circulation via a rich venous plexus which empties primarily into the internal iliac vein, thus absorbed drug avoids first-pass metabolism (Knuth *et al.*, 1993).

Although considered a mucosal tissue, vagina does not have secretory glands and lacks the direct release; a mixture of fluids originating from a number of different sources composes a moist surface film. The fluid is primarily transudate from vaginal and cervical cells (Alexander *et al.*, 2004; Valenta, 2005). This mucus coating has several important physiological functions and plays an important role in drug absorption (das Neves and Bahia, 2006).

The composition, volume and rheological properties of vaginal fluids are affected by age, the menstrual cycle or sexual arousal, thus influencing the drug release pattern of a vaginal delivery system (Hussain and Ahsan, 2005).

The pH of vagina in healthy, pre-menopausal women is 3.8 to 4.2. This naturally acidic environment is maintained by the action of bacteria, mainly *Lactobacillus*, converting glycogen into lactic acid (Alexander *et al.*, 2004). Hence, *Lactobacillus* plays an important role in the control of infection by common pathogens (das Neves and Bahia, 2006). The presence of semen, menstrual blood or disease, such as bacterial vaginitis, will increase the pH to levels closer to neutral range (Justin-Temu *et al.*, 2004; Mallipeddi and Rohan, 2010). Maintenance of low pH is necessary for the prevention of microbial growth and vaginal infections (Knuth *et al.*, 1993).

The different factors, in terms of microflora, pH and cyclic changes must be considered during the development and optimization of vaginal delivery systems (Acartürk, 2009).

[2.2 Vaginal drug delivery](#)

The vagina has been studied as a favorable administration site for drug delivery systems, in particular for drugs associated with female relevant diseases (Justin-Temu *et al.*, 2004). Traditionally, the vaginal route has been used for administration of locally acting drugs such

as antibacterial, antifungal, antiviral and spermicidal agents (Baloglu *et al.*, 2009). However, its large surface area, rich blood supply, avoidance of the first pass effect and relatively high permeability make vagina potentially attractive for systemic delivery as well (Acartürk, 2009).

Vaginal absorption of drugs have been characterized rather well (Benziger and Edelson, 1983); the absorption occurs in two main steps, namely drug dissolution in vaginal lumen followed by membrane penetration (Hussain and Ahsan, 2005). The vaginal membrane can be penetrated by the transcellular route, intracellular route or vesicular and receptor-mediated transport mechanisms. However, absorption of drugs targeted for local treatment is not desirable (Vermani and Garg, 2000). In parallel to the physiological properties, the physiochemical properties of drugs such as its molecular weight, lipophilicity and degree of ionization affect the absorption across the vaginal epithelium (Cicinelli, 2008).

The advantages of vaginal drug delivery, compared to other mucosal application sites, are summarized below (Vermani and Garg, 2000; Valenta, 2005; Baloglu *et al.*, 2009);

- Avoidance of hepatic first-pass metabolism.
- Reduction in the incidence and severity of gastrointestinal side effects.
- Reduction in hepatic side effects of steroids used in hormone replacement therapy or contraception.
- Overcoming inconvenience caused by pain, tissue damage and probable infection by other parenteral routes.
- Potential for self-insertion and removal of the dosage form.

Despite several advantages, systemic delivery via vaginal route has not been extensively explored due to gender specificity and cyclic variations (Valenta, 2005). Physiological factors such as cyclic changes in the thickness and porosity of the epithelium, volume, viscosity and pH of the vaginal fluid affect the drug absorption across the vaginal epithelium (Cicinelli, 2008).

The thickness of epithelium layer may affect the permeability, which signifies potential enhanced absorption in post-menopausal women (Justin-Temu *et al.*, 2004). On the contrary, changes in the hormone levels during the menstrual cycle increase the thickness of the epithelium resulting in decreased absorption (Cicinelli, 2008).

Furthermore, the change in volume, viscosity and pH of the vaginal fluid may influence the efficacy of drug delivery systems. An increased fluid volume may enhance the absorption of poorly water-soluble drugs. However, due to the self-cleansing action of the vaginal tract, excessive fluid volume may remove the drug and subsequently reduce absorption (Pavelić *et al.*, 1999; Hussain and Ahsan, 2005).

Additionally, the variations in enzyme activity due to hormonal changes further complicate the challenge of achieving consistent drug delivery (Valenta, 2005).

2.2.1 Vaginal dosage forms and delivery systems

Vaginal drug delivery systems include a large variety of pharmaceutical dosage forms such as solutions (foams, douches), aerosols, semisolids (cream, ointments, gels), tampons, tablets, capsules, pessaries, suppositories, vaginal films, intravaginal rings, sponges and powders (Justin-Temu *et al.*, 2004; das Neves and Bahia, 2006).

Ideally, vaginal delivery system should be easy to use, discreet, of reversible application, painless to the patient, cost-effective, non-toxic, non-irritating to the mucus membrane, widely available and safe for continuous administration (Hussain and Ahsan, 2005; das Neves and Bahia, 2006).

Creams, ointments, and gels are the most frequent used semisolid preparations for vaginal drug delivery (Vermani and Garg, 2000). Semisolids are affordable and easy to use, on the other hand related messiness, discomfort and leakage remain to be challenges with these preparations (Hussain and Ahsan, 2005; das Neves and Bahia, 2006). Moreover, the efficiency of currently available dosage forms is often limited by their poor retention time at the site of action due to the self-cleansing action of the vaginal tract (das Neves and Bahia, 2006).

It is generally accepted that the use of mucoadhesive polymers, either as vehicle for delivery systems or as surface modifying material for various types of particles, can prolong residence time at vaginal site.

2.3 Mucoadhesion

Mucoadhesion may be defined as a state in which a material binds to a mucosal tissue upon intimate contact and is held together for extended periods of time by interfacial forces (das Neves *et al.*, 2011). This property has been widely used to develop polymeric dosage forms for buccal, oral, nasal, ocular and vaginal drug delivery (Khutoryanskiy, 2011). Use of mucoadhesiveness in drug delivery shows several advantages like prolonged residence time, improved drug bioavailability, reduced administration frequency, simplified administration and the possibility of targeting and avoidance of first-pass metabolism (Khutoryanskiy, 2011; Shaikh *et al.*, 2011).

The mucoadhesion is generally divided into two steps (Figure 2); the contact stage and the consolidation stage (Boddupalli *et al.*, 2010). During the first stage, an intimate contact occurs between the mucoadhesive material and mucus membrane. In stage two, various physicochemical interactions occurs leading to strengthened connection, resulting in prolonged adhesion (Smart, 2005).

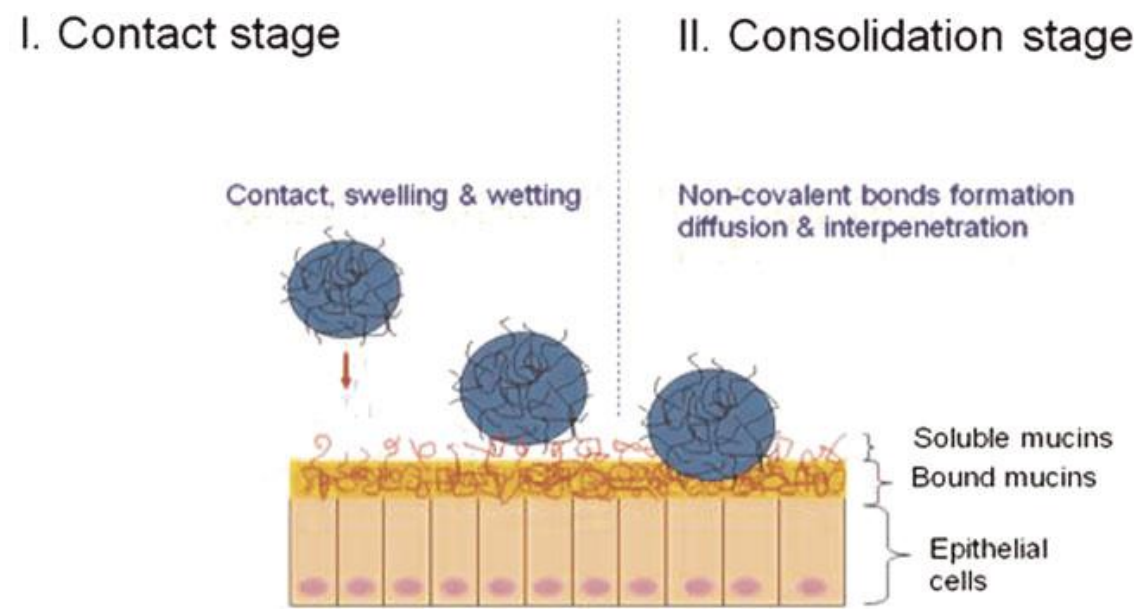


Figure 2: Contact and consolidation stages of mucosal adhesion (Khutoryanskiy, 2011)

2.3.1 Theories of mucoadhesion

Although mucoadhesion phenomenon is widely used in development of various novel drug delivery systems, mucohesion is a complex event, not yet fully understood (Khutoryanskiy, 2011). Various theories have been proposed to explain this phenomenon, and several general theories have been distinguished (Lee *et al.*, 2000; Edsman and Hägerström, 2005; Boddupalli *et al.*, 2010; Hombach and Bernkop-Schnürch, 2010; Shaikh *et al.*, 2011);

- Wetting theory – applies for liquid or low-viscosity systems. It considers the ability of a liquid to spread over a mucosal surface and explains the importance of contact angle and the energy needed to separate the two phases.
- Electrostatic theory – adhesion as result of electron transfer between mucus and the mucoadhesive system (given the mucus and the mucoadhesive polymer have different electronic characteristics). This results in the formation of an electrical double layer at the interface. Attractive forces within this double layer maintain contact between the two layers.
- Diffusion theory – interpenetration of glycoprotein mucin chain to sufficient depth, to form a semipermanent adhesive bond.
- Adsorption theory – adhesion to mucosa due to covalent bonds and/or van der Waals forces.
- The fracture theory – describes the force required for detachment of two surfaces after adhesion is established. It is regarded as being equal to the adhesive strength.
- The mechanical theory – considers the effect of irregularities on a rough surface.

Unfortunately, each of the proposed theories can only explain a limited number of interactions that constitute the bioadhesive bond. Mucoadhesion is probably achieved through a combination of several mechanisms (das Neves *et al.*, 2011; Khutoryanskiy, 2011).

2.3.2 Factors affecting mucoadhesion

The adhesiveness of a bioadhesive polymer is affected by its substantial polymer properties and environment-related factors (Lee *et al.*, 2000).

Bioadhesive polymer-related properties can be summarized as:

- Molecular weight – an optimum molecular weight for mucoadhesion have been proposed (Smart, 2005). Ideally, the chains should be small enough to allow easy interpenetration but still large enough for entanglements to occur (Edsman and Hägerström, 2005). However, the type of polymer is also important (Shaikh *et al.*, 2011).
- Concentration – an optimum concentration of polymer to produce the best mucoadhesion has been reported for several polymers (Lee *et al.*, 2000; Shaikh *et al.*, 2011).
- Chain flexibility – critical for interpenetration and entanglement. Cross-linked polymers show less mobility, therefore reduced desired entanglement with mucus, thus decreased mucoadhesion (Hombach and Bernkop-Schnürch, 2010).
- Particle size of mucoadhesive vesicular systems – influences the mucoadhesion, (Edsman and Hägerström, 2005).

Environment-related factors:

- pH - can influence the formal charge on the surface of mucus, due to the amino acid backbone; important for certain ionizable bio-adhesive polymers, for which the dissociation of the functional groups of the polymer is affected by pH (Lee *et al.*, 2000; Hombach and Bernkop-Schnürch, 2010).
- Initial contact time – between the bioadhesive and mucus layer. The strength of adhesion changes with the duration of initial contact time (Smart, 2005).
- Swelling characteristics – are related to the bioadhesive itself and its environment, depending on the polymer concentration, ionic strength and the presence of water (Lee *et al.*, 2000).
- Physiological variables – such as mucin properties, mucin turnover and disease states (Lee *et al.*, 2000).

2.3.3 Mucoadhesive polymers

Mucoadhesives are synthetic or natural polymers that interact with mucosal surfaces (Justin-Temu *et al.*, 2004). The polymers commonly used in mucoadhesive drug delivery may be divided into three broad classes (Asane *et al.*, 2008; Andrews *et al.*, 2009);

- Polymers that become sticky when placed in aqueous media and owe their mucoadhesion to stickiness.
- Polymers that adhere through nonspecific, noncovalent interactions that is primarily electrostatic in nature (although hydrogen and hydrophobic bonding may be significant).
- Polymers that bind to specific receptor site on the cell surface.

All three types of polymers can be used for drug delivery (Asane *et al.*, 2008).

Moreover, they can be classified according to their surface charge; anionic, cationic, non-ionic or amphiphilic, respectively. Their surface charge is important for the mechanism of their adhesion (Hombach and Bernkop-Schnürch, 2010).

The first generation mucoadhesives, hydrophilic macromolecules containing numerous hydrogen bond forming groups, are the most widely investigated group of mucoadhesives. The presence of hydroxyl, carboxyl or amine groups on the molecules shows good ability to stick to mucosal membranes, and strong adhesive bonds can form. Typical examples are carbomers (hydrogen bonds), chitosan (ionic bonds), sodium alginate and cellulose derivatives (Lee *et al.*, 2000; Smart, 2005).

Mucosal epithelia which is attractive for mucoadhesive drug delivery systems include eyes, nose, lungs, upper gastrointestinal tract and vagina (Cone, 2009). Our project aimed at development of vaginal mucoadhesive systems and vagina is the focus of this overview. The vaginal route appears to be highly appropriate for local drug treatment or for use in contraception (das Neves and Bahia, 2006; Acartürk, 2009).

2.3.4 Mucoadhesive dosage forms and systems for vaginal administration

Mucoadhesives have been, up to now, formulated into several dosage forms, including tablets, films, patches, gels and ointments (Boddupalli *et al.*, 2010). Gels are one of the most commonly studied and used mucoadhesive formulations for vaginal drug delivery (Edsman and Hägerström, 2005; Acartürk, 2009). Gels are easily manufactured, comfortable on use and have the ability to spread onto mucosal surface providing an intimate contact with vaginal mucosa (Ahuja *et al.*, 1997; Bonferoni *et al.*, 2006; Acartürk, 2009). Moreover, mucoadhesive formulations can, if properly formulated, control the release rate of incorporated drug (de Araújo Pereira and Bruschi, 2011).

As mentioned earlier, the physiological changes in vagina are one of the main challenges in developing mucoadhesive systems for vaginal administration. Very often the changes will have dual effect, for example an increased amount of vaginal fluid will facilitate the interaction between mucus and mucoadhesive molecules, and, at the same time, the degree of consolidation and strength of bonding will be decreased (das Neves *et al.*, 2011).

2.4 Hydrogels

Hydrogels are a cross-linked network of hydrophilic polymers with a three-dimensional configuration (Peppas *et al.*, 2000; Hoare and Kohane, 2008). They are capable of absorbing large amounts of water or biological fluids due to the presence of hydrophilic groups such as hydroxyl (-OH), amino (-CONH- or -CONH₂-) and sulfonic acid (-SO₃H-) forming hydrogel structures (Hamidi *et al.*, 2008). Despite their high affinity for water, hydrogels are prevented from dissolving due to the chemical or physical bonds formed between the polymeric chains (Bhattarai *et al.*, 2010). Thus, they maintain their three-dimensional structure even after swelling. This property distinguishes hydrogels from gels, which are already swollen to equilibrium and dissolve when more fluid is added (Gupta *et al.*, 2002).

The classification of hydrogels can be into natural polymer hydrogels, synthetic hydrogels and a combination of these two (Hoffman, 2002). Based on the presence of immunogenic/pathogenic fragments, natural hydrogels may exhibit immunogenicity or

provoke inflammatory responses. However, they are usually non-toxic and biocompatible (Hamidi *et al.*, 2008).

Hydrogels are one of the upcoming classes of polymer-based controlled-release drug delivery systems (Gupta *et al.*, 2002). Their hydrophilicity and potential biocompatibility makes them suitable as biomaterials. A property that makes hydrogels well suited as drug delivery vehicles is the mucoadhesive characteristics of many polymers used in hydrogel preparations. This adhesive property and the biocompatibility makes hydrogels suitable for vaginal administration (Bhattarai *et al.*, 2010).

However, the same polymer used to form hydrogels, can be used to modify liposomal surface through coating. This approach has been applied in our project.

A very important parameter in development of mucoadhesive delivery systems is the choice of polymer used for either coating or hydrogel formation. Among several potentially suitable polymers, we have focused on chitosan.

2.4.1 Chitosan

Chitosan is a natural cationic polysaccharide obtained from deacetylation of chitin (Filipović-Grčić *et al.*, 2001). It is the most extensively investigated, and the most abundant polysaccharide next to cellulose (Andrews *et al.*, 2009). Its common sources are the protective shells of crabs and shrimps (Bhattarai *et al.*, 2010).

Chitosan polymers consist of glucosamine and N-acetylglucosamine units (Figure 3). The presence of nitrogen and cationic groups distinguish chitosan from other available polysaccharides (Bhattarai *et al.*, 2010).

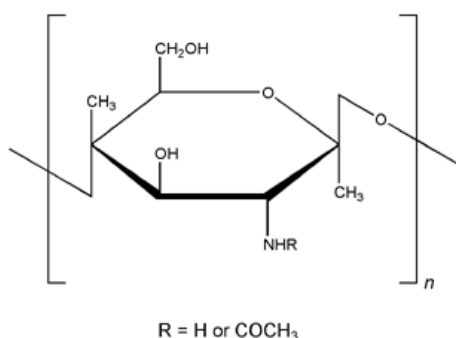


Figure 3: Structure of chitosan (Pharmaceutical Excipients)

Chitosan is sensitive to enzymatic degradation and does not induce an immune response (Bhattarai *et al.*, 2010). Moreover, electrostatic interactions between the cationic chitosan and the negatively charged mucosal surface result in remarkable mucoadhesive properties (Andrews *et al.*, 2009; Karn *et al.*, 2011).

The very low toxicity, good biocompatibility, and antimicrobial and mucoadhesive properties make chitosan suitable as a biomaterial for drug delivery systems (Perioli *et al.*, 2009; Karn *et al.*, 2011). Additionally, chitosan itself may provide controlled release of incorporated drugs (Kast *et al.*, 2002). Use of chitosan within pharmaceutical applications is versatile, and it is widely utilized in various formulations like powders, tablets, capsules, films, emulsions and gels (Kast *et al.*, 2002; Bhattarai *et al.*, 2010).

In addition, its intrinsic antimicrobial activity can present an advantage when treating mycotic infections in the oral cavity or the vagina (Kast *et al.*, 2002).

2.5 Liposomes as drug delivery system

2.5.1 Introduction

When phospholipids are placed in contact with water in aqueous medium, liposomes are formed spontaneously. The lipid head groups of phospholipids interact with water, forming a closed structure with an inner aqueous environment bounded by a membrane composed of lipid molecules. These spherical vesicles (Figure 4) are defined as liposomes (New, 1990; Vemuri and Rhodes, 1995; Samad *et al.*, 2007).

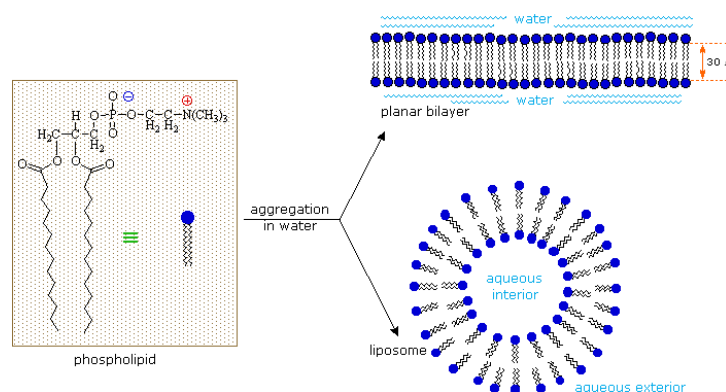


Figure 4: Liposomal structure (<http://morgencrewreise.de/dubai/liposome-bilayer>)

Liposomes can be prepared from a variety of natural and synthetic phospholipids (Lian and Ho, 2001), and be formulated to entrap various drugs both within their aqueous compartment and within the bilayers (New, 1990).

When prepared from natural lipids, the vesicle membrane forms a bilayer structure that resembles biological membranes (Goldberg and Klein, 2011).

2.5.2 Constituents and basic properties

Phospholipids are the major structural components of most biological membranes, the most common among them being phosphatidylcholine (Vemuri and Rhodes, 1995). Phosphatidylcholine (Figure 5), also known as lecithin, can be derived from both natural and synthetic sources (New, 1990), and is the most commonly used phospholipid for liposomes preparation (Ranade, 1989).

Lecithin is occurring in animal (bovine heart and spinal cord) and plants (egg yolk and soya beans) (New, 1990). Lipoid S100, which was used in this study, is lecithin from soybean containing more than 94 % phosphatidylcholine and is considered to be one of the purest lipids on the market.

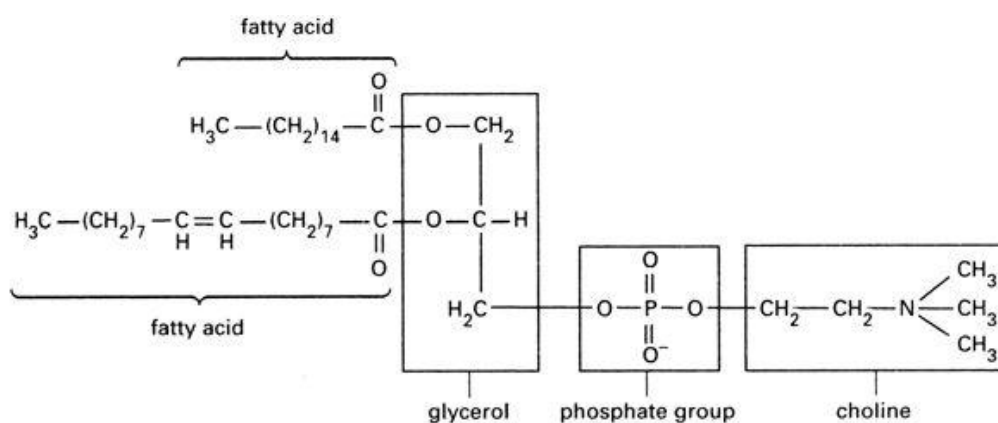


Figure 5: Structure of phosphatidylcholine

Liposome membranes are semipermeable, thus the diffusion rate of molecules across the membrane varies (New, 1990). Depending on number of carbon atoms and degree of unsaturation in the hydrophobic tail, the characteristic of the phospholipid change. Cholesterol may be added to improve the fluidity of the bilayer membrane, thus influencing the stability and permeability of the vesicle membrane (Vemuri and Rhodes, 1995).

The surface charge of the hydrophilic head affects the kinetics and biodistribution of liposomes (Vemuri and Rhodes, 1995).

Apart from their chemical constituents, which determine such properties as membrane fluidity, charge and permeability, liposomes can be characterized by their size and shape (New, 1990).

These liposomal properties are of clinical relevance and influence their performance in biological systems (Banerjee, 2001). Most of liposome formulations approved for human use contain phosphatidylcholine (Lian and Ho, 2001).

2.5.3 Preparation of liposomes

The physical structure of liposomes will be affected by the method of preparation (Vemuri and Rhodes, 1995). Several procedures have been developed and are classified as follows (Samad *et al.*, 2007);

- Mechanical methods.
- Methods based on replacement of organic solvent.
- Methods based on size transformations or fusion of prepared vesicle.

The original method of Bangham *et al.* (Bangham *et al.*, 1965), also referred to as the film method, is considered to be the simplest procedure. However, it has limitations such as low encapsulation efficiency for hydrophilic drugs and limitation for scale up as producing larger batch sizes is not readily feasible (Vemuri and Rhodes, 1995; Samad *et al.*, 2007).

This technique involves hydrating lipids in an organic solvent which then is removed by film deposition under vacuum. When all solvent is removed, the lipid film is dispersed in an aqueous medium followed by handshaking, causing the lipids to swell and form liposomes in size order of microns (New, 1990; Samad *et al.*, 2007).

2.5.4 Potential of liposomes in drug delivery

The lipid bilayer of liposomes has the ability to fuse with other bilayers, such as the cell membrane, thus discharging the liposome content. Drug molecules can be incorporated and/or encapsulated in the phospholipid vesicles, thus enabling drug delivery past cell membranes (Sharma, 2009).

Several properties make liposomes attractive as drug delivery systems and are summarized below (Torchilin, 2005):

- Biocompatibility.
- Ability to entrap both hydrophilic and hydrophobic pharmaceutical agents in the inner aqueous compartment and within the membrane, respectively.
- Ability to provide protection from external conditions that may inactivate incorporated pharmaceuticals, without causing unwanted side reactions.
- Property modification, in the sense of vesicle size, charge and surface properties, can easily be achieved by modification of composition or preparation method. Possibility for modification of their surface through coupling, coating or adherence of various types of molecules, leads to changed biological performance.

The major advantage concerning liposomal vaginal application is the ability to serve as carrier system for various types of drugs, regardless of drugs solubility. In parallel, the ability to provide sustained and/or controlled release of entrapped drug.

However, the liquid nature of the liposomal preparation is a challenge due to its limited retention time at the administration site. In order to assure suitable viscosity and desired formulation properties, liposomes can be incorporated in an adequate vehicle preserving the original structure of vesicles (Pavelić *et al.*, 2001; Patel and Patel, 2009).

2.5.5 Mucoadhesive liposomes

To overcome the limitation of liposomal liquid nature, resulting in their short residence time at the site of administration, inclusion of potentially mucoadhesive entities in their composition would be favorable (Chowdary and Srinivasa Rao, 2004). Coupling of mucoadhesive entities to liposomes provides additional advantages such as efficient drug

absorption and enhanced drug bioavailability due to a high contact surface to volume ratio, and much more intimate contact with the mucus layer. Moreover, specific targeting of drugs to the absorption site, whether vagina, buccal cavity, or intestinal mucosa, can be achieved by anchoring entities such as for example plant lectins to the surface of liposomes (Vasir *et al.*, 2003; Chowdary and Srinivasa Rao, 2004).

Mucoadhesive liposomes can be easily prepared by coating the liposomal surface with various types of polymers. Polymers with mucoadhesive properties, such as chitosan, Carbopol and Eudragit are among the most frequently studied (Karn *et al.*, 2011). Especially chitosan and modified chitosan have been used as polymer of choice to increase delivery system's stability in respect to drug release and for targeting purposes (Rengel *et al.*, 2002; Mehanna *et al.*, 2010). The mucoadhesiveness of chitosan-coated liposomes was confirmed in both *in vitro* and *in vivo* studies on rat intestines (Takeuchi *et al.*, 1996).

An appropriate combination of liposomal and chitosan characteristics may lead to development of liposomes with specific, prolonged and controlled drug release properties (Karn *et al.*, 2011). Chitosan provides an additional advantage of being safe for human use and is one of the most commonly studied polymer in drug delivery systems development (Bhattarai *et al.*, 2010).

In order to optimize mucoadhesive properties of chitosan-coated delivery systems, suitable mucoadhesive models have been proposed. The models rely on use of *ex vivo* animal tissue (Takeuchi *et al.*, 2005b).

We have focused on optimizing mucoadhesive liposomal system destined for vagina as site of administration. Clotrimazole was used as model vaginal antimicrobial.

2.6 Vaginal infections

Any condition causing an inflammation of the vagina is generally referred to as vaginitis, one of the most frequent gynecological diseases. Candidiasis and bacterial vaginosis are the two most common causes of vaginitis (Merabet *et al.*, 2005; Pavelić *et al.*, 2005). Also

trichomoniasis is a known cause of vaginitis, however, trichomoniasis is on the decline worldwide (Merabet *et al.*, 2005).

Vaginitis is one of the most frequent reasons for women visiting physicians (Eschenbach, 2004). As it is rarely considered to be a serious condition, it is consequently immensely understudied and poorly understood (Eschenbach, 2004) As bacterial vaginosis is not associated with vaginal inflammation, the term “vaginosis” is appropriate to be used instead of “vaginitis” (Mashburn, 2006).

Normally, vagina is colonized with lactobacilli. These lactobacilli produce hydrogen peroxide and convert glycogen to lactic acid, sustaining a low vaginal pH (Marrazzo, 2011). This acidic environment creates a hostile environment for other bacteria and common pathogens (das Neves and Bahia, 2006). In bacterial vaginosis, the normal vaginal flora is disturbed by a reduction in number of lactobacilli (Sobel, 1997; Owen and Clenney, 2004). The increase in pH favors commensal anaerobes, resulting in development of bacterial vaginosis. *Gardnerella vaginalis* and *Mycoplasma hominis* are the predominant organisms to cause bacterial vaginosis (Sobel, 1997).

The clinical presentation of bacterial vaginosis is variable. However, abnormal and increased vaginal discharge is the most common symptoms. The amount and viscosity of vaginal discharge can vary during the menstrual cycle and in relation to stress, and is easily mistaken for abnormal discharge. In addition to unusual vaginal discharge related to vaginal infections, discomfort and irritation or urinary symptoms such as dysuria or increased frequency may occur and are better indicators of vaginal discharge (Carr *et al.*, 1998).

As approximately 50 % of patients are asymptomatic, the prevalence of bacterial vaginosis is fairly underestimated (Carr *et al.*, 1998). Why some women develop symptoms and others do not, is still not fully understood (Marrazzo, 2011).

Local administration of metronidazole is the recommendable treatment of bacterial vaginosis (Pavelić *et al.*, 2005).

Candida-caused infections are normally caused by *Candida albicans*, a pathogenic fungus, yet a part of the normal vaginal microflora of reproductive aged women. Candida may cause infection when the normal microbial balance is disturbed or the host defenses are weakened

(Sobel *et al.*, 1998; Pavelić *et al.*, 2005). Up to 75 % of women will experience a vaginal candidiasis infection at least once in their lifetime (Kim and Sudbery, 2011).

The most commonly reported clinical symptoms are vulvar irritation or itching, vaginal burn, unusual appearance of vaginal discharge and dyspareunia (Bohbot *et al.*, 2011). However, 15-20 % are asymptomatic (Carr *et al.*, 1998).

Exact factors that generate the virulence of candida are somewhat unclear, yet different factors may increase perceptibility to vaginal yeast infection. For instance, pregnancy, use of oral contraceptives or broad-spectrum antibiotics and diabetes are confirmed to enhance the occurrence of candida infections (Carr *et al.*, 1998; Sobel, 2007).

Clotrimazole is known to be very effective in local treatment of candida infections (Kast *et al.*, 2002) and was the choice drug in this project.

2.6.1 Treatment of candidiasis

Topical azole antifungals can easily treat uncomplicated vaginal candidiasis, in single or short-time doses and show successful clinical cure rate in 80 – 90% of patients (Mashburn, 2006; Sobel, 2007). The treatment is reported to be well tolerated and notable safe (Sobel, 2007).

Several topical treatments are available over-the-counter, nevertheless many women prefer the convenience of prescribed oral treatment. A single dose of 150 mg fluconazole is the preferred oral therapy (Owen and Clenney, 2004).

Because vaginal candidiasis is not transmitted sexually, treatment of partners is not recommended (Mashburn, 2006).

Recurrent vaginal candidiasis is defined as four or more yeast infections during a 12 month period (Owen and Clenney, 2004; Mashburn, 2006). Factors that predispose for candidiasis should be considered and possibly eliminated, and treatment, either by oral or topical azole, should continue for at least 7-14 days (Sobel, 2007).

Use of efficient topical therapy is expected to reduce the need for oral therapy with antimicrobials, as side effects related to oral treatment are not negligible and rather common

2.7 Clotrimazole

2.7.1 General

Clotrimazole (C₂₂H₁₇ClN₂) (Figure 6) is a weak base that is freely soluble in methanol but practically insoluble in water (The Merck Index, 2011).

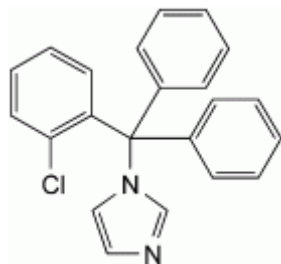


Figure 6: Structure of clotrimazole (Ph Eur)

It is an antimycotic imidazole derivative applied locally for the treatment of vaginal yeast infections and is formulated in conventional drug dosage forms such as creams, foams, tablets, gels or pessaries (Ceschel *et al.*, 2001).

Due to severe side effects, clotrimazole is unsuitable for oral use (Ning *et al.*, 2005a).

2.7.2 Mechanism of action

The azoles are a group of synthetic antifungal agents with a broad spectrum of activity based on the imidazole nucleus. Their mechanism of action (Figure 7) involves the inhibition of a fungal enzyme (14 α -demethylase) responsible for converting lanesterol to ergosterol, which is the main sterol in the fungal cell membrane. The subsequent reduction of ergosterol alters the fluidity of the membrane, interfering with the action of membrane-associated enzymes. The net effect is an inhibition of replication. Azoles also inhibit the transformation of candida yeast cells into hyphae – the invasive and pathogenic form of the parasite (Rang *et al.*, 2007).

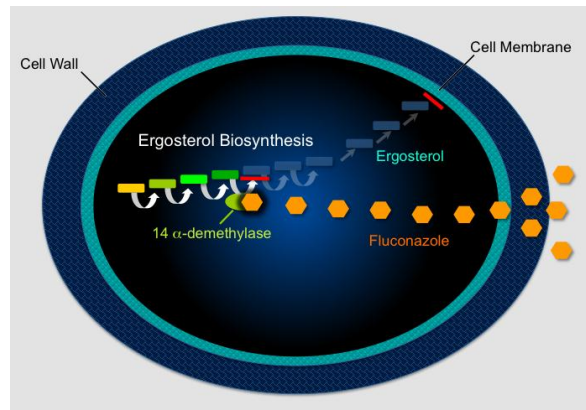


Figure 7: Mechanism of action of azoles (fluconazole)

(<http://depts.washington.edu/hiv aids/oral/case4/discussion.html>)

When treating uncomplicated vaginal candidiasis, clotrimazole for 7 days is an appropriate therapy (Carr *et al.*, 1998). Vaginal clotrimazole, in formulation of cream and vaginal tablets, are also available as single dose therapy. However, patients often report discomfort while in treatment and repeated treatment is often required (Owen and Clenney, 2004; Sobel, 2007).

3. AIMS OF THE STUDY

The main objective of this study was the development of chitosan-coated liposomes containing clotrimazole. Liposomes were expected to sustain clotrimazole release and assure prolonged residence time at vaginal site. The aim was divided in:

- Characterization of liposomes in respect to clotrimazole entrapment efficiency, size distribution and mucoadhesiveness.
- *In vitro* clotrimazole release testing in order to confirm sustained release properties.
- Evaluation of drug penetration into and through animal vaginal tissue *ex vivo*.
- Testing of mucoadhesiveness on animal vaginal tissue in the presence of simulated vaginal fluid.

4. MATERIALS AND METHODS

4.1 Materials

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

Ammonium acetate, BHD Prolab, Leuven, Belgium

Bovine serum albumin, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany

Calcium hydroxide, NMD, Oslo, Norway

Chitosan, low molecule weight, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany

Clotrimazole, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany

Distilled water

Glucose, NMD, Oslo, Norway

Glycerol, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany

Lactic acid, NMD, Oslo, Norway

Lipoid S 100 (soybean lecithin, > 94 % phosphatidylcholine), Ludwigshafen, Germany

Methanol CROMASOLV®, Sigma-Aldrich, Chemie GmbH, Ludwigshafen, Germany

Methanol, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany

Potassium hydroxide, NMD, Oslo, Norway

Potassium phosphate, Merck KGaA, Darmstadt, Germany

Propylenglycol, NMD, Oslo, Norway

Sodium chloride, Fluka Analytical, St. Louis, USA

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

Sodium hydrogen phosphate, Merck KGaA, Darmstadt, Germany

Sodium hydroxide, NMD, Oslo, Norway

Ureum, NMD, Oslo, Norway

4.2. Tissues

Vaginal tissue from newly sacrificed cow was obtained through Mydland, Tromsø, from their slaughterhouse.

4.3 Instruments

Agilent 8453 UV-visible spectroscopy system, Agilent Technologies, Santa Clara, USA

Beckman model L8-70M preparative Ultracentrifuge, Beckman Instruments, Palo Alto, USA

Beckman SW 60 Ti rotor, Beckman Coulter, Beckman instruments, Palo Alto, USA

Branson 5510 E-MT, Branson ultrasonic Cleaner, Danbury, USA

Branson1510E-MT, Danbury, USA

Büchi rotavapor R-124, Büchi water bath B-480, Büchi vacuum controller B-721, Büchi Vac® V-500, Büchi labortechnik, Flawil, Switzerland

Jubalo heating circulator, Jubalo laboratechnik, F12-ED, Seclback, Germany

Perme Gear Ink, Diffusion cells and Systems, 12 ml, Hellertown, USA

Submicron particle sizer model 370, Nicomp, Santa Barbra, USA

Ultrasonic processor 500 watt, Sigma-Aldrich, St. Louis, USA

4.4 Computer programs

Nicomp Particle Sizing System, CW 388 version 1.68

UV-Visible ChemStation Software, Agilent technologies 95-03

4.5 Methods

4.5.1 Preparation of liposomes with clotrimazole

Clotrimazole (20 mg) and Lipoid S 100 (200 mg) were dissolved in methanol in a round bottom flask. The solvent was evaporated using rotoevaporator, for at least 1 hour at 50 mm Hg and 50 °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.

4.5.2 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 center of the beaker containing liposomal suspension. The sonicator was set to 40 % amplitude and the liposomes were exposed to ultrasonic irradiation for 1, 2 or 2 x 2 minutes, respectively. The sonicated liposomes were stored in the refrigerator for at least 6 hours prior to further use.

4.5.3 Particle size analysis

The particle size distributions of liposomes were determined by photon correlation spectroscopy on Nicomp model 370. 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. All analyses were run in vesicle mode and the intensity distribution. Three parallels were run of each sample; with a run time of 10 minutes.

4.5.4 Entrapment efficiency determination

To separate free drug from liposomally entrapped drug, the samples were ultracentrifuged for 30 minutes, at 10 °C and 85 000 g. The pellet was separated from the supernatant, re-suspended in 500 µl of distilled water and finally diluted to 2 ml with methanol. From both the supernatant and the pellet, aliquots of 100 and 200 µl were taken and diluted to a final volume of 2 ml with methanol. Drug content in both supernatant and pellet was determined spectroscopically as described below.

4.5.5 Calibration curve

Standard solutions were prepared by diluting the stock solution of clotrimazole in methanol (2 mg/ml). Solutions of 20, 50, 75, 100, 150, 200, 250 and 300 µg/ml were prepared and their absorbance was measured at 261 nm. Standard curve was prepared based on the spectrophotometrical determination. The measurements were performed in triplicates.

4.5.6 Coating of liposomes

The 0.1 and 0.6 % (w/v) chitosan solutions were prepared in 0.1 % (v/v) glacial acetic acid. The chitosan solution was added drop-wise to an equal volume of liposomes (1.5 ml), under controlled magnetic stirring at room temperature for 1 hour, followed by incubation in the refrigerator overnight. The rate of stirring was kept constant for all preparations (Karn *et al.*, 2011).

Due to difficulties to separate liposomally entrapped drug from free drug after coating, the coating was performed after ultracentrifugation. Hence, the liposomes were not coated in the presence of free drug.

4.5.7 Preparation of acetate buffer

Acetate buffer (pH 4.5) was prepared by dissolving 77.1 g/l CH₃COONH₄ in distilled water, 70 ml glacial acetic acid was added and the volume adjusted to 1000 ml with distilled water (Ph.Eur). Measured pH was 4.6.

4.5.8 In vitro drug release study

The Franz cell manual diffusion system was properly cleaned with methanol, demineralized water and distilled water, respectively, each cleaning procedure lasting for half an hour. The heating circulation was set to 37 °C. The reception chamber (12 ml) was filled up with the acetate buffer (pH 4.6) and the membrane used, cellophane, was pre-soaked in the same

buffer for half an hour before use. Samples (300 μ l) were added in the donor cells and the system was properly sealed. Samples of 750 μ l were collected after 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 hours (for the sample of free clotrimazole), and after 1, 2, 3, 4, 8 and 24 hours (for the samples of liposomes containing drug). An equal amount of buffer was added as replacement for each sample extracted. Drug content was determined spectroscopically. The experiments were performed in triplicates for free drug and in duplicates for the liposomally entrapped drug.

4.5.9 Preparation of vaginal tissue

The vaginal mucosa was carefully removed from the underlying tissue and cleaned with acetate buffer (pH 4.6). 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 hours prior to experiments.

4.5.10 Preparation of phosphate buffer

Phosphate buffer (pH 7.4) was composed of 8 g/l NaCl, 0.19 g/l KH_2PO_4 , and 2.38 g/l Na_2HPO_4 (Pavelić *et al.*, 2005). Its pH was adjusted to be 7.4 using 1 M NaOH.

4.5.11 *Ex vivo* penetration study

Before experiment, the defrosted vaginal tissue was cut to fit the Franz diffusion cells surface (1.77 cm^2).

The receptor chamber was filled up with phosphate buffer (pH 7.4, 12 ml), and the vaginal tissue fixed as membrane. Samples (600 μ l) were added in the donor cells and the system was properly sealed. Samples of 750 μ l were collected after 1, 2, 3, 4, 8 and 24 hours and replaced with an equal amount of buffer. Drug content was determined spectroscopically.

4.5.12 Preparation of vaginal fluid simulant

Vaginal fluid simulant (pH 4.5) was prepared from 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. pH was adjusted to 4.5 using 0.1 M HCl (Owen and Katz, 1999).

4.5.13 Mucoadhesion testing

Vaginal tissue was cut into desirable pieces (approximately 50 mm in diameter). An adequate amount of phosphate buffer (pH 7.4) was added to four beakers (10 ml). The prepared tissue was placed on the top of each beaker with a sunken area immersed into the buffer, and fixed to remain in the position.

Samples (500 µl) were placed on top of the tissue, and 100 µl of vaginal fluid simulant was added to each sample to mimic exposure to vaginal fluid. The preparations were covered with aluminum foil and left in room temperature. Samples of 100 µl were collected after 1, 2, 4 and 8 hours. When all samples were collected, the remaining sample mixture was transferred to a test tube, and the tissues were placed in beaker containing 5 ml of methanol. After 24 hours, aliquots from samples were collected and measured. Drug content was determined spectoscopically of all samples.

5. RESULTS AND DISCUSSION

5.1 Liposome characterization

5.1.1 Particle size analysis of uncoated vesicles

The vesicle size and size distributions were determined using NICOMP distribution, by using scattering-intensity-weighted evaluation of particles in vesicle mode. Particle size and size distribution of liposomes are important for liposomes as drug carriers in topical drug delivery. Most of the work so far has been focused on the effect of liposomal size on the efficacy of liposomal delivery to skin (Cevc, 2004). Relatively little is known about the effect of vesicle size on the delivery of drugs intended for mucosal targeting. Takeuchi *et al.* have found that the amount of liposomal particles 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). The vesicle particle size shown in Table 1 indicates that our liposomes sonicated for 4 minutes were in the desired size range.

Table 1: Representative particle size distribution – sonicated samples

Time of sonication (min)	Vesicle size				Polydispersity
	Peak 1 (nm)	Intensity (%)	Peak 2 (nm)	Intensity (%)	Index (PI)
1	317 ± 47	56.8	40 ± 5	41.0	0.58
2	234 ± 31	53.9	36 ± 5	43.8	0.46
4	111 ± 16	83.1	29 ± 4	16.9	0.46

The values denote the average of three cycles of determination ± SD.

The polydispersity index (PI, Table 1) is a measure of the width of unimodal size distributions (Verma *et al.*, 2003). An acceptable polydispersity index should have the value below 0.70. Most of our samples showed two distinguished peaks in particle distributions, indicating

bimodal distribution which results in rather high polydispersity (Table 1). Some liposomal samples sonicated for 1 or 2 minutes have exhibited trimodal distributions, but majority of liposomal formulations exhibited bimodal distribution, indicating that two distinguished populations of vesicles were observed. The polydispersity was rather high for all samples; however, with the increase in sonication time the polydispersity index values decreased (Table 1). Sonication as vesicle size reduction method is expected to result in rather high polydispersity of vesicles (New, 1990) as the sonication time, power and position of probe used in sonication affect the performance of whole process and bimodal distributions are rather common. We tried to use minimum time of exposure of liposomes to the sonication force, as extensive sonication is known to cause release of originally entrapped drug (di Cagno *et al.*, 2011). The desired vesicle size needs to be compromised with respect to the amount of drug associated within liposomes.

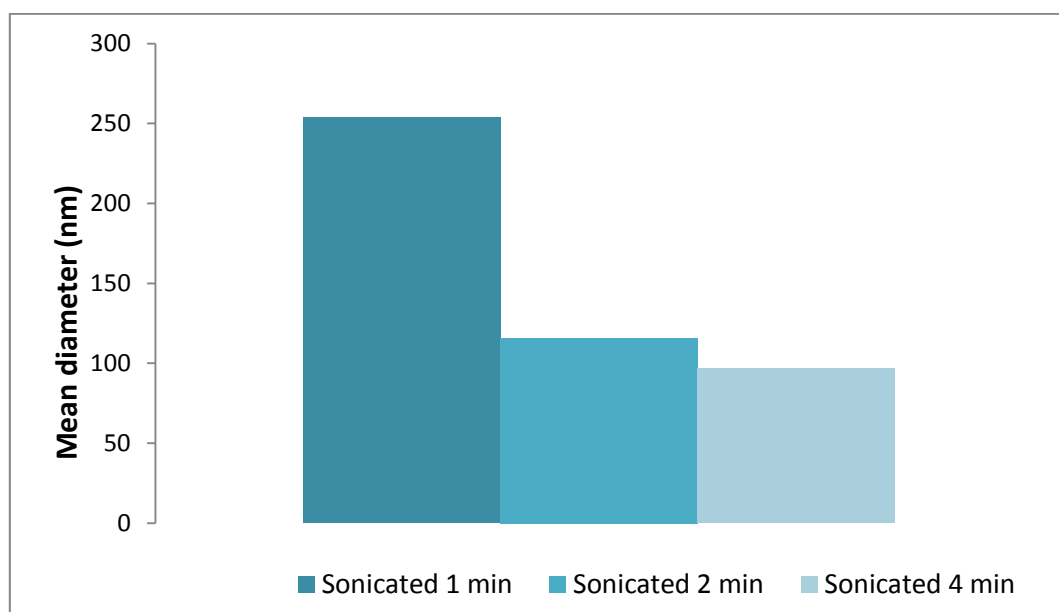


Figure 8: Mean diameter of sonicated liposomes

When a mean diameter was calculated in correlation to sonication time (Figure 8), it was evident that the increase in sonication time resulted in smaller vesicles, as expected. We have to make clear that mean diameter in this case is a rough estimate, as high polydispersity of the samples prevented us from using Gaussian distribution. The mean diameter was calculated based on the intensity percentage of vesicles grouped in two populations, for three separate formulations (Figure 8). However, as this is an estimate, standard deviations do not have real meaning and are therefore excluded.

5.1.2 Clotrimazole entrapment efficiency

The calibration curve for clotrimazole in methanol (Figure 9) was expressed as absorbance of standard clotrimazole at 261 nm versus corresponding concentrations of the standard clotrimazole.

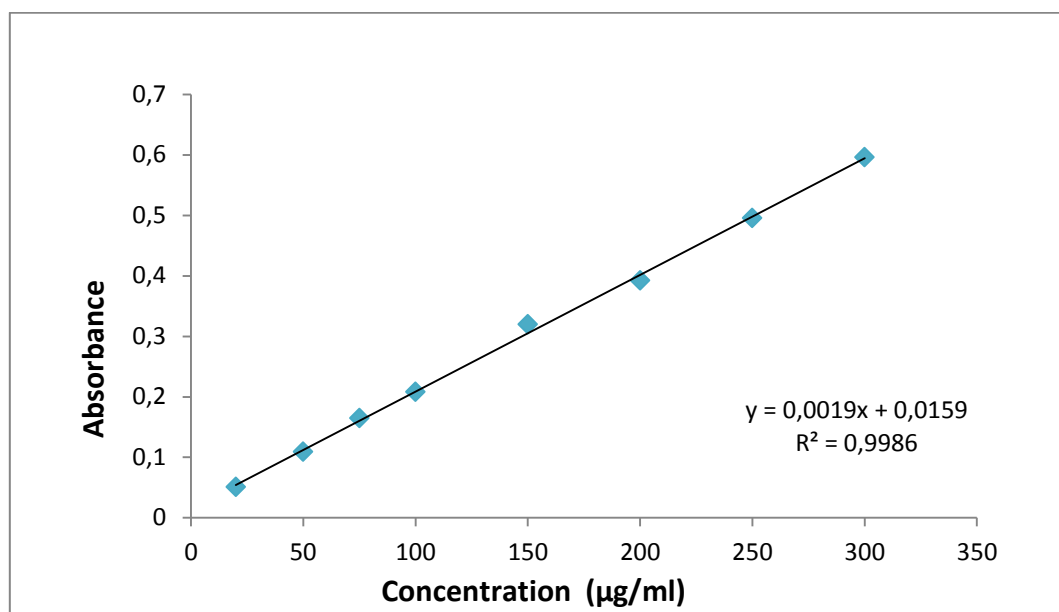


Figure 9: Calibration curve of clotrimazole in methanol

The concentration range of 20-300 µg/ml was found to result in linear relationship between drug concentration and absorbance. The correlation coefficient was determined to be 0.9986.

In order to achieve desired therapeutic effect, adequate entrapment efficiency is required. The efficacy of drug entrapment in liposomes will be affected by the physicochemical properties of the drug, especially its solubility (Mura *et al.*, 2007). Due to the lipophilic nature of the drug (logP of 3.5 (Wulff *et al.*, 2001)), clotrimazole was dissolved in the organic solvent together with lipid during the liposomal preparation and was expected to incorporate itself within lipid bilayers of liposomes.

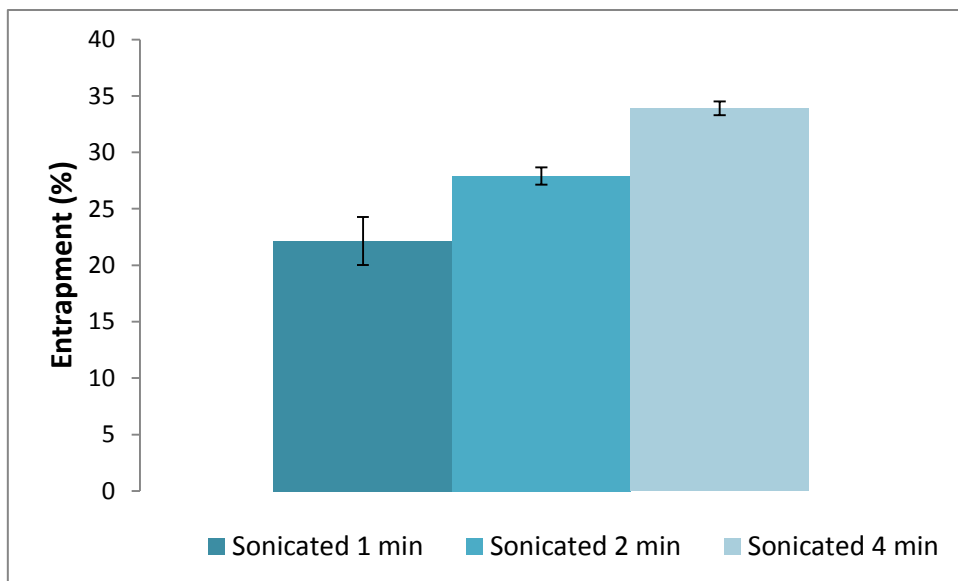


Figure 10: Clotrimazole entrapment efficiency

The values represent the mean of three separate experiments \pm SD.

Figure 10 indicates entrapment efficiencies in correlation to sonication time. It would be reasonable to expect that shorter sonication time results in higher drug entrapment values than longer sonication time as sonication is linked to loss of originally incorporated drug. The fact that we observed almost similar entrapment efficiencies (25-35 %) for liposomes sonicated for different time, or even higher entrapment for smaller liposomes, can be attributed to the limitation of ultracentrifugation as separation method. Namely very large liposomes, over 200 nm, will also precipitate down together with untrapped drug; therefore, the values for entrapment in larger liposomes seem to be underestimated. The particle size distributions before and after ultracentrifugation, shown in Table 2, confirm that the larger liposomes have precipitated.

Table 2: Particle size distribution – before and after ultracentrifugation

Sonication (min)	Ultra- centrifugation	Vesicle size				PI
		Peak 1	Intensity	Peak 2	Intensity	
		(nm)	(%)	(nm)	(%)	
2	Before	234 ± 31	53.9	36 ± 5	43.8	0.46
	After	72 ± 9	71.6	23 ± 3	28.4	0.31
4	Before	111 ± 16	83.1	29 ± 4	16.9	0.46
	After	79 ± 8	66.8	31 ± 3	33.2	0.32

Ultracentrifugation was performed at 85000 g for 30 minutes.

As we have focused on preparation of smaller vesicles, sonicated over longer period of time (2 and 4 minutes), we did not replace ultracentrifugation method by other methods of separations. We could have used gel filtration of Sepharose or Sephadex columns, but those methods would be more time consuming and expensive. Moreover, those methods could not be used to separate coated liposomes and we wanted to apply the same methods throughout of the project.

The entrapment efficiencies (Figure 10) were consistent and acceptable to proceed with coating. In comparison to literature data, our entrapment was found to be lower; however none of the published articles describes use of same liposome preparation method and same phospholipid composition. Although Ning *et al.* (2005a) reported a very high entrapment of clotrimazole (over 90 %) using the thin-film method, they used dialysis to separate untrapped drug from liposomal drug. The fact that clotrimazole is practically insoluble in water and that no data is available on whether or not the sink conditions were assured, 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 method and polyol dilution method were reported to have rather high entrapment of clotrimazole (Pavelić *et al.*, 1999). However, the methods of preparations were different and the vesicle size was bigger than in our case. Pavelić *et al.* (2005) also observed an entrapment efficiency of 64-71 % for larger liposomes. In their case gel chromatography was used to separate free from liposomally entrapped clotrimazole. Additional differences between their work and current work are that our liposomal composition included pure

phosphatidylcholine, whereas they used mixture of phosphatidylcholine and egg-phosphatidylglycerol-sodium.

As it is known that coating of liposomes results in increase in vesicle size (Karn *et al.*, 2011) we have accepted that the entrapment values were lower than desired, but that vesicle size was optimal for the purpose of development of mucoadhesive liposomal delivery system.

5.2 Coating of liposomes

5.2.1 Size characteristics

Coating of liposomes with various mucoadhesive polymers is expected to prolong their residence time once they come into the contact with mucosal tissues, whether in buccal, intestinal, colon or vaginal site. The choice of polymer used for coating of liposomes will be depended on the targeted mucosal site and drug characteristics (Karn *et al.*, 2011). We selected chitosan as model coating polymer, as it has excellent mucoadhesiveness and safety profile (Perioli *et al.*, 2009; Karn *et al.*, 2011).

There are several indicators which can be used to confirm that the coating was successful, such as increase in vesicle size, change in zeta potential on the surface of vesicles and similar (Karn *et al.*, 2011). One of the easiest and most commonly used indicators is the increase in original vesicle size, and we have used this parameter in formulation development.

Our experiments confirmed the expectations and the results in Table 3 can serve as a proof that coating of liposomes indeed took place.

Table 3: The effect of chitosan coating on particle size distribution

Sonication (min)	Coating (%, w/v)	Vesicle size				Polydispersity Index
		Peak 1 (nm)	Intensity (%)	Peak 2 (nm)	Intensity (%)	
2	-	87 ± 9	62.3	30 ± 3	37.2	0.34
	0.1	135 ± 24	41.3	54 ± 7	55.4	0.27
	0.6	148 ± 16	63.3	51 ± 5	35.8	0.25
4	-	91 ± 12	52.5	11 ± 1	47.5	0.32
	0.1	121 ± 13	49.2	31 ± 4	50.1	0.25
	0.6	180 ± 16	51.2	51 ± 5	47.4	0.29

The values denote the mean of three separate experiments, determined through three measurement cycles, ± SD.

Coating indeed increased the original size of vesicles and coating with higher concentration of chitosan resulted in formation of bigger vesicles (Table 3). Although coating of vesicles results in formation of bigger vesicles, they were more homogenous in size and the polydispersity of such populations was decreased. It might seem unexpected that coated vesicles, which are bigger in size, are more homogenous in size distribution than the uncoated vesicles, however, the results are explainable by specificity of the vesicle size distributions as determined by NICOMP size evaluation systems. Polydispersities of coated vesicles were found to be satisfactory, particularly for vesicles coated with 0.1 % (w/v) chitosan. We have not calculated the degrees of significance as the vesicle size presented is an estimate based on the intensity of the subpopulations of particles.

Considering the actual therapeutic potential of liposomally incorporated drug and its ability to penetrate the mucous layer, the size of our coated liposomes was corresponding well to the optimized size for coated liposomes proposed by Takeuchi et al. (2001). They suggested that nanoparticles in size range of 100 nm have greatest potential to penetrate deeper into mucosal layer and induce therapeutic response to the drug associated to nanoparticles.

The same group conducted several experiments on mucoadhesiveness of coated liposomes and confirmed that it is dependent on the particle size, and submicron-sized chitosan-coated liposomes were found to be more effective than the liposomes of the same compositions but of bigger size (Takeuchi *et al.*, 2005a). However, these results were obtained in rat intestine

after oral administration of liposomes containing fluorescent dye and entrapped drug was calcitonin.

5.2.2 Clotrimazole entrapment in coated liposomes

Chitosan-coating performed in the presence of liposomally untrapped drug may increase the entrapment efficiency for some drugs (Filipović-Grčić *et al.*, 2001; Karn *et al.*, 2011). When we have tried to perform the coating of liposomes in the presence of untrapped clotrimazole, we experienced serious problems in separating liposomes from drug, as centrifugation resulted in semi-gel formation in centrifugation tube. We tried to use gel chromatography as mean to separate vesicles from free drug, but due to the mucoadhesiveness of coated vesicles, the vesicles were stacked on the top of the gel column (data not shown).

Due to the low solubility of clotrimazole, we did not apply dialysis and decided to perform coating in the absence of untrapped clotrimazole.

We have checked whether we can still detect the entrapped clotrimazole in coated liposomes and determine the entrapment efficiency and the values were found to be in the exact range as determined for entrapment yield in non-coated liposomes, namely 34.4 ± 13.9 % (Figure 10). The only difference was in rather larger SD determined for coated liposomes as compared to uncoated.

5.3 In vitro clotrimazole release

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).

It is known that the pH value of the healthy human vagina ranges between 4.0 and 5.0 (Ning *et al.*, 2005a). Hence, an acetate buffer (pH 4.6) was chosen as receptor medium. Ning *et al.* (2005b) reported that the release rate for clotrimazole from proliposomes in acetate buffer (pH 4.5) was faster than release in phosphate buffer (pH 7.4). Due to the fact that clotrimazole is a weak base (pKa 4.7), this finding was as expected (Ning *et al.*, 2005c).

In order to confirm that the delay in release is indeed contributable to the effect of liposomes as carrier system, clotrimazole dissolved in propylene glycol (2 mg/ml) served as control.

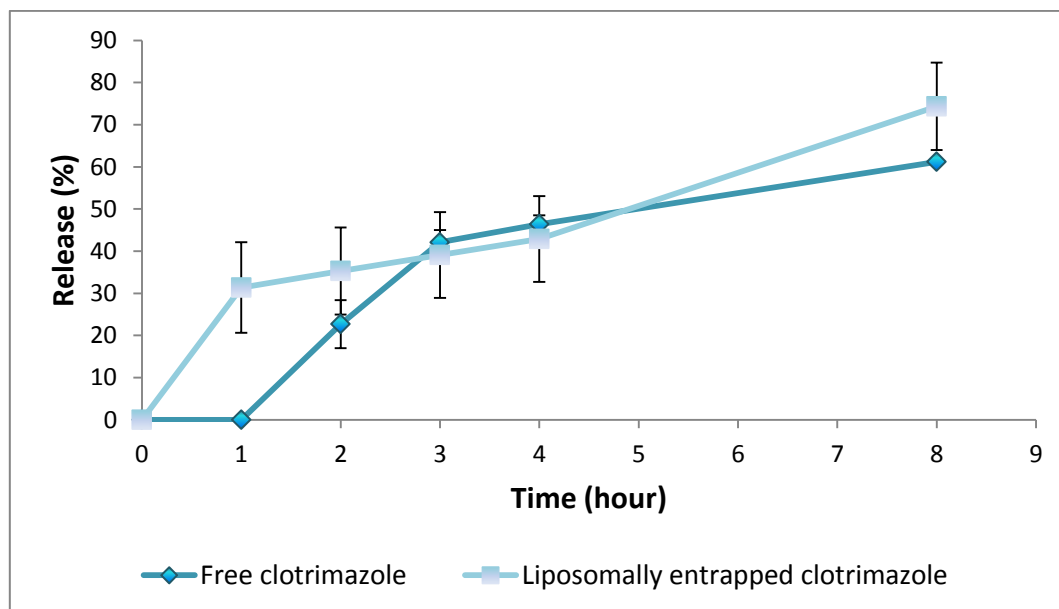


Figure 11: *In vitro* clotrimazole release

The values denote the mean of three separate experiments \pm SD.

The release rate of free clotrimazole (clotrimazole in propylene glycol) was slow in the first 1.5 h, but showed an increased release after 1.5 hours. However, the release flattened after 4 hours (Figure 11). Within 4 hours almost 50 % of drug is released. Compared with free clotrimazole, initially faster release of drug was observed from non-coated liposomes. This might seem surprising as liposomes are expected to delay the release of drug. However, once should take into the consideration that liposomes serve as solubilizers for clotrimazole and the first burst can be attributed to the presence of clotrimazole in out bilayers and its release. After 2 hours the release from liposomes slowed, indicating expected delay in release of drug. We have also taken the samples after 24 hours release studies but have found that the drug recovery was over 130 %. The drug recovery for *in vitro* release studies in first 8 hours was between 87 and 96 %. Another reason for focusing our experimental set up on first 8 hours was the fact that vaginal discharge and urination, menstruation or presence of semen is known to assist in fast clearance of formulation from vaginal site (das Neves *et al.*, 2011).

The total amount and rate of drug release determined in our experiments corresponds well to the findings by Pavelić *et al.* (2005) and Ning *et al.*, (2005b) where clotrimazole release from proliposomes was investigated (Pavelić *et al.*, 2005; Ning *et al.*, 2005c).

After 8 hours the remaining samples were collected from the donor chambers in order to determine the drug recovery. Surprisingly, the volume was found to increase for samples containing clotrimazole in propylene glycol, indicating that some acetate buffer had diffused through the membrane from acceptor chamber; the fact that may interfere with the levels observed and could contribute to observed equilibrium.

The same experimental set up was used in experiments performed with coated liposomes. The drug release was only studied for liposomes coated with 0.6 % w/v chitosan as they were expected to have stronger mucoadhesiveness.

Initially the two parallels show equal release, although after 24 hours the amount of released drug was not similar.

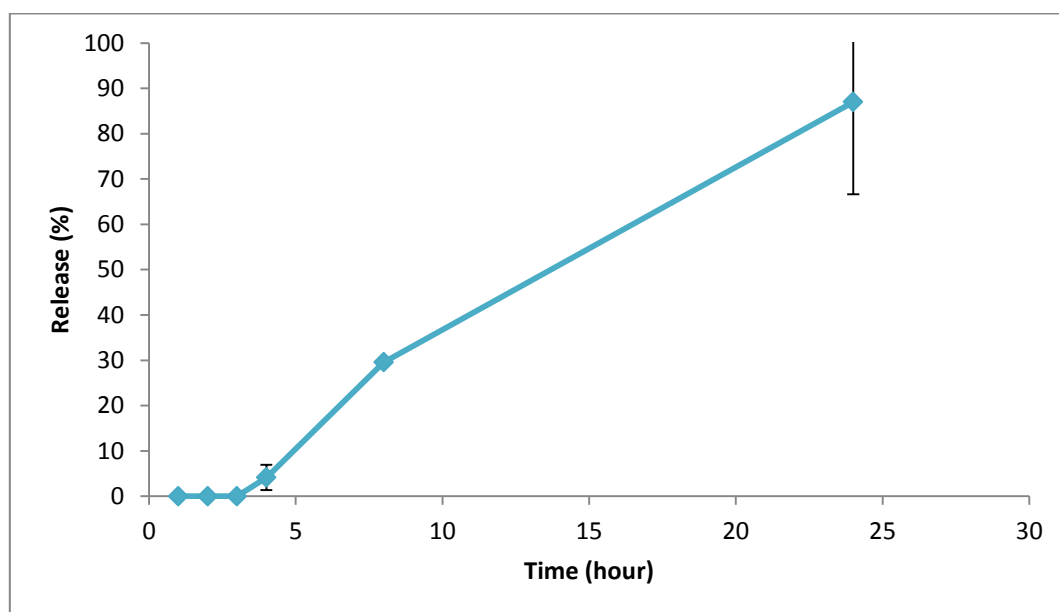


Figure 12: *In vitro* clotrimazole release from coated (0.6 %, w/v) liposomes

Unlike the non-coated liposomes, the release of clotrimazole from chitosan-coated liposomes was delayed for first 4 hours. Figure 12 indicates that chitosan-coated liposomes have ability to prolong the release of clotrimazole to greater extent than non-coated liposomes.

Mucoadhesive liposomes are expected to adhere to the vaginal surface and retain at the site over longer period of time. The release pattern observed in our experiments from chitosan-coated vesicles correspond well to the release of clotrimazole from liposomes incorporated in Carbopol[®] hydrogels, as reported by Pavelić *et al.* (Pavelić *et al.*, 2005). This indicates that our coated liposomes have equal potential to delay the release of clotrimazole as liposomal hydrogels. There are no reports available on release of clotrimazole from chitosan-based hydrogels.

5.4 *Ex vivo* penetration

Cow vaginal mucosa was reported to have characteristics similar to human vaginal mucosa, thus it has been widely used as a model membrane in various types of mucoadhesion testing (das Neves *et al.*, 2008). Accordingly, cow mucosa was also used as model mucosa in our penetration and mucoadhesiveness studies.

Samples of free clotrimazole (2 mg/ml in propylene glycol), non-coated liposomes, 0.1 % chitosan-coated liposomes and 0.6 % chitosan-coated liposomes were added to donor chambers. The same experimental set up was used as in experiments for *in vitro* drug release, except for the receptor medium which in this experiment was phosphate buffer (pH 7.4).

Surprisingly, we were unable to determine any clotrimazole present in receptor chamber at given time intervals, indicating that neither free clotrimazole nor liposomal clotrimazole nor clotrimazole from coated liposomes were able to penetrate the mucosal tissue. This would indirectly indicate that no systemic absorption from this delivery systems applied vaginally would take place, favorable finding for topically applied therapy.

However, when we determined the amount of drug retained in the donor chamber of Franz diffusion cells, we realized that less clotrimazole was present in any of the chambers than the amount we have used at the start of the experiments. The logical conclusion would be that drug was in the vaginal mucosa itself. We have tried to determine the amount retained in the mucosa by extracting the tissue in methanol, but faced serious obstructions due to the interference from proteins and other molecules present in mucosal tissue.

In order to confirm that the tissue was the source of the interference we experienced, a control experiment with pure phosphate buffer (pH 7.4) in the donor chamber under the same experimental setup was performed under the same conditions as earlier experiments. The same disturbance in measurements was observed for this experiment in which no drug was present.

Similar findings for lipophilic drugs have been reported earlier (Justin-Temu *et al.*, 2004) and similar release pattern for curcumin has also been observed, a highly lipophilic molecule, from liposomes applied onto vaginal tissue, namely that curcumin was retained within vaginal tissue and only negligible amount detected in receptor chamber (Berginc *et al.*; unpublished data). We have to confirm the findings by using more sophisticated methods of drug determination, such as HPLC or mass spectroscopy, and this part is included in our perspectives.

However, we have to mention that *Ning et al.* reported that liposomal/niosomal delivery systems has the ability to deliver clotrimazole into and through rabbit vaginal mucosa, using vertical Franz diffusion cells (*Ning et al.*, 2005b). Whether the different mucosa or different experimental set up are responsible for this discrepancy, is hard to further elaborate on.

Considering that clotrimazole is most effective in local treatment, systemic effect is not required. Moreover, absorption of drugs targeted for local action in the vagina is not desirable (Valenta, 2005).

5.5 Preliminary mucoadhesion testing

When a mucoadhesive formulation is applied vaginally, the durability of contact will be significantly affected by the vaginal environment. The contact with low-pH vaginal fluid is known to result in viscosity loss and erosion of gels for example (*Geonnotti et al.*, 2005). Changes in rheological properties of gels in the presence of simulated vaginal fluid have been observed for several formulations (*Chang et al.*, 2002; *Geonnotti et al.*, 2005; *Lai et al.*, 2008; *Andrews et al.*, 2009). Hence, when evaluating mucoadhesive properties, the formulation should be exposed to vaginal fluid or simulated vaginal fluid

(das Neves and Bahia, 2006; das Neves *et al.*, 2008). Consequently, a vaginal fluid simulant (pH 4.5(Owen and Katz, 1999)) was used in this study when investigating mucoadhesion of the chitosan-coated liposomes. Moreover, literature data suggest that significant differences in mucoadhesion have been observed between testing of formulations at body (37 °C) and room temperature (20 °C). Das Neves *et al.* (2008) demonstrated the importance of experimental set up and temperature in particular.

Our experiment was performed at room temperature (24 °C) and we evaluated the mucoadhesiveness of chitosan-coated liposomes compared to non-coated liposome and free drug. Samples of free clotrimazole (2 mg/ml in propylene glycol), non-coated liposomes, 0.1 % chitosan-coated liposomes and 0.6 % chitosan-coated liposomes were applied to tissue and exposed to vaginal fluid simulant.

It was expected that the contact of the coated liposomes would be superior to non-coated liposomes and that the entrapped drug would still be present in liposomes. Due to disturbance in the absorbance at 261 nm, the amount of originally entrapped drug still present in liposomes (in donor chamber) could not be determined. Very high absorbance shown at 209 nm was interfering with the measurements at 261 nm. This is thought to be the interference from proteins and other molecules present in mucosal tissue, similar to what we observed in the *ex vivo* penetration experiment, namely high absorbance at 209 nm. In an attempt to eliminate the disturbance, the samples were filtrated prior to determination but it did not remove interference. However, the disturbance was still present. This is additional proof that HPLC method should be applied in determination of clotrimazole.

Our preliminary findings suggest that clotrimazole applied to vaginal tissue tends to penetrate into the tissue, rather than penetrate through the tissue, which is preferable considering that aim is the local therapy of vaginal infections. In order to be able to confirm these interesting findings, we need to apply more sensitive method for drug determination, such as HPLC for example.

6. CONCLUSION

Topical application of phospholipid vesicles may improve the delivery of incorporated drug and is applicable in treatment of local vaginal infections. Use of mucoadhesive polymers to modify liposomal surface is expected to increase their retention time at vaginal site.

Incorporation of clotrimazole in liposomes by the mechanical dispersion method yields consistent and acceptable entrapment efficiency. The procedure is simple and reproducible. The original particle size can be reduced to desired size by varying the sonication conditions. Coating of liposomal surface by chitosan, a known mucoadhesive polymer, results in increased vesicle size.

Chitosan-coated liposomes provided sustained release properties for clotrimazole and were shown to penetrate into but not through vaginal tissue. Clotrimazole is known to be effective in local treatment of vaginal infections, and its systemic absorption is not desirable. Chitosan-coated phospholipid vesicles appear to be promising as a delivery system for clotrimazole, however more penetration and mucoadhesion studies are required to optimize this novel delivery system.

7. PERSPECTIVES

Chitosan-coated mucoadhesive liposomes developed through this project will serve as base to further optimize mucoadhesive delivery system for clotrimazole. We have experienced problems in determination of clotrimazole in the presence of tissue material and alternative, more sensitive method of detection, should be applied. The newly developed system should be evaluated for its stability in the presence of vaginal fluid simulant and its safety in both cell model and suitable *in vivo* model confirmed. The final evaluation needs to be performed in *in vivo* animal studies, where the system would be compared to commercially available dosage forms of clotrimazole.

8. REFERENCES

- Acartürk, F. (2009). "Mucoadhesive Vaginal Drug Delivery Systems." *Recent Patents on Drug Delivery & Formulation* **3**(3): 193-205.
- Ahuja, A., Khar, R. K. and Ali, J. (1997). "Mucoadhesive Drug Delivery Systems." *Drug Development and Industrial Pharmacy* **23**(5): 489-515.
- Alexander, N. J., Baker, E., Kaptein, M., Karck, U., Miller, L. and Zampaglione, E. (2004). "Why consider vaginal drug administration?" *Fertility and Sterility* **82**(1): 1-12.
- Andrews, G. P., Donnelly, L., Jones, D. S., Curran, R. M., Morrow, R. J., Woolfson, A. D. and Malcolm, R. K. (2009). "Characterization of the rheological, mucoadhesive, and drug release properties of highly structured gel platforms for intravaginal drug delivery." *Biomacromolecules* **10**(9): 2427-2435.
- Andrews, G. P., Laverty, T. P. and Jones, D. S. (2009). "Mucoadhesive polymeric platforms for controlled drug delivery." *European Journal of Pharmaceutics and Biopharmaceutics* **71**(3): 505-518.
- Asane, G. S., Nirmal, S. A., Rasal, K. B., Naik, A. A., Mahadik, M. S. and Rao, Y. M. (2008). "Polymers for Mucoadhesive Drug Delivery System: A Current Status." *Drug Development and Industrial Pharmacy* **34**(11): 1246-1266.
- Baloglu, E., Senyigit, Z. A., Karavana, S. Y. and Bernkop-Schnurch, A. (2009). "Strategies to prolong the intravaginal residence time of drug delivery systems." *Journal of Pharmacy & Pharmaceutical Sciences* **12**(3): 312-336.
- Banerjee, R. (2001). "Liposomes: applications in medicine." *Journal of Biomaterials applications* **16**(1): 3-21.
- Bangham, A., Standish, M. and Weissmann, G. (1965). "The action of steroids and streptolysin S on the permeability of phospholipid structures to cations." *Journal of molecular biology* **13**(1): 253-259.
- Benziger, D. P. and Edelson, J. (1983). "Absorption from the Vagina." *Drug Metabolism Reviews* **14**(2): 137-168.
- Bhattacharai, N., Gunn, J. and Zhang, M. (2010). "Chitosan-based hydrogels for controlled, localized drug delivery." *Advanced Drug Delivery Reviews* **62**(1): 83-99.
- Boddupalli, B., Mohammed, Z., Nath, R. and Banji, D. (2010). "Mucoadhesive drug delivery system: An overview." *Journal of Advanced Pharmaceutical Technology & Research* **1**(4): 381-387.
- Bohbot, J. M., Sednaoui, P., Verriere, F. and Achhammer, I. (2011). "Diversité étiologique des vaginites." *Gynécologie Obstétrique & Fertilité*: <http://dx.doi.org/10.1016/j.gyobfe.2011.1008.1001>.
- Bonferoni, M., Giunchedi, P., Scalia, S., Rossi, S., Sandri, G. and Caramella, C. (2006). "Chitosan gels for the vaginal delivery of lactic acid: Relevance of formulation parameters to mucoadhesion and release mechanisms." *The American Association of Pharmaceutical Scientists* **7**(4): 141-147.
- Carr, P., Felsenstein, D. and Friedman, R. (1998). "Evaluation and management of vaginitis." *Journal of General Internal Medicine* **13**(5): 335-346.
- Ceschel, G. C., Maffei, P., Borgia, S. L., Ronchi, C. and Rossi, S. (2001). "Development of a Mucoadhesive Dosage Form for Vaginal Administration." *Drug Development and Industrial Pharmacy* **27**(6): 541-547.

- Cevc, G. (2004). "Lipid vesicles and other colloids as drug carriers on the skin." *Advanced Drug Delivery Reviews* **56**(5): 675-711.
- Chang, J. Y., Oh, Y. K., Choi, H. G., Kim, Y. B. and Kim, C. K. (2002). "Rheological evaluation of thermosensitive and mucoadhesive vaginal gels in physiological conditions." *International Journal of Pharmaceutics* **241**(1): 155-163.
- Chowdary, K. P. R. and Srinivasa Rao, Y. (2004). "Mucoadhesive microspheres for controlled drug delivery." *Biological and Pharmaceutical Bulletin* **27**(11): 1717-1724.
- Cicinelli, E. (2008). "Intravaginal oestrogen and progestin administration: advantages and disadvantages." *Best Practice & Research Clinical Obstetrics & Gynaecology* **22**(2): 391-405.
- Cone, R. A. (2009). "Barrier properties of mucus." *Advanced Drug Delivery Reviews* **61**(2): 75-85.
- das Neves, J., Amaral, M. H. and Bahia, M. F. (2008). "Performance of an in vitro mucoadhesion testing method for vaginal semisolids: Influence of different testing conditions and instrumental parameters." *European Journal of Pharmaceutics and Biopharmaceutics* **69**(2): 622-632.
- das Neves, J., Amiji, M. and Sarmiento, B. (2011). "Mucoadhesive nanosystems for vaginal microbicide development: friend or foe?" *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology* **3**(4): 389-399.
- das Neves, J. and Bahia, M. F. (2006). "Gels as vaginal drug delivery systems." *International Journal of Pharmaceutics* **318**(1-2): 1-14.
- de Araújo Pereira, R. R. and Bruschi, M. L. (2011). "Vaginal mucoadhesive drug delivery systems." *Drug Development and Industrial Pharmacy* **38**(6): 1-10.
- di Cagno, M., Styskala, J., Hlaváč, J., Brandl, M., Bauer-Brandl, A. and Skalko-Basnet, N. (2011). "Liposomal solubilization of new 3-hydroxy-quinolinone derivatives with promising anticancer activity: a screening method to identify maximum incorporation capacity." *Journal of Liposome Research* **21**(4): 272-278.
- Edsman, K. and Hägerström, H. (2005). "Pharmaceutical applications of mucoadhesion for the non-oral routes." *Journal of Pharmacy and Pharmacology* **57**(1): 3-22.
- Eschenbach, D. A. (2004). "Chronic Vulvovaginal Candidiasis." *New England Journal of Medicine* **351**(9): 851-852.
- Filipović-Grčić, J., Škalko-Basnet, N. and Jalšienjak, I. (2001). "Mucoadhesive chitosan-coated liposomes: characteristics and stability." *Journal of Microencapsulation* **18**(1): 3-12.
- Friend, D. (2011). "Advances in vaginal drug delivery." *Drug Delivery and Translational Research* **1**(3): 183-184.
- Geonotti, A. R., Peters, J. J. and Katz, D. F. (2005). "Erosion of microbicide formulation coating layers: Effects of contact and shearing with vaginal fluid or semen." *Journal of Pharmaceutical Sciences* **94**(8): 1705-1712.
- Goldberg, R. and Klein, J. (2011). "Liposomes as lubricants: beyond drug delivery." *Chemistry and Physics of Lipids* **165**(4).
- Gupta, P., Vermani, K. and Garg, S. (2002). "Hydrogels: from controlled release to pH-responsive drug delivery." *Drug Discovery Today* **7**(10): 569-579.
- Hamidi, M., Azadi, A. and Rafiei, P. (2008). "Hydrogel nanoparticles in drug delivery." *Advanced Drug Delivery Reviews* **60**(15): 1638-1649.
- Hoare, T. R. and Kohane, D. S. (2008). "Hydrogels in drug delivery: Progress and challenges." *Polymer* **49**(8): 1993-2007.
- Hoffman, A. S. (2002). "Hydrogels for biomedical applications." *Advanced Drug Delivery Reviews* **54**(1): 3-12.
- Hombach, J. and Bernkop-Schnürch, A. (2010). "Mucoadhesive Drug Delivery Systems

- ". *Drug Delivery*. M. Schäfer-Korting. Springer Berlin Heidelberg, Berlin. **197**: 251-266.
- Hussain, A. and Ahsan, F. (2005). "The vagina as a route for systemic drug delivery." *Journal of Controlled Release* **103**(2): 301-313.
- Justin-Temu, M., Damian, F., Kinget, R. and Mooter, G. V. D. (2004). "Intravaginal gels as drug delivery systems." *Journal of Women's Health* **13**(7): 834-844.
- Karn, P. R., Vanić, Z., Pepić, I. and Škalko-Basnet, N. (2011). "Mucoadhesive liposomal delivery systems: the choice of coating material." *Drug Development and Industrial Pharmacy* **37**(4): 482-488.
- Kast, C. E., Valenta, C., Leopold, M. and Bernkop-Schnürch, A. (2002). "Design and in vitro evaluation of a novel bioadhesive vaginal drug delivery system for clotrimazole." *Journal of Controlled Release* **81**(3): 347-354.
- Khutoryanskiy, V. V. (2011). "Advances in Mucoadhesion and Mucoadhesive Polymers." *Macromolecular Bioscience* **11**(6): 748-764.
- Kim, J. and Sudbery, P. (2011). "Candida albicans , a major human fungal pathogen." *The Journal of Microbiology* **49**(2): 171-177.
- Knuth, K., Amiji, M. and Robinson, J. R. (1993). "Hydrogel delivery systems for vaginal and oral applications: Formulation and biological considerations." *Advanced Drug Delivery Reviews* **11**(1-2): 137-167.
- Lai, B. E., Xie, Y. Q., Lavine, M. L., Szeri, A. J., Owen, D. H. and Katz, D. F. (2008). "Dilution of microbicide gels with vaginal fluid and semen simulants: Effect on rheological properties and coating flow." *Journal of Pharmaceutical Sciences* **97**(2): 1030-1038.
- Lee, J. W., Park, J. H. and Robinson, J. R. (2000). "Bioadhesive-based dosage forms: The next generation." *Journal of Pharmaceutical Sciences* **89**(7): 850-866.
- Lian, T. and Ho, R. J. Y. (2001). "Trends and developments in liposome drug delivery systems." *Journal of Pharmaceutical Sciences* **90**(6): 667-680.
- Mallipeddi, R. and Rohan, L. C. (2010). "Nanoparticle-based vaginal drug delivery systems for HIV prevention." *Expert Opinion on Drug Delivery* **7**(1): 37-48.
- Marrazzo, J. M. (2011). "Interpreting the epidemiology and natural history of bacterial vaginosis: Are we still confused?" *Anaerobe* **17**(4): 186-190.
- Mashburn, J. (2006). "Etiology, Diagnosis, and Management of Vaginitis." *The Journal of Midwifery & Women's Health* **51**(6): 423-430.
- Mehanna, M. M., Elmaradny, H. A. and Samaha, M. W. (2010). "Mucoadhesive liposomes as ocular delivery system: physical, microbiological, and in vivo assessment." *Drug Development and Industrial Pharmacy* **36**(1): 108-118.
- Merabet, J., Thompson, D. and Saul Levinson, R. (2005). "Advancing vaginal drug delivery." *Expert Opinion on Drug Delivery* **2**(4): 769-777.
- Mura, P., Maestrelli, F., González-Rodríguez, M. L., Michelacci, I., Ghelardini, C. and Rabasco, A. M. (2007). "Development, characterization and in vivo evaluation of benzocaine-loaded liposomes." *European Journal of Pharmaceutics and Biopharmaceutics* **67**(1): 86-95.
- New, R. R. C. (1990). "Liposomes: A practical approach". Oxford University Press, New York.
- Ning, M., Guo, Y., Pan, H., Chen, X. and Gu, Z. (2005a). "Preparation, in Vitro and in Vivo Evaluation of Liposomal/Niosomal Gel Delivery Systems for Clotrimazole." *Drug Development and Industrial Pharmacy* **31**(4-5): 375-383.
- Ning, M., Guo, Z., Pan, H., Yu, H. and Xiao, K. (2005b). "Preparation and in vitro evaluation of liposomal/niosomal delivery systems for antifungal drug clotrimazole." *Indian Journal of Experimental Biology* **43**(2): 150-157.

- Ning, M. Y., Guo, Y. Z., Pan, H. Z., Yu, H. M. and Gu, Z. W. (2005c). "Preparation and evaluation of proliposomes containing clotrimazole." *Chemical and Pharmaceutical Bulletin* **53**(6): 620-624.
- Owen, D. H. and Katz, D. F. (1999). "A vaginal fluid simulant." *Contraception* **59**(2): 91-95.
- Owen, M. K. and Clenney, T. L. (2004). "Management of vaginitis." *American Family Physician* **70**(11): 2125-2132.
- Patel, D. B. and Patel, J. K. (2009). "Liposomal drug delivery of metronidazole for the local treatment of vaginitis." *International Journal of Pharmaceutical Science Nanotechnology* **2**(1): 421-427.
- Pavelić, Ž., Škalko-Basnet, N. and Jalšenjak, I. (1999). "Liposomes containing drugs for treatment of vaginal infections." *European Journal of Pharmaceutical Sciences* **8**(4): 345-351.
- Pavelić, Ž., Škalko-Basnet, N. and Jalšenjak, I. (2005). "Characterisation and in vitro evaluation of bioadhesive liposome gels for local therapy of vaginitis." *International Journal of Pharmaceutics* **301**(1-2): 140-148.
- Pavelić, Ž., Škalko-Basnet, N. and Schubert, R. (2001). "Liposomal gels for vaginal drug delivery." *International Journal of Pharmaceutics* **219**(1-2): 139-149.
- Peppas, N. A., Bures, P., Leobandung, W. and Ichikawa, H. (2000). "Hydrogels in pharmaceutical formulations." *European Journal of Pharmaceutics and Biopharmaceutics* **50**(1): 27-46.
- Perioli, L., Ambrogi, V., Pagano, C., Scuota, S. and Rossi, C. (2009). "FG90 chitosan as a new polymer for metronidazole mucoadhesive tablets for vaginal administration." *International Journal of Pharmaceutics* **377**(1-2): 120-127.
- Ranade, V. (1989). "Drug delivery systems. 1. site-specific drug delivery using liposomes as carriers." *The Journal of Clinical Pharmacology* **29**(8): 685-694.
- Rang, H. P., Dale, M. M., Ritter, J. M. and Flower, R. J. (2007). "Rang and Dale's Pharmacology". Churchill Livingstone Elsevier, New York.
- Rengel, R. G., Barišić, K., Pavelić, Ž., Žanić Grubišić, T., Čepelak, I. and Filipović-Grčić, J. (2002). "High efficiency entrapment of superoxide dismutase into mucoadhesive chitosan-coated liposomes." *European Journal of Pharmaceutical Sciences* **15**(5): 441-448.
- Samad, A., Sultana, Y. and Aqil, M. (2007). "Liposomal Drug Delivery Systems: An Update Review." *Current Drug Delivery* **4**(4): 297-305.
- Shaikh, R., Raj Singh, T., Garland, M., Woolfson, A. and Donnelly, R. (2011). "Mucoadhesive drug delivery systems." *Journal of Pharmacy and Bioallied Sciences* **3**(1): 89-100.
- Sharma, S. (2009). "Liposomes: a review." *Journal of Pharmacy Research* **2**(7): 1163-1167.
- Siewert, M., Dressman, J., Brown, C., Shah, V., Aiache, J. M., Aoyagi, N., Bashaw, D., Brown, W., Burgess, D., Crison, J., DeLuca, P., Djerki, R., Foster, T., Gjellan, K., Gray, V., Hussain, A., Ingallinera, T., Klancke, J., Kraemer, J., Kristensen, H., Kumi, K., Leuner, C., Limberg, J., Loos, P., Margulis, L., Marroum, P., Moeller, H., Mueller, B., Mueller-Zsigmondy, M., Okafo, N., Ouderkirk, L., Parsi, S., Qureshi, S., Robinson, J., Uppoor, R. and Williams, R. (2003). "FIP/AAPS guidelines to dissolution/in vitro release testing of novel/special dosage forms." *The American Association of Pharmaceutical Scientists* **4**(1): 43-52.
- Smart, J. D. (2005). "The basics and underlying mechanisms of mucoadhesion." *Advanced Drug Delivery Reviews* **57**(11): 1556-1568.
- Sobel, J. D. (1997). "Vaginitis." *New England Journal of Medicine* **337**(26): 1896-1903.
- Sobel, J. D. (2007). "Vulvovaginal candidosis." *The Lancet* **369**(9577): 1961-1971.

- Sobel, J. D., Faro, S., Force, R. W., Foxman, B., Ledger, W. J., Nyirjesy, P. R., Reed, B. D. and Summers, P. R. (1998). "Vulvovaginal candidiasis: Epidemiologic, diagnostic, and therapeutic considerations." *American Journal of Obstetrics and Gynecology* **178**(2): 203-211.
- Takeuchi, H., Matsui, Y., Sugihara, H., Yamamoto, H. and Kawashima, Y. (2005a). "Effectiveness of submicron-sized, chitosan-coated liposomes in oral administration of peptide drugs." *International Journal of Pharmaceutics* **303**(1-2): 160-170.
- Takeuchi, H., Thongborisute, J., Matsui, Y., Sugihara, H., Yamamoto, H. and Kawashima, Y. (2005b). "Novel mucoadhesion tests for polymers and polymer-coated particles to design optimal mucoadhesive drug delivery systems." *Advanced Drug Delivery Reviews* **57**(11): 1583-1594.
- Takeuchi, H., Yamamoto, H. and Kawashima, Y. (2001). "Mucoadhesive nanoparticulate systems for peptide drug delivery." *Advanced Drug Delivery Reviews* **47**(1): 39-54.
- Takeuchi, H., Yamamoto, H., Niwa, T., Hino, T. and Kawashima, Y. (1996). "Enteral Absorption of Insulin in Rats from Mucoadhesive Chitosan-Coated Liposomes." *Pharmaceutical Research* **13**(6): 896-901.
- Torchilin, V. P. (2005). "Recent advances with liposomes as pharmaceutical carriers." *Nature Reviews Drug Discovery* **4**(2): 145-160.
- Valenta, C. (2005). "The use of mucoadhesive polymers in vaginal delivery." *Advanced Drug Delivery Reviews* **57**(11): 1692-1712.
- Vasir, J. K., Tambwekar, K. and Garg, S. (2003). "Bioadhesive microspheres as a controlled drug delivery system." *International Journal of Pharmaceutics* **255**(1-2): 13-32.
- Vemuri, S. and Rhodes, C. T. (1995). "Preparation and characterization of liposomes as therapeutic delivery systems: a review." *Pharmaceutica Acta Helveticae* **70**(2): 95-111.
- Verma, D. D., Verma, S., Blume, G. and Fahr, A. (2003). "Particle size of liposomes influences dermal delivery of substances into skin." *International Journal of Pharmaceutics* **258**(1-2): 141-151.
- Vermani, K. and Garg, S. (2000). "The scope and potential of vaginal drug delivery." *Pharmaceutical Science & Technology Today* **3**(10): 359-364.
- Wulff, H., Gutman, G. A., Cahalan, M. D. and Chandy, K. G. (2001). "Delineation of the clotrimazole/TRAM-34 binding site on the intermediate conductance calcium-activated potassium channel, IKCa1." *Journal of Biological Chemistry* **276**(34): 32040-32045.

PhEur online database - 7th Edition 2011 (7.2), http://online6.edqm.eu/ep702/#doc_1_of_21
http://www.medicinescomplete.com/mc/merck/current/02417.htm?q=clotrimazole&t=search&ss=text&p=1#_hit
<http://morgencrewreise.de/dubai/liposome-bilayer>
<http://depts.washington.edu/hiv aids/oral/case4/discussion.html>