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Novel chitosan-containing liposomes as mucoadhesive delivery system for vaginal administration

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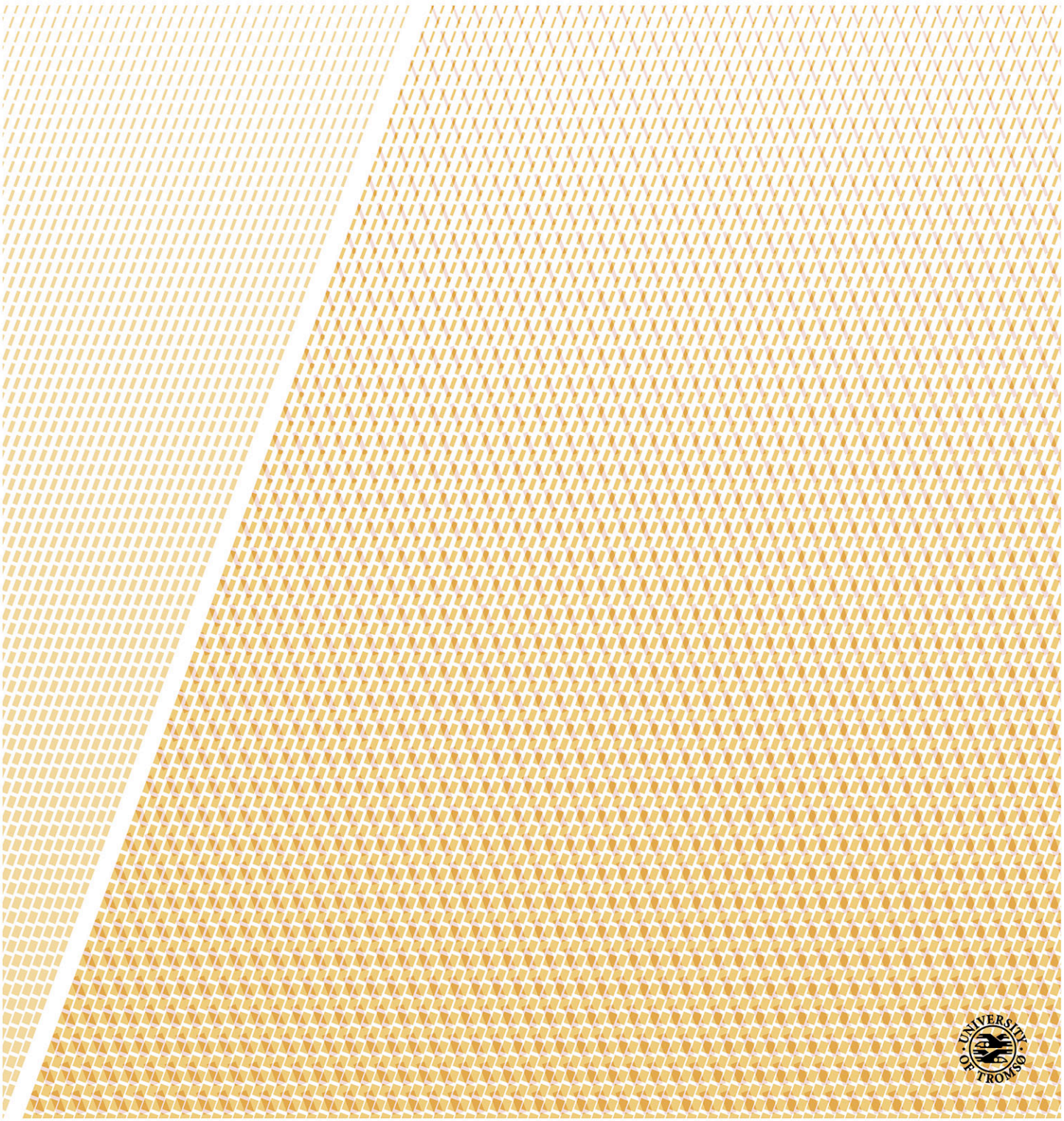
Toril Andersen

A dissertation for the degree of Philosophiae Doctor – July 2015



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Novel chitosan-containing liposomes as mucoadhesive delivery system for vaginal administration

Toril Andersen

Tromsø 2015

Drug Transport and Delivery Research Group
Department of Pharmacy
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UiT The Arctic University of Norway
Norway

To my Family

" If you trust in yourself … and believe in your dreams … and follow your star…
you´ll still get beaten by people who spent their time working hard and learning things and weren´t so lazy"

From "The Wee Free Men", GNU Terry Pratchett (1948-2015)

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Tromsø, July 2015

Table of content

Abstract

Treatment of vaginal infections remains unsuccessful due to recurrent infections and limited patient compliance. Current therapies are unable to penetrate bacterial and fungal biofilms, allowing the survival of microorganisms, which often leads to new infections. Local administration of drug is able to provide a higher drug concentration at the vaginal site; enabling the administration of lower drug doses and reduced administration frequency that again decreases the risk of adverse reactions. A successful local treatment should assure a sufficient amount of a drug at the vaginal site for a sufficient period of time. This can be achieved by a mucoadhesive drug delivery system.

Coating of nanopharmaceuticals with mucoadhesive polymers has been proposed as a mean to assure longer residence time at the vaginal site. However, conventional coating techniques may reduce the concentration of the loaded drug due to the subsequent coating of preformed liposomes and are in addition time consuming. The aim of this work was the development of novel mucoadhesive polymer-containing liposomes that would allow for a straightforward and simple one-pot preparation method. Chitosan was chosen as a promising polymer based on its known mucoadhesiveness, biological activity and general safety profile.

The resulting chitosan-containing liposomes were characterized for their size distribution, zeta potential, entrapment efficiency and *in vitro* release of a drug (metronidazole) and model substances (FITC-dextrans of varying molecular weights). The characterization of surface-available chitosan in the novel system has shown that the system contains both chitosan as an outer coating as well as embedded within the liposomes, and the mucoadhesive effect of the surface-available chitosan has further been confirmed. The system provides sustained release of entrapped drug or model substances, and is expected to assure sufficiently high drug concentrations at vaginal site. The chitosan-containing liposomes exhibited a potent ability to inhibit the growth of *Candida albicans* independently of the presence of loaded drug. This could be utilized in the treatment of vaginal infections of complicated natures, such as mixed bacterial and fungal infections.

Abbreviations

DD	degree of deacetylation
DE	degree of esterification
FITC	fluorescein isothiocyanate dextran
MTZ	metronidazole
PI	polydispersity index
PL	plain liposomes
PM	pig mucin
SPC	soy phosphatidylcholine

List of papers

The present thesis is based on the following publications and manuscripts:

Paper I

Toril Andersen, Željka Vanić, Gøril Eide Flaten, Sofia Mattsson, Ingunn Tho, Nataša Škalko-Basnet Pectosomes and chitosomes as delivery systems for metronidazole: The one-pot preparation method. *Pharmaceutics* **2013**, *5*, 445-456.

Paper II

Toril Andersen, Stefan Bleher, Gøril Eide Flaten, Ingunn Tho, Sofia Mattsson, Nataša Škalko-Basnet Chitosan in mucoadhesive drug delivery: Focus on local vaginal therapy. *Mar. Drugs* **2015**, *13*, 222-236.

Paper III

Toril Andersen, Ekaterina Mishchenko, Gøril Eide Flaten, Johanna U. Ericson Sollid, Sofia Mattsson, Ingunn Tho, Nataša Škalko-Basnet. Chitosomes: novel chitosan-containing liposomes with antifungal activity against *Candida albicans*. *Manuscript*.

1 Introduction

Local treatment of illness related to the female genitalia and local contraception has been used throughout history from the ancient cultures of Egypt, Greece, and Rome to the Middle Ages. It was used in the Arabic and Oriental cultures, through the Renaissance and continues to be used in our time. Some of the oldest records of pharmaceutical preparations such as the Kahun Papyrus, the oldest surviving medical papyrus dating back to 1850 B.C., contain descriptions of preparations intended for vaginal administration. These contained ingredients such as mud, frankincense, oil, malachite, donkey urine, myrrh, crocodile dung, honey, and sour milk (O'Dowd, 2001). As das Neves *et al.* states in their chapter on the topic of vaginal drug delivery (2007) "although the understanding we currently have of vaginal anatomy, physiology, and pathophysiology is very extensive, it stands in contrast to the limited knowledge we have of the possibilities within the field of vaginal drug delivery."

1.1 Vaginal anatomy and physiology

The word vagina (Figure 1.1) comes from Latin and means sheath. It is an important organ in the female reproductive tract, and has different functions involving the menstrual cycle, sexual intercourse, and conception. The vagina is a fibro muscular tube ranging from 6 to 10 cm long in the adult female. It is positioned between the rectum, bladder, and urethra, and extends outwards from the cervical end of the uterus. Figure 1.2 (1) illustrates the position of the vagina relative to its surroundings (Washington *et al.*, 2000). Lining the inner walls of the vagina is a mucosal tissue that forms a series of transverse folds named rugae, marked R in Figure 1.2. The rugae increase the surface area of the vagina allowing for stretching during penile penetration and birth (Misra, 2014). The surface area of the vagina has been shown to range between 103.9-165.0 mm^2 (Barnhart *et al.*, 2006).

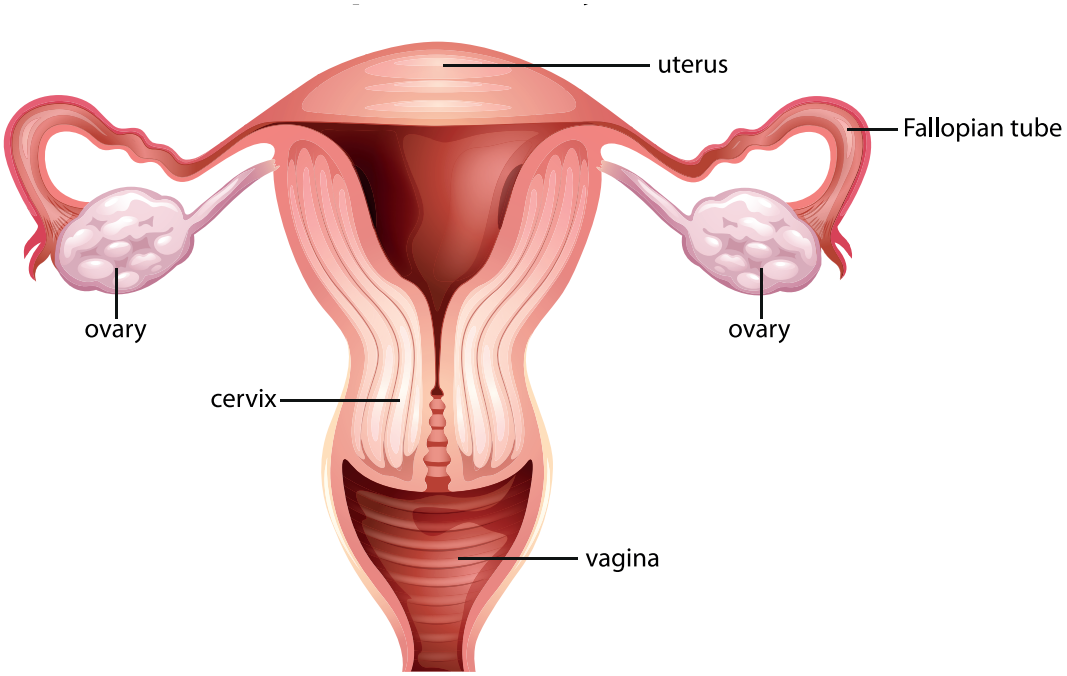


Figure 1.1: schematic illustration of the female reproductive system. Photo: colourbox.com

Beneath the epithelial layer lies a muscular coat and the tunica adventia (Figure 1.2 (2)). Because of the presence of smooth elastic fibers in the muscular coat and loose connective tissue in the tunica adventia the vagina has an excellent elasticity. The walls of the vagina are rich in blood vessels and lymphatic vessels that bypass the liver (Siddique, 2003; Hussain, 2005).

Vaginal mucosal tissue is comprised of a nonkeratinized, stratified squamous epithelium, depicted in Figure 1.2 (3), which unlike the other mucosal surfaces of the body does not contain mucus-secreting glands, or goblet cells. The fluids of the vagina, or cervicovaginal mucus, mainly consists of cervical secretion and transudations from the blood vessels, it consists of 90-95 % water, 1-2 % mucin, and other lesser constituents such as inorganic and organic salts, urea, carbohydrates, glycerol, fatty acids, albumin, immunoglobulin, enzymes, leukocytes, and epithelial debris. The amount secreted, viscosity, pH, and composition of fluid will vary with the menstrual cycle and onset of menopause. Normal daily production of cervicovaginal mucus is estimated at around 6 mL, increasing in midcycle, and decreasing around the menstruation period. Post-menopausal women will produce about half the amount (das Neves and Gad, 2007; Washington *et al.*, 2000).

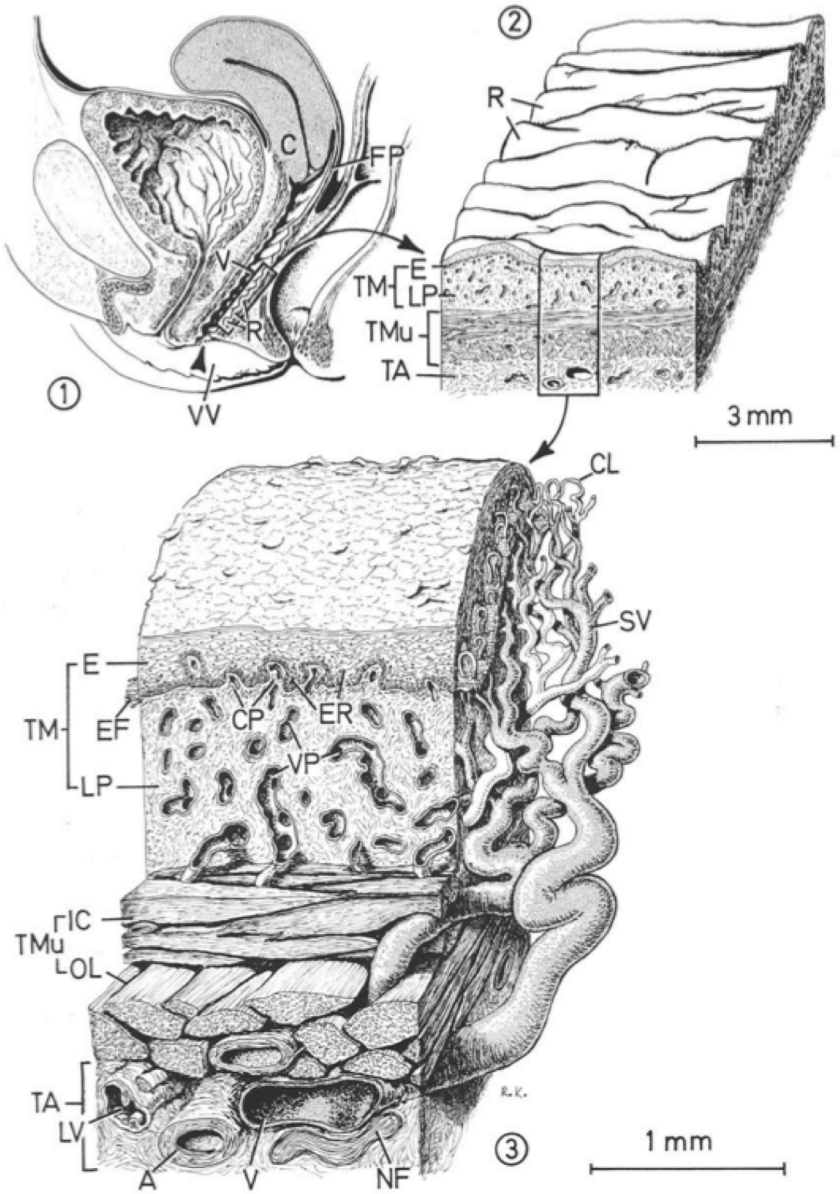


Figure 1.2: schematic illustrations of the vagina. Reprinted with permission from Springer (Krstić, 1991).

Although the cyclical variations of the vagina are not as dramatic as that of the endometrium, thickness of the vaginal epithelium varies up to 200-300 μm with hormonal activity and age. The thickness is highest in the proliferative stage, with the highest glycogen content and thickness during ovulation, as can be seen in Figure 1.3 (Valenta, 2005).

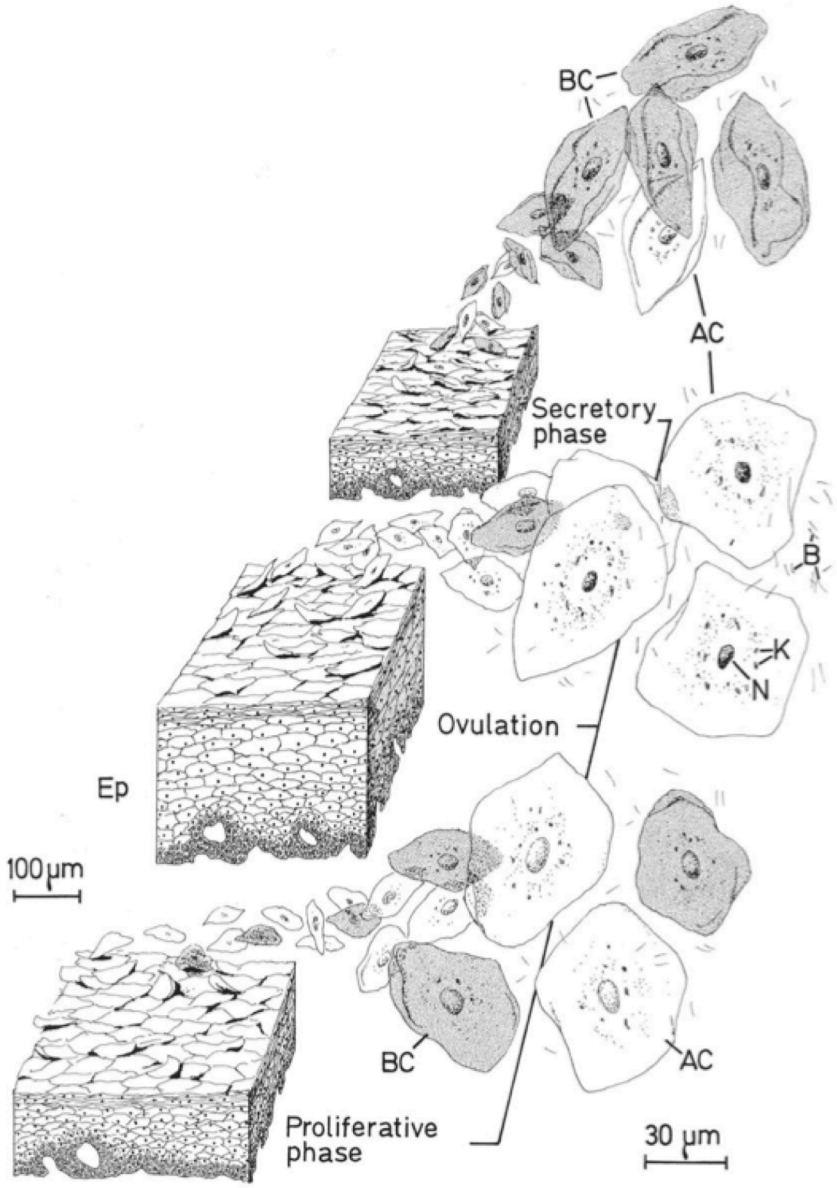


Figure 1.3: Illustration of the variation of epithelial thickness throughout the menstrual cycle. Reprinted with permission from Springer (Krstić, 1991).

Vaginal pH is maintained at an acidic level, pH ranging from 3.5 to 4.9, by of lactic acid producing bacteria, mainly *Lactobacillus acidophilus*. The *Lactobacilli* convert glycogen to lactic acid from exfoliated epithelial cells; this produces a bacteriostatic pH level. At birth, a transfer of bacteria and maternal hormones leads to an initial low pH for the first weeks of life. After the hormone level decreases the pH increases to around 7 where it is quite stable until puberty. This can be associated with an increased risk of infection. In post-pubescent

women, the pH decreases in response to the increased hormonal levels, but can be increased periodically by menstruation, and frequent acts of coitus, because seminal fluid and vaginal transudate are both alkaline. Post-menopausal women have less hormonal activity leading to an increased pH because the cervical secretions decrease in volume and have a higher pH (Washington *et al.,* 2000; Linhares *et al.*, 2011; Misra, 2014).

1.1.1 Vaginal infections

Vaginal infections is a widespread problem, it is reported as one of the most common reasons that women seek professional health care. The most common of the vaginal infections are bacterial vaginosis, trichomoniasis, and vulvovaginal candidiasis. Factors that increase the risk of infections are agents that have an impact on the normal microflora and therefore the acidity of the environment. Such factors include stress, frequent changes in sexual partner, sexually transmitted disease, and medical agents, both oral and topical, such as contraceptives, hormones and antibiotics (Chiaffarino, 2004; Andrist, 2001; Mashburn, 2012).

Bacterial vaginosis is the most common vaginal infection in women of reproductive age. The most frequently reported symptoms include vaginal discharge and foul odor, although as many as 60 % of women with bacterial vaginosis are asymptomatic. Although the risk of infection is increased by a frequent change in sexual partners, it is not considered a sexually transmitted disease. The etiology of bacterial vaginosis is not completely understood but there is a shift from the domination of *Lactobacillus* species to other bacteria such as *Gardinella vaginalis, Ureaplasma, Mycoplasma, Prevotella* and *Mobiluncus.* These bacteria produce enzymes that degrade the mucus layer; some species are also known to produce inflammatory proteins that are associated with risk of complications. In pregnancy, there is a risk of preterm labor and birth, and late fetal loss. There is also a risk of development of further infections such as pelvic inflammatory disease, endometritis and a increased susceptibility of sexually transmitted diseases such as HIV type 1, herpes simplex virus, *Neisseria gonorrhoeae* and *Chlamydia trachomatis* (Brotman, 2011; van der Pol, 2010).

The recommended treatment of bacterial vaginosis by the Center for Disease Control in the USA is metronidazole, orally or intravaginally, both being equally efficient. As metronidazole is not teratogenic the treatment of symptomatic cases in pregnancy is recommended. Screening for non-symptomatic infection is not recommended as the beneficial effect of treatment on pregnancy outcome has been shown to be inconclusive. Recurrence of infection after treatment with the recommended regiment of metronidazole is common, and has been shown as high as 69 % (Riggs and Klebanoff, 2004; Mashburn, 2012; Sobel and Sobel, 2015).

Trichomonas vaginalis is a parasitic, pear-shaped protozoan that adheres to the vaginal epithelium and causes inflammation and destruction of the epithelial cells by releasing cytotoxic substances. It is the most common curable and non-viral sexually transmitted disease worldwide. The symptoms of trichomoniasis are an increase of in malodorous, greenish-yellow discharge and vaginal irritation, although many women are non-symptomatic. In pregnancy trichomoniasis is associated with preterm birth, low birth weight and preterm rupture of membranes. (Mashburn, 2012; Van Der Pol, 2010). Recommended treatment for trichomoniasis in the United States is either metronidazole or tinidazole, both orally. Both treatment options have a cure rate of around 90 %. Recurrent infection is often a result of lack of treatment of the sexual partner. Treatment of infection in pregnancy is recommended, as there is no data that indicates that metronidazole is teratogenic (Mashburn, 2012).

In the treatment of these metronidazole-sensitive vaginal infections with metronidazole there is an additional advantage. While many antimicrobial agents are damaging to the natural microflora, metronidazole has been demonstrated to not affect the lactobacillus strains in the vagina. This leaves them able to maintain the beneficial pH in the vaginal environment protecting from further infection from opportunistic pathogens (Melkumyan *et al.*, 2015).

Approximately 75 % of healthy women in the USA experience an episode of vulvovaginal candidasis during their reproductive years, and 40-45 % will

have recurrent episodes. Most cases are caused by the strain *Candida albicans*, with *C. glabrata* as the second most common. The cases not caused by *C. albicans* are more treatment resistant. Symptoms of vulvovaginal candidasis are vaginal and vulvar itching, pain, burning and soreness, although these symptoms are not specific. Many women reporting to have a "yeast" infection may be suffering from bacterial vaginosis or trichomoniasis; a self-diagnosis has an accuracy only of 35 % (Gaur *et al.*, 2010; Johnson, 2010). There are a variety of imidazole preparations available over the counter. These are local treatment options of creams and vaginal suppositories. For uncomplicated infections these are sufficient (Mashburn, 2012).

Although these infections increases the risk of complications in pregnancy, there has not been shown any benefit of treating the infections during pregnancy on the risk of preterm birth or low birth weight (Riggs and Klebanoff, 2004). The recommended treatment given for the United Stated agrees with the treatment recommended for Norway (Folkehelseinstituttet, 2012).

1.2 Vaginal drug delivery

1.2.1 The vagina as a site for drug delivery

Vaginal drug delivery has traditionally been used for local acting drugs such as anti-microbial, anti-inflammatory, and contraceptive drugs. Local application gives higher concentration on the site of action compared to systemic oral delivery, making the administration of lower doses possible and decreasing the risk of adverse reactions (Machado *et al.*, 2015).

If the aim of vaginal administration is that the drug reach systemic circulation there are also many advantages. The large surface area in vaginal walls leads to rapid absorption of low molecular weight drugs to an area highly perfused with blood. Absorbed drugs through the vaginal walls avoid first pass metabolism, because the blood supply bypasses the liver. Other disadvantages of oral administration, such as the harsh climate of the gastrointestinal tract, are avoided (Vermesh *et al.*, 1988), which can lead to

increased bioavailability in some drugs, like propranolol (Patel *et al.*, 1983). After administration the drug needs to be dissolved in the vaginal cavity and absorbed across the epithelial membrane. The absorption of drugs through the mucosa occurs by three mechanisms. Either by passive diffusion across the epithelial cells, paracellularly via tight junctions, or through receptor-mediated transport (Woolfson *et al.*, 2000; Katz *et al.*, 2015).

In spite of these advantages there is limited use of the vagina as an administration route. Factors such as gender specificity, individual sensitivities, beliefs in personal hygiene, cultural sensitivities, local irritation, and influence of sexual intercourse limits the use of this route of administration (Srikrishna and Cardozo, 2013).

In vaginal delivery of drugs there are many factors that can influence the rational design of drug formulation. Purely physically the vagina can be seen as a tube extending at a 45° angle upwards in a standing woman before the angle increases to almost vertical, so there is a gravitational challenge in retention of administered drug. Many dosage forms is prone to leakage, leading to messiness and the need for increased dosing frequency (Baloglu *et al.*, 2009). The presence of cervicovaginal mucus will also affect the administered drugs penetration, distribution and residence time. There are challenges like the variation in the composition, amount, and viscosity of the cervicovaginal mucus depending on the stage of the menstrual cycle of a woman of fertile age. During ovulation the cervicovaginal mucus is more watery and the proteins are aligned in order to facilitate the passage of sperm through to the uterus, but the secretion is also considerable and facilitates a rapid clearance of administered drug. In a non-ovulating state the cervicovaginal mucus form a tighter meshwork, acting as a barrier protecting the epithelium. After the onset of menopause the amount of cervicovaginal mucus is reduced, bringing its own set of challenges (Ensign, 2014).

Hormonal changes through the menstrual cycle and in postmenopausal women also have an impact on the effect of administered drugs beyond the level of mucus secretion. The cyclical variations in the wall thickness and vascularity can affect the absorption of drugs. Hormonal changes can be both

natural changes in hormonal levels, such as cyclical variations and onset of menopause, and external changes, such as the administration of contraceptive hormones (Ashok *et al.*, 2012). Estradiol for instance drives the thickening and subsequent shedding of layers of the epithelium, providing a glycogen source that promotes the growth of *Lactobacilli* spp. In return, these bacteria secrete lactic acid, producing the acidic environment that hinders competition from other species and serves as protection from vaginal infection by pathogenic bacteria. A disruption of this reciprocal relationship by administered drugs or dosage forms can increase the risk of development of infections (Wong *et al.*, 2014; O´Hanlon, 2013).

Table 1.1: Physiological and physiochemical factors affecting vaginal delivery systems (Kale and Ubgade, 2013).

Vaginal physiology	Formulation factor affected	Effect at administration site
Vaginal axis due to woman's posture	Residence time Drug distribution	The API may leak out due to gravity. Entire vaginal cavity not accessible difficulty in homogeneous distribution.
Surface area (rugae)	Drug absorption rate	Increase in the surface area and hence the absorption rate.
Vaginal fluid	Amount of soluble drug	It varies through menstrual cycle and with onset of menopause.
Presence of mucus	Drug permeability	Thick mucus less permeable and thin mucus more permeable.
Epithelium thickness	Drug permeability	Thick epithelium less permeable and thin epithelium more permeable.
pH	Solubility and stability of drug	Ionic state of drug may change due to change in pH, affecting the solubility, absorption and stability of drugs.

Table 1.1 represents a summary of factors that may influence the effectiveness of drugs administered vaginally. In considering these factors that can affect the efficacy of the administered drug it is important to consider whether the drug is intended for local effect or if the drug is aimed for systemic treatment of some ailment. In some cases it can be very important to avoid any absorption of the drug such as in treating vaginal infection in pregnant women (Jøraholmen *et al.*, 2014).

1.2.1.1 Vaginally administered drugs

The drugs that are most commonly administered by the vaginal route are antimicrobial drugs and hormone therapy, either as contraceptive or hormone replacement therapy (Alexander *et al*., 2004). If the goal is systemic treatment via vaginal administration the permeability through the epithelium is highest for small lipophilic drugs. Any drug administered vaginally needs to have some degree of water solubility because vaginal fluids main constituent is water (Hussain and Ahsan, 2005). Commonly administered drugs for local treatment are antimicrobial drugs; antifungal drugs, such as imidazoles, and antibacterial drugs, such as metronidazole (Kale and Ubgade, 2013). In this work we chose to use metronidazole (Figure 1.4) as a model drug. Metronidazole has a bactericidal effect against susceptible organisms; it exerts its effect through production of free radicals by reduction of the nitro group (Sobel and Sobel, 2015). Metronidazole is poorly soluble in both aqueous and organic solvents, which makes it difficult to formulate (Kelly, 2012). It has a pKa of 2.5 and is neutral at the physiological pH of the vagina (Shalaeva *et al*., 2008).

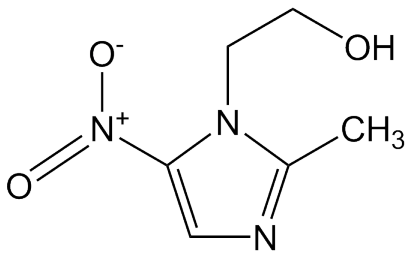
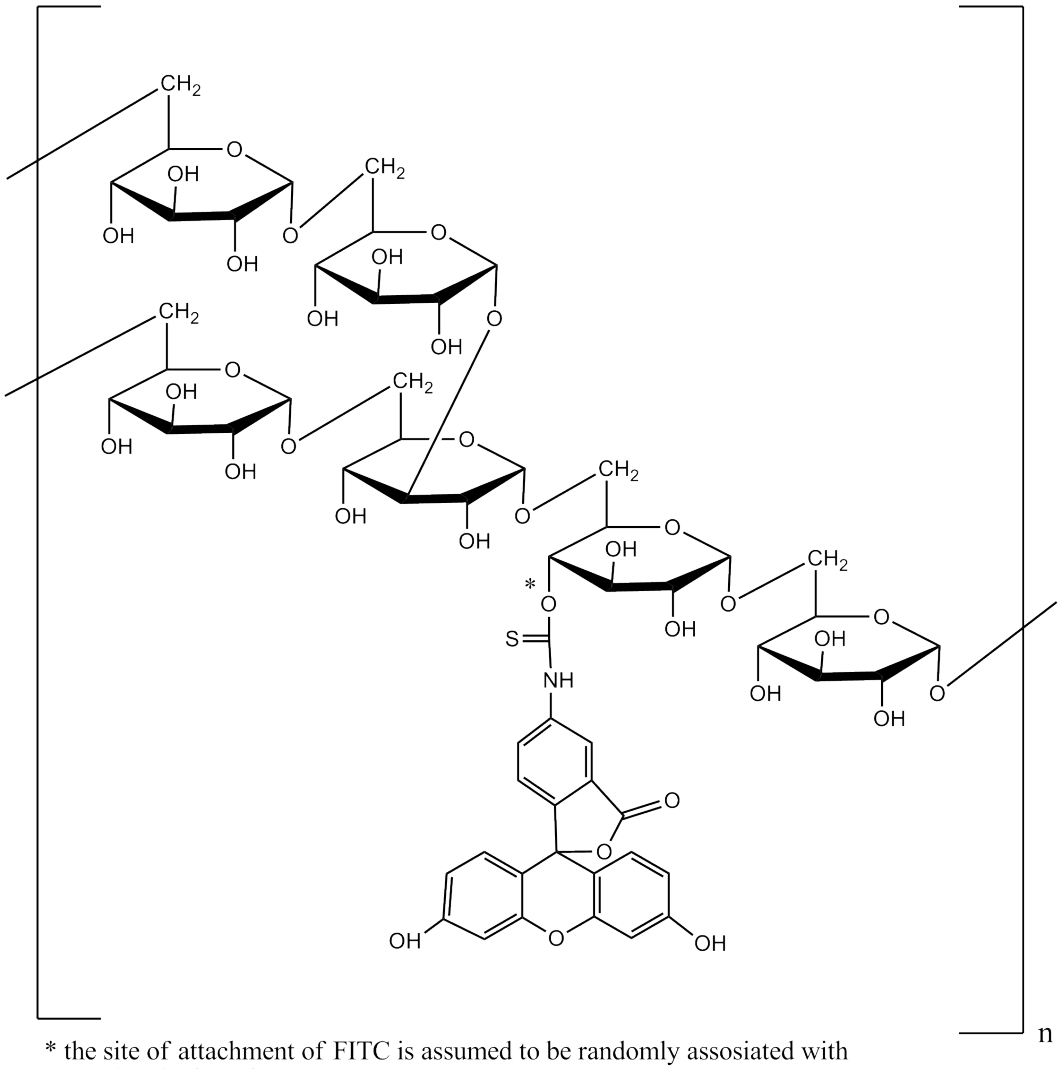


Figure 1.4: the structure of metronidazole.

In addition, in one part of this work we wanted to demonstrate the ability of our novel system to incorporate larger drug molecules. For this we used fluorescein isothiocyanate dextran (FITC-dextran; Figure 1.5) as a model substance, which is a long-chained polymer of anhydroglucose (dextran) with a fluorescent marker (FITC) attached (Dimler *et al*., 1955). It varies in chain length and molecular weight; in our work we employed two different FITC-dextrans with molecular weights of 4 and 20 kDa. FITC-dextran is hydrophilic and is extensively used to test cell permeability (Rutili and Arfors, 1976).



* the site of attachment of FITC is assumed to be randomly assosiated with any free hydroxyl group

Figure 1.5: Structure of FITC-dextran.

1.2.2 Conventional drug dosage forms

In their article on vaginal drug delivery Srikrisma and Cardozo (2013) suggests criteria for an ideal vaginal drug based on a selection of consumer surveys. The drug dosage form should not have a negative effect on coitus, be odorless and colorless, be suitable for application several hours before intercourse, not be associated with leakage, messiness, or feeling of vaginal fullness, cause no local irritation, and be amendable to use with or without an applicator. Srikrisma and coauthors comments further that the choice to use such dosage forms and the choice of type varies between individuals and their partner's preference, cultural norms, age, and economic, social and climatic conditions. Conventional vaginal drug dosage forms include tablets,

capsules, suppositories, intravaginal rings, and topical drug products such as creams, gels and ointments (Kale and Ubgade, 2013; das Neves *et al.*, 2014b).

Vaginal gels are the most established vaginal drug delivery system and they have the benefit of being easy to manufacture. Gels can be homogeneously applied on the mucosal surface, and the aqueous character provides hydration and lubrication. This can be considered highly beneficial because with the onset of menopause and also in some pathological condition, vaginal dryness is characteristic. However they can be characterized by a general messiness in application, being uncomfortable, and having a tendency to leak into the undergarments. Gels have the additional drawback that it can sometimes be difficult to achieve an exact dosage (das Neves and Bahia, 2006; Hussain, 2005).

Vaginal rings are delivery devices designed to have a controlled release of drug after insertion. They are mainly used to deliver hormones as contraceptive or hormone replacement devices. They are generally rings of 5-6 cm in diameter where the active ingredient is homogeneously dispersed throughout, and have a sandwich or reservoir delivery system (Alexander *et al.*, 2004).

Vaginal suppositories (pessaries) are similar to rectal suppositories. They are mainly used for local infection, but occasionally used to achieve systemic absorption of the active substance. They are, unlike rectal suppositories, formulated with water-soluble or water-miscible bases, like glycerol-gelatin or polyethylene glycols. After insertion in the vagina they dissolve due to the high solubility of the polymers (Hussain and Ahsan, 2005).

Vaginal tablets are designed to rapidly disintegrate in the small volume of the vaginal cavity and rapidly release the active drug; many of the vaginal tablets on the marked are antibiotics. Formulations of tablets for vaginal administration often contain the same ingredients as oral tablets, although mucoadhesive polymers can be used to improve the residence time (Kale and Ubgade, 2013).

1.3 Novel drug delivery systems promising for vaginal therapy

1.3.1 Nanomedicine in drug delivery

Nanomedicine represents a great potential in improved drug delivery of drugs characterized by low bioavailability, either from low solubility or low permeability (Cho *et al.,* 2008). Nano means one billionth, it is a prefix that denotes 10^{-9}, is often used is science and electronics to describe time and length. It is used to describe particles and vesicles in the nanometer size range, often limited in nanotechnology to the range of 0-100 nm, but in applied in medicine the range is extended to include particles up to 1 micron. Nanomedicine includes a wide range of different carriers such as liposomes, polymeric micelles, dendrimers, nanosuspensions, nanoemulsions, nanospheres, and nanotubes (Karn *et al.,* 2011; Nguyen *et al.,* 2011; Zaru *et al.,* 2009, Vanić and Škalko-Basnet, 2014; Berginc *et al., 2014;* Casettari and Illum, 2014; Calderon *et al.,* 2013; Li *et al.,* 2009). Nanomedicinal drug delivery systems can be used to deliver a wide range of substances ranging from small drug molecules to larger molecules such as proteins, nucleic acids, and antigens. Use of nanomedicine can, in addition to solubilizing poorly water-soluble drugs, enable drug targeting, reduce dosage and thereby toxicity of drugs, and improve absorption across epithelial and endothelial barriers (Wong *et al.,* 2014; das Neves *et al.,* 2014a).

1.3.1.1 Liposomes

These spherical structures of bilayers formed by the suspension of egg yolk lecithin in water, known as liposomes where first described by Alec Bangham and co-workers in the mid 60' (Bangham *et al.,* 1965). The phospholipids of the lecithin have a tendency to, because of their amphiphilic nature, automatically form bilayers upon agitation in an aqueous media (Figure 1.6). Initially liposomes where studied as a model system for biological membranes, but their potential in drug delivery was soon recognized (Gregoriadis, 1981) for their innate abilities such as self assembly to a closed relatively permeable membrane, and their potential for easy manipulation of surface characteristics and size, and ability to carry both lipophilic and hydrophilic substances (Janoff, 1999).

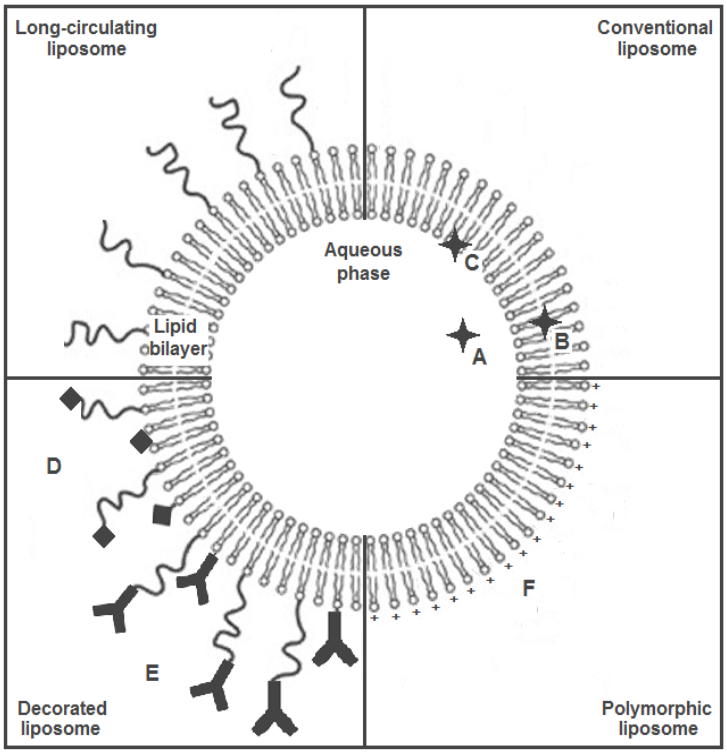


Figure 1.6: Structural composition of liposomes with different surface characteristics. Reprinted with permission from InTech (Lopes *et al.*, 2013).

Liposomes are classified according to their size and the number of bilayers (i.e. lamellarity), which can vary with the method of preparation, and later be altered by size reduction techniques. Liposomes can consist of either a single lipid bilayer or several concentric bilayers. Unilamellar liposomes can range from the smallest liposomes, small unilamellar vesicles, which can be as small as 100 nm, to large unilamellar vesicles, ranging from 200 to 800 nm. Multilamellar vesicles are larger, in the size range of 500 to 5000 nm (Torchilin, 2005). The most common methods of production of liposomes include the film hydration method, reverse phase evaporation, ultra sonication, or detergent removal from mixed lipid-detergent micelles by dialysis, gel filtration, or freeze-thawing (Torchilin, 2012).

In addition to the manipulation of size of the liposomes by method of manufacture or by subsequent size reduction, there are various ways to manipulate the liposomes in regards to surface characteristics, stability and targeting as illustrated in Figure 1.6 (El-Hammadi and Arias, 2015). The lipid

composition can be altered to change the membrane fluidity. Addition of cholesterol will increase the membrane stability (Kirby *et al.,* 1980), and the addition of polyethylene glycol on the surface can prolong the circulation of the liposomes (Gabizon, 2001). Because liposomes are able to increase the solubilization of poorly soluble drugs, they are investigated for the use in a wide variety of applications. As mentioned above, both lipophilic and hydrophilic drugs can be entrapped in liposomes, hydrophilic drugs (A, in Figure 1.6) are incorporated in the inner aqueous phase of liposomes; lipophilic drugs (B) are incorporated in the liposomal bilayer; amphiphilic drugs (C) can be found in the interface between the lipid bilayer and the inner aqueous phase. Long-circulating liposomes present a hydrophilic polymer attached to the liposome surface. The decorated liposomes can be subdivided as surface-modified liposomes (D) or immunoliposomes (E). Ligands can be directly attached to the liposome surface or to the extremity of a hydrophilic polymer. The cationic liposomes (F) are a type of polymorphic liposome used in the intracellular delivery of DNA (Lopes *et al.,* 2013).

In order to improve the applicability of the liposomal formulations and improve the stability of the liposomes, liposomes have been incorporated in bioadhesive hydrogels, Carbopol is one such which is frequently used (Pavelić *et al*., 2005a; Pavelić *et al*., 2005b). Incorporation of liposomes in hydrogel has been shown to result in prolonged retention time of the formulation at the site of administration (Pavelić *et al*., 2001; Vanić and Škalko-Basnet, 2013).

There are a limited amount of pharmaceutical preparations approved for clinical use, such as AmBisome, Mycet and DaunoXome, liposomal formulations of amphotericin B, levocetirizine and daunorubicine respectively (Torchilin, 2012).

1.3.1.2 Preparation of liposomes by solvent injection

The solvent-injection method of Gentine *et al.,* (2012), which was the basic method from which the one-pot preparation method was developed in this study, is in it self a modification of the more frequently used ethanol injection method (Batzri and Korn, 1973). In this original method a lipid film is

redispersed in a small amount of ethanol and injected into an aqueous solution (Figure 1.7).

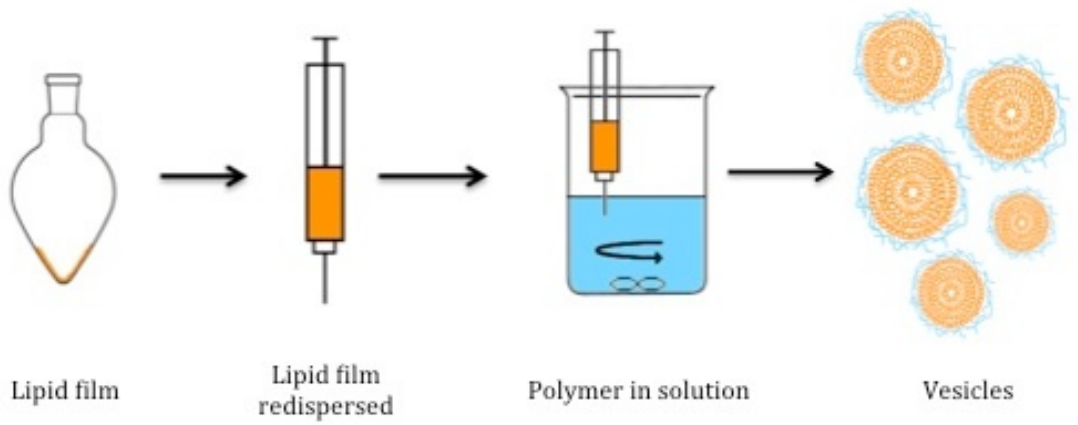


Figure 1.7: the solvent injection method (Andersen *et al.*, 2013, Graphical abstract).

This is a method where factors such as lipid type and concentration, type of aqueous media, stirring speed of the aqueous media, injection speed and volume will affect the vesicle size and lamellarity. Although a great advantage of this method is the possibility to upscale the method to an industrial level, there is also great variability on the vesicles produced, varying with the factors described above (Pons *et al.*, 1993; Wagner *et al.*, 2002). Gentine and coworkers performed a study to investigate the possibility of improving this method by changing the solvent used from ethanol to a different organic solvent. After testing a selection of short-chained alcohols and ethyl acetate, and evaluating the stability of the resulting liposomes in regards to size, lamellarity and permeability, they found isopropanol to be the best candidate. Similarly, different solvents were tested in the start of this study. Characterizing the resulting liposomes with regards to stability of size, n-propanol was chosen as the best solvent under the conditions used in our study.

1.3.2 Mucoadhesion

Bioadhesion can be defined as the state where two materials, where at least one is of a biological nature, comes in contact and stay together for a substantial amount of time due to the establishment of interfacial bonding.

When the biological surface is a mucosal surface it is referred to as mucoadhesion (Sosnik *et al.*, 2014). Cervicovaginal mucus contains mucin, like all mucosal gels, which is the main constituent responsible for mucoadhesion. Mucins are a family of heavily O-glycosylated macromolecules that forms a mesh that allows the exchange of nutrients, but will inhibit the passage of particles (Vanić and Škalko-Basnet, 2014). There are several different theories that are suggested to explain the mechanisms of adhesive interactions; these are summarized in Table 1.2.

Table 1.2: Theories used to explain the mucoadhesive interactions (Sosnik *et al.*, 2014).

Theory	Mode of adhesion
Electronic theory	Adhesion is established due to the electrostatic attraction between the negatively charged mucin and positively charged materials.
Adsorption theory	Adhesive interactions are related to the establishment of hydrogen and van der Waals bonding; hydrophobic effects and chemisorption may also contribute.
Wetting theory	Adhesion is related with the ability of a mucoadhesive (when in liquid form) to spread over the mucus layer.
Diffusion theory	Adhesion is established by the interpenetration of macromolecular mucoadhesives (either polymeric or other) with mucin fibers, as driven by a contraction gradient differential.
Fracture theory	Regulates adhesion with the force required for interfacial detachment of two previously joint solid surfaces.
Mechanical theory	Adhesion is dependent on the roughness of two different surfaces and the available area for interaction.

None of these theories explains fully the mechanisms of adhesive interactions; a combination of them must be used as an explanation depending on the characteristics of the substances interacting. Mucoadhesion starts with a stage where the dosage form wets and swells (wetting theory), then physical, non-covalent bonds forms with the mucus-polymer interface (electronic and adsorption theories). After this the polymer and protein chains interpenetrate (diffusion theory) and entangle and form more non-covalent (physical) and covalent (chemical) bonds (electronic and adsorption theory) (Khutoryanskjy, 2011; Smart, 2005).

For a drug delivery system to be efficient in delivering its drug to the mucosal surface it is dependent on several factors. Firstly, the nanosystem must have good distribution and retention at the mucosal site. Secondly, the drug delivery system must have a good infiltration and penetration through the mucus mesh. Finally, the release profile of the drug should be optimal for a good concentration of the drug at the active site. These factors are determined by the characteristics of the nanosystem, such as size, shape, and surface characteristics (Vanić and Škalko-Basnet, 2014).

The mucus is arranged in a mesh that is a continuously renewed, semipermeable, viscoelastic barrier. It has been shown to have pores in the range of 50-1800 nm with an average pore size of 340 ± 70 nm; this mesh is shown in Figure 1.8 (Lai *et al.*, 2007; Lai *et al.*, 2010). Particles and vesicles in the nanosize range have the ability to 'fit' into the pores of the mucus mesh, so the diameter of the particles influences the interaction with the mucosal surface in addition to the surface characteristics of the particles, which influences the repulsion or attraction with the mucin chains (Cone, 2009).

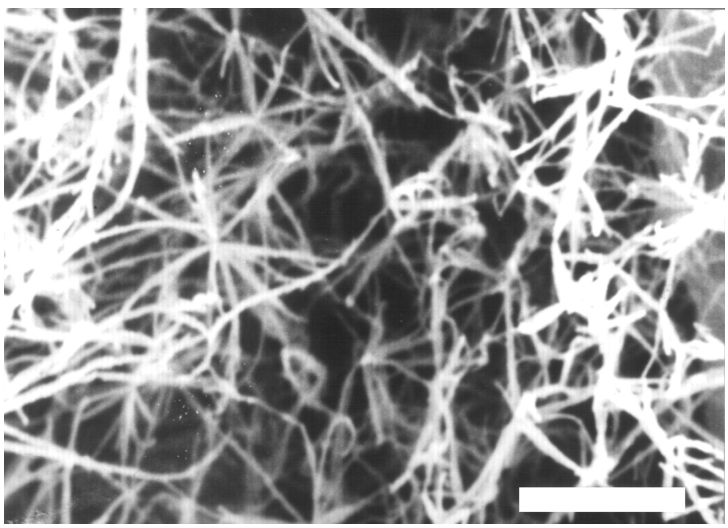


Figure 1.8: Network-shaped mesh of oestrogenic mucus with showing the pore structure (Ceric et al., 2005 Printed with permission from Oxford University press).

It is also possible that the retention of particles is related to physical entrapment within the mucus. Wang and colleagues has demonstrated that non-adherent particles in the size range of 100 nm are more hindered than larger particles in the range of 200-500 nm. This has been explained by the

idea that smaller particles have the ability to pass through more narrow channels in the mucus and can end up hindered in dead ends, while larger particles must diffuse through larger pores that generally are more unhindered (Wang *et al.,* 2008).

1.3.3 Mucoadhesive materials

The main strategy of increasing the mucoadhesion on liposomes is based on the use of materials capable of interacting with mucin in mucoadhesive bonds (Caramella *et al.,* 2015). The use of polymeric materials in the surface modification of nanopharmaceuticals has been shown to be advantageous. Both the large surface area of the nanovesicles and the interaction of the surface-tethered polymer will substantially enhance the adhesive interaction (das Neves *et al.,* 2011).

1.3.3.1 Chitosan

Chitosan is obtained by n-deacetylation of chitin; a natural, linear polysaccharide found in the shells of crustaceans and insects, and in fungi. The main source of chitosan is as a waste product of the shellfish industry. The structure consists of β(1-4) linked glucosamine and N-acetylglucosamine subunits. The structure of chitosan is shown in Figure 1.9. Chitosan is biocompatible, biodegradable, and non-toxic making it an interesting substance as a pharmaceutical excipient (Kean and Thanou, 2010; Pal *et al.,* 2013). It has also shown excellent mucoadhesive properties making it interesting for the development of a controlled release drug delivery system (Baldrick, 2010; Singla and Chawla, 2001).

Chitosan can be classified by the degree of deacetylation (DD), which determines the physiochemical and biological properties. Some physiochemical properties such as solubility, viscosity and biocompatibility has a proportional relationship with the DD, while others like biodegradability and crystallinity have an inversely proportional relationship with the DD. Most biological properties such as mucoadhesion, antimicrobial effect, and the permeation enhancing effect all have a proportional relationship with the DD (Dash *et al.,* 2011).

Chitosan has a potent antimicrobial effect. There are two proposed mechanisms for this antibacterial and antifungal activity. One is that the positively charged chitosan can interact with the negatively charged groups on the surface of cells, and thus alter their permeability and lead to leakage of essential molecules or solutes from the cells. The other proposed mechanism involves binding of chitosan with the cell DNA, leading to the inhibition of the RNA synthesis in the microbes. Probably there is a combination of these two mechanisms (Croisier and Jérôme, 2013).

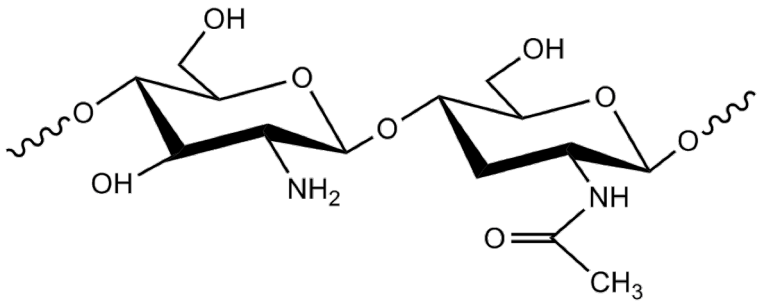


Figure 1.9: Chemical structure of chitosan.

As other cationic polymers chitosan has a permeation enhancing ability. It opens the tight junctions through interactions between the positively charged amines of the polymer and the negatively charged sialic groups on the membrane bound glycoproteins (Laffleur and Bernkop-Schnurch, 2013).

Chitosan has demonstrated higher mucoadhesive properties after the introductions of thiol groups, while still maintaining its biodegradability. This has been utilized in the formulation of controlled release vaginal tablets for the cationic drug clotrimazole for improved treatment of *C. albicans* (Kast *et al.*, 2002).

In drug delivery chitosan is widely used as an excipient in emulsions, tablets, gels and films where it can provide controlled release of the incorporated drugs; it has also been extensively used as a constituent in nanomedicine; chitosan-based nanoparticles, nanoemulsions and as a coating material for liposomes (Bernkop-Schnurch and Dunnhaupt, 2012; Perioli *et al.*, 2008; Li *et al.*, 2009; Zaru *et al.*, 2009; Calderon *et al.*, 2013; Casettari and Illum, 2014;

Vanić *et al.*, 2014; Jøraholmen *et al.*, 2014; Berginc *et al.*, 2014; Abruzzo *et al.*, 2012).

1.3.3.2 Pectin

Pectin is another natural polysaccharide that exists in the cell wall of higher plants; it is commercially obtained from the peel of citrus fruits and apple pomace. It is a linear polysaccharide consisting of α(1-4) D-galacturonic acid with a carboxyl group that can be esterified with a methyl group, the structure is shown in Figure 1.10. The degree of esterification (DE) can vary with the source and condition applied during isolation, and is used to classify the pectins; together with the molecular weight the DE this determines the gelling ability and viscosity of pectin (Sriamornsak, 2003). Pectin is biodegradable, biocompatible and non-toxic, which together with the low price and easy availability makes it a promising polymer in drug delivery (Klemetsrud *et al.*, 2013).

Pectin has well known mucoadhesive properties, and the mucoadhesive mechanism is well characterized. Adsorption to mucin through hydrogen bonding seems to be the main mechanism, with a contribution of electrostatic repulsion in an aqueous media that unfolds the polymer chains so that there is more interaction with mucin (Sosnik *et al.*, 2014).

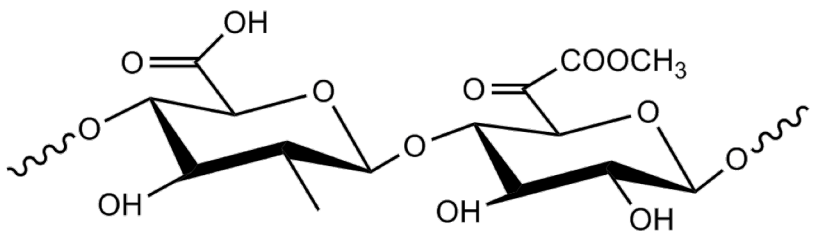


Figure 1.10: Chemical structure of pectin.

1.3.3.3 Carbopol

Carbopols are high molecular weight polymers of acrylic acid, Figure 1.11, and have been used in liquid and semisolid pharmaceutical formulations, as thickening and viscosity increasing agents, in gels, suspensions and emulsions. It has also been used for its mucoadhesive properties.

Formulations include ophthalmic, nasal, buccal, topical, intestinal and vaginal (Bonacucina *et al.*, 2004).

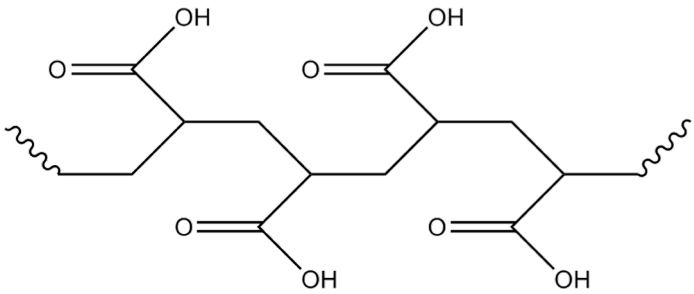


Figure 1.11: Chemical structure of Carbopol.

There are many types of Carbopol; all similar on the chemical levels as high molecular weight polyacrylic acid polymers. They differ by the density of cross-linking and whether they are homopolymers or copolymers with different chain length (C10 – C30) alkyl acrylate cross-linked with allyl pentaerythritol. There are also differences in the cosolvent used in polymerization; benzene being the "traditional" solvent is not desirable from a toxicological point of view. Carbopol 974P NF (used in this study) is a homopolymers where ethyl acetate is used in the polymerization process, so it has a better toxicity profile (Lubrizol, 2015).

Polyacrylic acids display high bioadhesive bond strength upon contact with biological tissues. The presence of unionized carboxylic acid in such numbers, one per monomer in the polymeric chains, gives the ability to form many hydrogen bonds between the polymer and mucin (Sosnik *et al.*, 2014).

2 Aims of the thesis

The overall aim of this thesis was to develop a novel mucoadhesive delivery system able to improve the treatment of vaginal infections.

The specific aims were:

- To develop a novel mucoadhesive system that would allow for a straightforward and simple preparation procedure with potential for scale up.
- To demonstrate the ability of the novel system to serve as a carrier for both small drugs, as well as larger drug molecules such as biologicals.
- To characterize the new system for its mucoadhesiveness, as well as confirm that the preparation method can be applied for various polymers.
- To demonstrate the sustained drug release characteristics of the new system.
- To demonstrate the antifungal activity of the novel chitosan-containing system against *Candida albicans* based on the antifungal activity of chitosan.

3 Summary of papers

3.1 Paper I

In this paper we developed novel polymer-containing liposomes prepared by a simple preparation procedure for mucoadhesive liposomes.

Using chitosan and pectin, both of two different degrees of deacetylation and esterification respectively, in the aqueous phase of the solvent injection method we achieved a one-pot preparation method of the polymer-containing liposomes. These were named chitosomes[1], for the chitosan-containing liposomes, and pectosomes[1], for the pectin-containing liposomes.

Both chitosomes and pectosomes were characterized for their size, zeta potential, and entrapment efficiency of the model drug, metronidazole. Results showed a strong indication of the polymers presence both on the surface of the vesicles and embedded inside the vesicles. This indicated a novel type of coating with improved properties as compared to plain liposomes.

Liposomes were furthermore characterized for the stability of the entrapment and size. This indicated an ability of the polymer-containing liposomes to retain more of the entrapped drug compared to the plain liposomes.

[1] The name was changed in the second article to *polymer*-containing liposomes.

3.2 Paper II

In the second paper we wanted to further study the novel polymer-containing liposomes. As the liposomes formulated with chitosan with 77 % degree of deacetylation showed the most promise we chose to use this in the further characterization. In this study the chitosan-containing liposomes (also known as chitosomes) were compared to chitosan-coated liposomes, to show the improvement of the novel coating technique compared to conventional coating, in addition to plain liposomes.

To show the chitosan-containing liposomes ability to entrap a larger variety of drugs, such as biologicals, the model substances chosen in this study was fluorescein isothiocyanate dextran of molecular weight 4 and 20 kDa. These demonstrated the size range of the substances that the chitosan-containing liposomes were capable of entrapping.

In addition the liposomal formulation were characterized for their surface availability of chitosan, mucin-binding properties and the *in vitro* release on Franz diffusion cells of the FITC-dextrans from the vesicles. The lower percentage of surface-available chitosan in the chitosan-containing liposomes, when comparing to the chitosan-coated liposomes, served as a confirmation that a portion of the chitosan was embedded within the liposome. This difference between the formulations was also shown to affect the mucin-binding ability of the vesicles.

Tests of the *in vitro* release showed a sustained release of the incorporated drug from all formulation compared to the free drug, with the chitosan-containing liposomes having a quite stable release profile for both of the model substances.

3.3 Paper III

In the final paper we continued to characterize the chitosan-containing liposomes. As a comparison Carbopol-containing liposomes was used as Carbopol is a polymer frequently used in vaginal drug delivery.

In this paper we wanted to investigate the biological activity of the chitosan-containing liposomes. As chitosan is known to have an antifungal effect, we wanted to investigate if the chitosan-containing liposomes had an inhibiting effect on the growth of *Candida albicans* a pathogen that is responsible for many vaginal "yeast" infections. Both empty and drug loaded chitosan-containing liposomes was tested for their antifungal activity, with Carbopol-containing liposomes and plain liposomes as a comparison.

In addition we wanted to characterize the *in vitro* release of a antimicrobial drug, metronidazole, as this would be a beneficial addition to a system that had an antifungal activity on vaginal infections, which can often be caused by several pathogens or lead to secondary infections by other pathogens.

4 Materials and methods

4.1 Materials

Soy phosphatidylcholine (SPC; Lipoid S100 Lipoid GmbH, Ludwigshafen, Germany) was the generous gift by Lipoid GmbH. Chitosan of varying degree of deacetylation, Fiske-SubbaRow reducer, fluorescein isothiocyanate dextran of two different molecular weights (FITC-dextran 4 and FITC-dextran 20 corresponding to Mw 4 and 20 kDa respectively), metronidazole, methanol, mucin from porcine stomach type II, *n*-propanol, phosphorus standard, and Triton X solution were purchased from Sigma Aldrich Inc. (St. Luis, MO, USA). Pectins of various degree of esterification were the product of Herbstreith and Fox KG (Neuenbürg, Germany). Ammonium molybdate and peroxide were purchased from Merck KGaA (Darmstadt, Germany), while sulfuric acid was purchased from May and Baker LTD (Dagenham, England). Cibacron Brilliant Red 3B-A was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Sepharose CL-4b gel was ordered from Pharmacia Bioteck AB (Uppsala, Sweden). Potato dextrose broth was purchased from Difco (BD, Franklin Lakes, NJ, USA) All other chemicals used in the experiments were of analytical grade.

4.2 Viscosity of polymer solutions

The viscosity of the aqueous solutions of polymers was measured using a rotational viscometer (Haake Viscotester 7 plus, Thermo Electron GmbH, Karlsruhe, Germany) with a TL 5 spindle. The polymer concentrations were adjusted to the viscosity of the aqueous solutions in the range of 0.65 ± 0.20 mPa*s.

4.3 Preparation of vesicles

4.3.1 Preparation of polymer-containing vesicles

Liposomes were prepared by adapting the solvent injection method originally described by Gentine *et al.* (2012). Lipid (200 mg) and 20 mg of metronidazole (for Paper I and III) were dissolved in methanol. For liposomes formulated with FITC-dextran (Paper II) the hydrophilic model substance (42.0 mg) was dissolved in the water phase. The solvent was evaporated using a vacuum rotary evaporation system (Büchi rotavapor R-124 with vacuum controller B-721, Büchi Vac V-500, Büch Labortechnik, Flawil, Switzerland). The resulting lipid film was redispersed in 100 μL of *n*-propanol. The dispersion was injected via needle into 2 mL of aqueous polymer solution of chitosan, pectin, or Carbopol, and stirred for 2 hours at room temperature. Two grades of chitosan with two different degrees of deacetylation (77 % DD and 95 % DD, respectively) were used, as well as two grades of pectin with two different degrees of esterification (35 % DE and 50 % DE, respectively). The dispersions were left in a refrigerator overnight prior to vesicle size reduction and characterization. For comparison in the *candida* assay drug-free chitosan-containing liposomes were prepared under the same conditions as the polymer-containing liposomes with metronidazole, except with the omission of metronidazole in the lipid film.

4.3.2 Plain liposomes

Plain, non-mucoadhesive liposomes, were prepared under the same conditions using the same lipid concentration, and the same metronidazole concentration (for Paper I and III) to prepare the film, which was subsequently redispersed and injected into distilled water, containing hydrophilic model substance; FITC-dextran 4 or 20 (for Paper II). The dispersions were left in a refrigerator overnight prior to vesicle size reduction and characterization.

4.3.3 Vesicle size reduction

The polymer-containing and plain (non-mucoadhesive) liposomes were reduced to a smaller size by sonication using a Sonics High Ultrasonic Processor (Sigma Aldrich Chemie GmbH, Steinheim, Germany). Prior to sonication, the samples were diluted to a suitable volume with distilled water and sonicated for an appropriate time to achieve the desired size range of the vesicles.

4.3.4 Chitosan coated liposomes

Coating of sonicated plain liposomes was performed following a method previously described (Jøraholmen *et al.*, 2014). In brief, chitosan solution (0.1 % w/v) in acetic acid (0.1 % v/v) was added drop-wise to an equal volume of liposomes under controlled magnetic stirring at room temperature for 1 hour. The dispersions were left in a refrigerator overnight before characterization.

Table 2.1: Constituents of prepared formulations by location during vesicle preparation.

Paper	Type of liposomes	Film composition		Aqueous phase	
I	Chitosomes[1,2]	SPC	MTZ	Chitosan	
	Pectosomes[1,2]	SPC	MTZ	Pectin	
	Plain	SPC	MTZ		
II	Chitosan-containing	SPC		Chitosan	FITC[1]
	Chitosan-coated	SPC		[3]	FITC[1]
	Plain	SPC			FITC[1]
III	Chitosan-containing	SPC	MTZ	Chitosan	
	Carbopol-containing	SPC	MTZ	Carbopol	
	Plain	SPC	MTZ		

1. Two different formulations, with either different qualities of the polymer or different Mw of the model substance. 2. The name was changed in the second article to *polymer*-containing liposomes. 3. Formulation also contains chitosan but this was added in a subsequent coating step. SPC: Soy phosphatidylcholine, MTZ: metronidazole, FITC: FITC-dextran 4 or 20.

4.4 Entrapment efficiency

4.4.1 Entrapment of metronidazole

To remove unentrapped metronidazole from the liposomal dispersion, the liposomes were dialyzed against distilled water for 4 hours at room temperature (Cellulose hydrate barrier, Mw cut off: 12-14 kDa; Medicell International Ltd, London, UK). The volume was adjusted to assure sink conditions.

The amount of drug entrapped in the liposomal formulations was quantified by UV-spectrophotometry (Agilent Technologies, Santa Clara, CA, USA). Liposomal samples were dissolved in methanol and metronidazole concentrations measured at 311 nm.

4.4.2 Entrapment of FITC-dextran

In order to remove the unentrapped FITC-dextrans from liposomes two different separation methods were used, depending on the molecular weight of the model substance. For the liposomes containing FITC-dextran 4 dialysis was performed for 24 hours at room temperature. For liposomes containing FITC-dextran 20 column separation on a Sepharose CL-4B gel was used.

The entrapment efficiency of the liposomal formulation was determined of fluorescence spectroscopy using a Polarstar fluorimeter (Fluostar, BMG Technologies, Offenburg, Germany) on excitation and emission wavelengths of 485 and 520 nm, respectively. To dissolve lipid, liposomal samples were pretreated by addition of 10 % (v/v) of Triton X in a volume ratio of 1:1.

4.5 Characterization of vesicles

4.5.1 Phosphorous assay

The content of phosphatidylcholine was measured following a method previously described (Bartlett, 1959). In brief, the samples were diluted to

appropriate concentration in distilled water and an aliquot (1 mL) was mixed with 0.5 mL of 10 N H_2SO_4 and heated at 160 °C for a minimum of 3 hours. After cooling, two drops of H_2O_2 were added and the mixture heated to 160 °C for 1.5 hour. Then ammonium molybdate (4.6 mL; 0.22 % v/v) and 0.2 mL of Fiske-SubbaRow reagent were added after cooling, vortexed, and the mixture was heated for 7 min at 100 °C. All samples were analyzed by UV spectrophotometry at 830 nm.

4.5.2 Particle size analysis

The particle size distributions of the non-sonicated liposomes were determined by photon correlation spectroscopy on a Zetasizer 3000HS (Malvern Instruments, Oxford, UK). The measurements were performed at a scattering angle of 90° and a temperature of 25 °C. The dispersions were diluted with 1 mM NaCl, which was previously filtered though 200 nm Minisart filters, to achieve a count rate between 100 and 300 kcps (Vanić *et al.*, 2013).

The morphology and particle size distributions (based on the number of particles) of the non-sonicated liposomes were also estimated with the aid of and Olympus BH-2 microscope equipped with a computer-controlled image analysis system (Optomax V, Cambridge, UK) In all measurements 1000 particles were examined (Škalko *et al.*, 1998).

The size distributions of sonicated liposomes were measured by photon correlation spectroscopy using a Submicron Particle-sizer (Model 370, Nicomp, Santa Barbara, CA, USA). Both Gaussian and Nicomp algorithms were fitted to the experimental data to find the distribution the best describes the particle population (di Cagno *et al.*, 2011). As the fit error was found to be smaller than 1.5 and the residual error was smaller than 10, Nicomp distribution was selected. The volume-weighted distribution was used to determine the mean diameter and polydispersity index of all samples.

4.5.3 Zeta potential determination

The zeta potential of all liposomes was measured on a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd, Oxford, UK). The instrument was calibrated throughout the measurements using Malvern zeta potential transfer standard (-50 ± 5 mV and -42 ± 4 mV). The samples were diluted in filtered water until an appropriate count rate was achieved and measured in a disposable folded capillary cell. All measurements were performed at room temperature and the results represent an average of at least three independent measurements (Jøraholmen *et al.,* 2014).

4.5.4 pH measurement

pH was determined both in the polymer solutions and the dispersions of polymer-containing liposomes in order to elucidate whether, and to what extent, an interaction between polymer and liposomes took place. A change in pH of the polymer solution after the injection of dissolved lipids may be interpreted as a ”loss” of dissolved polymer, i.e., polymer could be entrapped or closely associated with the liposomes. pH was measured at room temperature using a calibrated pH meter (Metrohm AG, Herisau, Switzerland).

4.5.5 Determination of surface-available chitosan

To determine the surface-available chitosan the colorimetric method originally reported by Muzzarelli (1998) was applied. Glycine buffer (pH 3.2) was prepared by dissolving 1.87 g of glycine and 1.46 g of NaCl in 250 mL of distilled water; an aliquot of 81 mL was further diluted with 0.1 M HCl to a final volume of 100 mL. Cibacron Brilliant Red 3B-A (150 mg) was dissolved in 100 mL of distilled water. The dye solution (5 mL) was further diluted to 100 mL with the glycine buffer. Liposomal suspensions were diluted with distilled water to desirable concentration (1:2, v/v) before 3 mL of the final dye solution was added. UV absorbance was measured photometrically at 575 nm (Agilent technologies, Santa Clara CA, USA). The surface-available chitosan was calculated using equation 1:

$$Surface - available\ chitosan = \frac{C_s}{C_C} \times 100 \qquad \text{Eq. 1}$$

where C_S is the concentration of surface-available chitosan in the sample and C_C is the concentration of chitosan used to prepare the liposomal formulation. A standard curve was made by suspending chitosan powder (0.5 g) in 50 mL of distilled water. After 30 min stirring at room temperature, 2.0 mL of acetic acid was added. And additional 50 mL of distilled water was added to the acidic chitosan solution, the final dilution with distilled water provided a final concentration of 0.5 g/L. Standard solutions were made by diluting the chitosan solution with glycine buffer to desired concentrations.

4.5.6 Mucin–binding test as an indicator of mucoadhesiveness

The mucoadhesive properties were determined by the method developed by Pawar *et al.* (2012) and modified in our group (Jøraholmen *et al.*, 2014). The pig mucin (PM; 400 μg/mL) was hydrated in phosphate buffer (0.05 M, pH 7.4), the suspension mixed with the vesicle suspension (1:1, v/v) and the mixture incubated at room temperature (23 °C) for 2 h prior to centrifugation for 60 min at 216,000x *g* and 10 °C (Optima LE-60; Beckman instruments, Pao Alto, CA, USA). Absorbance of the remaining free PM in the supernatants was measured by UV spectrophotometer (Microtiter plate reader; Spectra Max 190 Microplate, Spectrophotometer molecular Devices, Sunnyvale, CA, USA) at 251 nm. The mucoadhesiveness was expressed as PM binding efficiency calculated by equation 2:

$$PM\ binding\ eff. = \left(\frac{C_0 - C_S}{C_0}\right) \times 100 \qquad \text{Eq. 2}$$

where C_0 is the initial concentration of the PM used for incubation (400 μg/mL) and C_S is the measured concentration of the free PM in the supernatant after removal of the liposome-bound PM. The standard curve was determined from the standard PM solutions in the phosphate buffer made by diluting the PM stock solution in a range from 40 to 320 μg/mL.

4.6 Stability testing

The stability of the newly developed polymer-containing liposomes was determined after one month of storage in a refrigerator (4 °C). All liposomes were tested for entrapment (retention of the originally entrapped metronidazole), size distribution, and zeta potential.

4.7 Activity and release studies

4.7.1 *In vitro* release studies

Release studies were performed using Franz diffusion cells (PermeGear, Hellertown, PA, USA) with the heating circulator (Julabo Labortechnik F12-ED, Seelback, Germany) maintaining the temperature at 37 °C. The cells with 12 mL volume acceptor chambers and a diffusion area of 1.77 cm^2 were used in *in vitro* studies based on the method by Hurler *et al.* (2012). Polyamide membranes (0.2 μm pore size; Sartorius polyamide membrane; Sartorius AG, Gröttingen, Germany) were used. Liposomal formulations were added to the donor compartment in a volume of 600 μL. The acceptor chambers were filled with distilled water and kept at 37 °C. Samples (500 μL) from the acceptor chamber were taken at different time intervals, and replaced with fresh medium. Both the sampling port and the donor chamber were covered with quadruple layers of parafilm to prevent evaporation. Quantification of released model substance was either determined by fluorimetric measurements at excitation and emission wavelengths of 485 and 520 nm, respectively, when the model substance used was either of the FITC-dextrans (Paper II), or by spectrophotometry, where the aqueous solution of metronidazole was measure at 319 nm (Paper III).

4.7.1 Antifungal activity assay

Antifungal activity was tested according to the method previously described (Sperstad *et al.*, 2009) using the yeast strain *Candida albicans*. Fungal spores were dissolved in potato dextrose broth and the cell concentration was determined and adjusted after counting in a Bürker chamber. An aliquot of 50 μL of fungal spores (final concentration 2×10^5 spores/mL) was inoculated in

96-well Nunc™ microtitre plate (Thermo Fisher Scientific Inc. Waltham, MA, USA) along with 50 μL of liposomal formulation dissolved in Milli-Q water. Liposomal formulations were diluted in a two-fold sequence. Cultures were grown in a dark chamber without shaking for 24 hours at 37 °C. Growth inhibition was determined by plating an aliquot of the *C. albicans* on agar plates allowing growth for 24 hours in a dark chamber at 37 °C. Growth inhibition was defined as the concentration where no growth was seen on the agar plate after 24 hours incubation. In addition, the negative controls containing neither *C. albicans* nor liposomal sample, or just the liposomal formulations were also tested for growth.

4.8 Statistical evaluation

Student's *t*-test was used for comparison of two means. A *p* value greater than 0.05 was considered a significant difference.

5 Results and discussion

The aim of this study was to develop a novel mucoadhesive delivery system for the improved treatment of local vaginal infections. In order to achieve a successful treatment it is important to maintain a sufficient amount of drug at the vaginal site for a sufficient periode of time (Hainer and Gibson, 2011). Traditionally coating of liposomal vesicles is performed in a subsequent step to their preparation. This is performed in a one-to-one ratio between the preformed liposomes and the polymer, reducing the concentration of the entrapped drug significantly (Jøraholmen *et al.*, 2014). This would be undesirable for poorly soluble drugs (e.g. metronidazole), which are difficult to formulate in high concentrations (Vanić *et al.*, 2013). The focus of the first part of this study was therefore to develop a novel mucoadhesive system, with a straightforward and simple preparation procedure, such as a one-pot preparation method. For this purpose the coating polymer was added to the aqueous phase during the preparation of liposomes. This was done under the assumption that coated liposomes would form in the initial preparation step; in addition it was thought that the polymer would also be embedded within the aqueous compartments of the liposomes.

5.1 Preparation of vesicles

Initially 6 different pectin types with different degrees of esterification were tested with the new method. Of these the pectin with 35 % DE and the pectin with 50 % DE were chosen for further study because they showed the most promising results in regards to entrapment efficiency and physical characteristics such as size and zeta potential. In addition two types of chitosan, high molecular weight (77 % DD) and low molecular weight (95 % DD) was chosen.

Before the preparation of vesicles, the viscosity of the pectin and chitosan solutions were measured and the concentration adjusted so all the polymer solutions were of the same range of viscosity (0.65 ± 0.2 mPa*s). An overview of the resulting viscosities and corresponding concentrations can be seen in

Table 5.1. Appropriate concentration varied greatly between the different polymers and grades of polymers.

Table 5.1: Characteristics of polymer solutions (n=3).

Polymer type	Conc. (%, w/w)	Viscosity (mPa*s)
Pectin (35 % DE)	0.50	0.85
Pectin (50 % DE)	0.50	0.67
Chitosan (77 % DD)	0.17	0.75
Chitosan (95 % DD)	0.05	0.49

5.2 Entrapment efficiency

5.2.1 Entrapment of metronidazole

Figure 5.1 shows the entrapment efficiency of metronidazole in pectin-containing liposomes and chitosan-containing liposomes, presented as the amount of drug per lipid, normalized after the determined amount of lipid in each formulation as determined by the phospholipid assay. Because of the role that the size of the vesicles play in the vesicles ability to penetrate through the mucosa and reach the underlying tissue (das Neves *et al.*, 2011) the formulations where subjected to different degrees of size reduction. Non-sonicated liposomes were compared to liposomes that had been sonicated for either 1 or 2 minutes. Both pectin-containing liposomes and chitosan-containing liposomes showed higher entrapment efficiency than plain liposomes. However, because of the high standard deviation it is difficult to draw more than trends from the results.

Sonication for 1 minute did not result in a loss of the originally entrapped metronidazole, but a decrease was seen after 2 minutes of sonication. There was a reduction in size as well (see section 5.3.1 for discussion of size of vesicles), so the decrease in entrapment is as expected. Chitosan-containing liposomes appears to have a superior ability to entrap metronidazole compared to pectin-containing liposomes, however not at a significant level. Pectin with 50 % DE on the other hand appears to give the most stable entrapment, with small degree of loss of entrapped drug upon sonication.

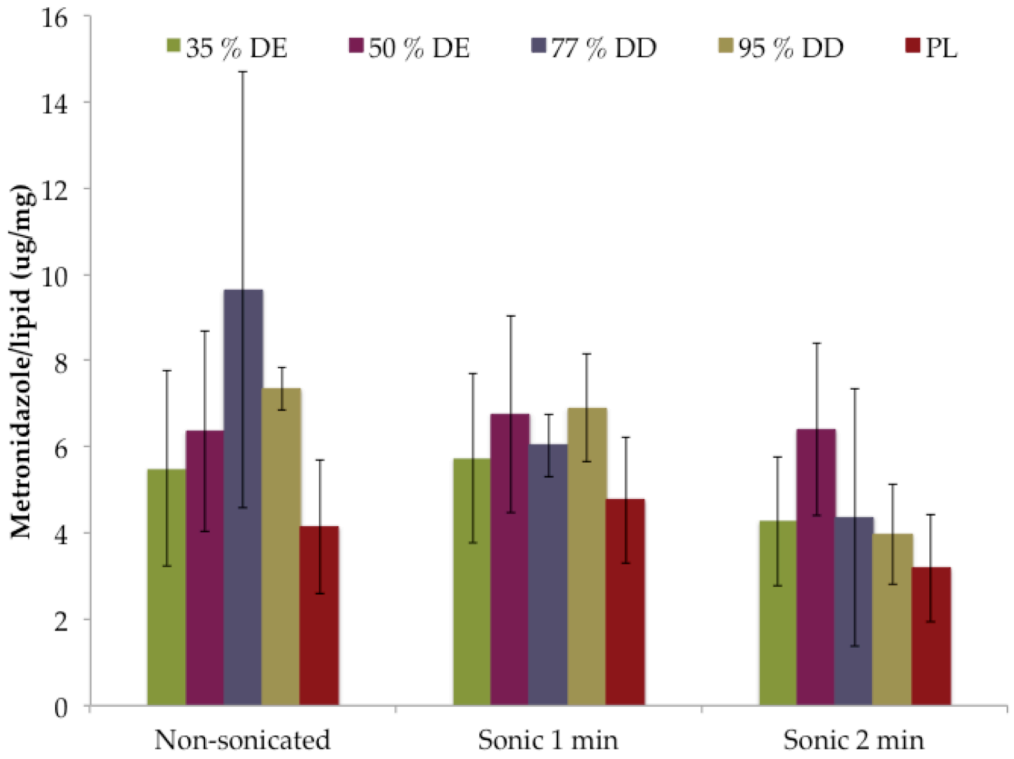


Figure 5.1: Entrapment efficiency of metronidazole in the different liposomal formulations; Pectin-containing liposomes (35 % DE and 50 % DE), chitosan-containing liposomes (77 % DD and 95 % DD) and plain liposomes. The values represent the mean ± SD (n=3).

At the pH of the liposomal suspensions, around 3-4, the different polymers used have different degree of ionization. Chitosan, which has a pK_a in the range of 6.0-6.5 depending on the molecular weight and degree of deacetylation (Wang *et al.*, 2006), will be fully charged at the pH of the solutions. Pectin on the other hand, with its pK_a of 3.5-4.0 depending on the degree of esterification (Plaschina *et al.*, 1978) will be only partially ionized. Metronidazole with its low pK_a of 2.5 (Shalaeva *et al.*, 2008) may be partially ionized at pH of the suspensions. This may also affect the interaction between the polymer and the drug. Positively charged chitosan will retain more of the negatively charged metronidazole, while there could be a repelling force between the portions of charged metronidazole and pectin because both are negatively charged.

Later metronidazole was also used as the model drug in the comparison of chitosan-containing liposomes and Carbopol-containing liposomes. Figure 5.2

shows the entrapment efficiency of metronidazole in the different liposomal formulations expressed as the amount of drug per lipid, normalized after the determined amount of lipid in each sample by the phosphorus assay. As observed previously plain liposomes have the least amount of entrapped drug, at approximately 6 μg/mg lipid, which is significantly different from the polymer-containing liposomes ($p < 0.05$). The chitosan-containing liposomes and both of the Carbopol-containing liposomes, sonicated 1 and 2 minutes, have an entrapment of approximately 11-12 μg/mg lipid, and are not significantly different from each other. Chitosan-containing liposomes shows entrapment efficiency after one minute sonication that is higher than was seen previously, with a more narrow standard deviation, which may be attributed to a change in the sonication method; in the previous work samples were not diluted prior to sonication. This could lead to harsher condition leading to the loss of entrapped drug.

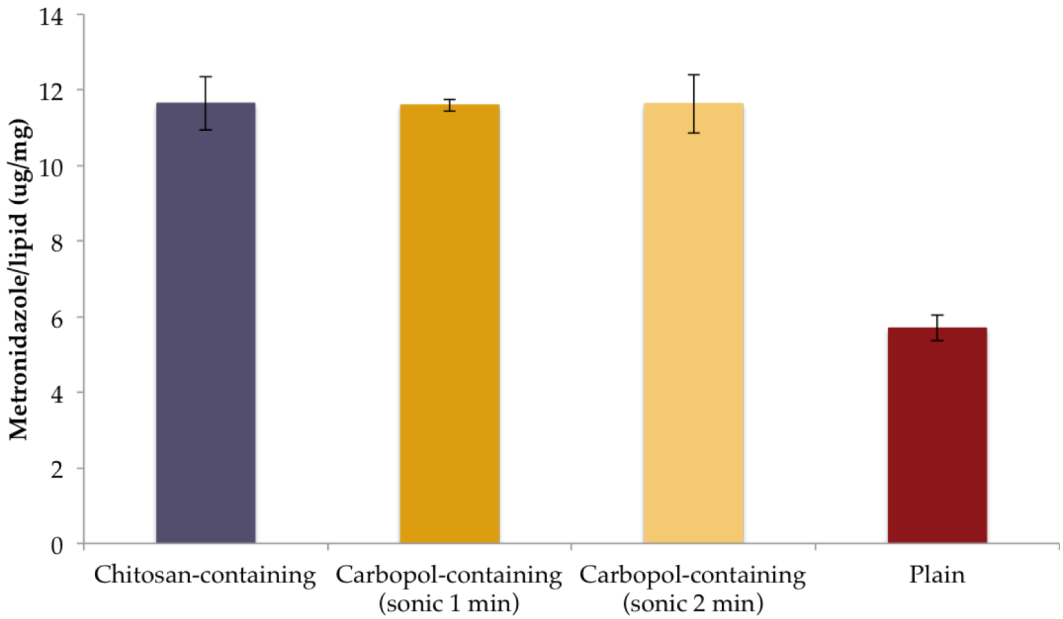


Figure 5.2: Entrapment efficiency of metronidazole in chitosan containing liposomes, Carbopol-containing liposomes (sonic 1 min), Carbopol-containing liposomes (sonic 2 min) and plain liposomes. All values represent the mean ± SD (n=3).

The lack of difference in entrapment efficiency between the two Carbopol-containing liposomes, which have been sonicated for 1 and 2 minutes respectively, is unexpected because there is a difference in size (see section

5.3.1). One possible explanation is that because of the branched structure of Carbopol the polymer could be more able to retain the drug.

5.2.2 Entrapment of FITC-dextran

In order to evaluate the chitosan-containing liposomes ability to entrap larger substances, FITC-dextran of two different molecular weights, 4 and 20 kDa named FITC-dextran 4 and FITC-dextran 20 respectively was used as models for high molecular weight hydrophilic substances to be encapsulated in the liposomes. The entrapment efficiency of these model substances in the three types of liposomes, chitosan-containing liposomes, chitosan-coated liposomes and plain liposomes, is shown in Figure 5.3. Both the low and the high molecular weight FITC-dextran showed the highest entrapment in the chitosan-containing liposomes. Chitosan-coated liposomes and plain liposomes had similar entrapment efficiency for both the high and the low molecular weight FITC-dextrans. An explanation for this could be that the liposomes that are the basis for the chitosan-coated liposomes are the plain liposomes, which undergoes an additional coating step. The entrapped FITC-dextran has been entrapped prior to the addition of the polymer coating, and remains at the same level. The higher entrapment of the chitosan-containing liposomes can be explained by the difference in the production step, these liposomes are formed in the presence of the polymer. With the polymer present both as a coating on the outside of the liposomes and also in the aqueous compartments of the liposomes, it is possible that the chitosan pulls the FITC-dextran into the aqueous compartments of the liposomes. Both FITC-dextran and chitosan have a high capacity for forming hydrogen bonds, which may have a positive contribution on pulling the model substance into the liposomes. The chitosas presence between the lipid bilayers may also disorganize the structure of the bilayers, just enough to make more room for the FITC-dextran, but not enough to disrupt their formation. At the pH of the liposome suspension both the chitosan and the FITC-dextrans are charged; the chitosan negatively and the FITC-dextrans positively, so it is possible that the increased entrapment efficiency compared to the other formulations is partly ionic interactions. As expected the FITC-dextran 4 had higher entrapment efficiency than the larger FITC-dextran 20 for all the vesicles,

however both the FITC-dextran showed a high percentage of entrapment demonstrating the chitosan-containing liposomes ability to entrap sufficient amount of larger molecules, such as biologicals, within their structure.

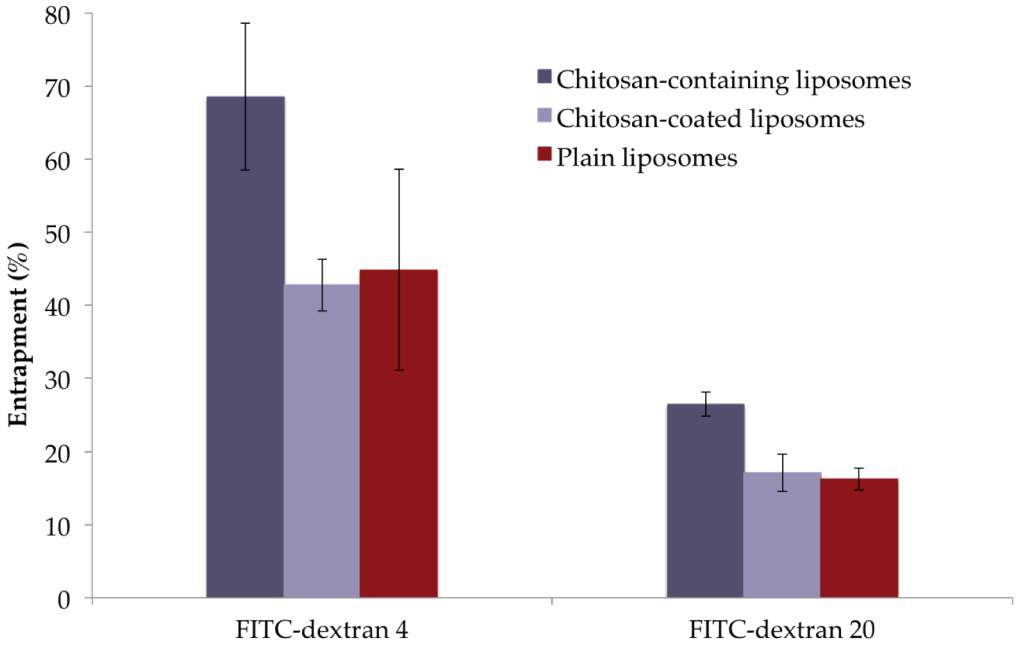


Figure 5.3: Entrapment of the two FITC-dextrans in chitosan-containing liposomes, chitosan-coated liposomes, and plain liposomes. All values represent the mean ± SD (n=3).

5.3 Characterization of vesicles

5.3.1 Particle size analysis

Size determination of non-sonicated liposomes prepared with this solvent injection method revealed that the vesicles where larger than one micron. Because the polydispersity indexes for these formulations were over 0.70 the sizes were only taken as an estimate and the assumption was made that these were multilamellar vesicles. Although the solvent injection method was made for the production for small unilamellar vesicles, the method is sensitive to many factors such as stirring speed, amount of lipid and solvent, type of solvent, and injection speed among them (Gentine *et al.*, 2012). These parameters were kept as constant as possible. In addition the presences of polymer into/onto the vesicles at production may have contributed to the larger size.

Because of the high polydispersity index of the vesicle suspensions image analysis was applied to gain a deeper insight on the possibility of aggregation and the overall structure. However, the image analysis only confirmed that the vesicles were larger than one micron. The shape and vesicle structure was found to be similar for all formulations, although one has to take into account that image analysis is flattening the 3D structure and that all vesicles appear spherical.

In order to reduce the polydispersity of vesicle size, and to prepare liposomes of size suitable for vaginal administration (Vanić and Škalko-Basnet, 2013), sonication was applied for an appropriate amount of time. Smaller, more uniformly sized liposomes are expected to have a better distribution in the vaginal cavity and to penetrate deeper into the mucosal layer (das Neves *et al.*, 2011). This will improve the treatment and the residence time as the vesicles can penetrate deeper than the rapidly clearing upper layer of the mucosa (Laffleur and Bernkop-Schnurch, 2012).

Because of the large size of the vesicles and high polydispersity index, the vesicles were subjected to sonication for an appropriate amount of time. The size distribution of all the following data on size expressed as a NICOMP distribution, *i.e.* bimodal distribution where the particles of similar size are grouped in populations and presented as the percentage of particles with the specific mean diameter. The NICOMP distribution gave the best fit for the measured data; Fit error < 1.5 and residual error < 10.

The size and size distribution of the sonicated vesicles are shown in Table 5.2. These are the initial formulations of chitosan-containing liposomes, pectin-containing liposomes, and plain liposomes with metronidazole as the model drug. Here the liposomal formulations were subjected to sonication in two rounds, first 1 minute and then for an additional minute. After being subjected to sonication all vesicles resulted in vesicles of a smaller size. However, pectin-containing liposomes (50 % DE) did not undergo further size reduction upon being subjected to sonication for the second time, unlike the vesicles formulated with the other polymers. These liposomes were also

different from the other liposomes, in that they retained more of the entrapped metronidazole after sonication (Figure 5.1), indicating that this grade of pectin is able to stabilize the vesicles during the strain of sonication. Chitosan-containing liposomes formulated with chitosan of 77 % DD were the smallest of the polymer-containing liposomes.

Table 5.2: Size and polydispersity index (PI) of the polymer-containing liposomes (n=3).

Type of liposomes	Sonication time	Peak 1		Peak 2		PI
		Size (nm)	%	Size (nm)	%	
Pectin-containing [1] (35% DE)	1 min	148	13	626	86	0.373
	2 min	91	14	324	84	0.287
Pectin-containing [1] (50% DE)	1 min	222	11	847	82	0.446
	2 min	166	12	718	86	0.397
Chitosan-containing [1] (77% DD)	1 min	62	30	239	72	0.315
	2 min	58	21	193	76	0.384
Chitsan-containing [1] (95% DD)	1 min	194	14	733	86	0.442
	2 min	67	10	290	91	0.421
Plain	1 min	91	18	450	83	0.329
	2 min	82	12	415	89	0.446

[1] the names was changed from pectosomes and chitosomes respectively in the second article.

Table 5.3 shows the size and polydispersity index of the liposomal vesicles, formulated with FITC-dextran 4 and 20, after size reduction (45 seconds of sonication). Here the chitosan-coated liposomes (77 % DD was used both in Paper II and III) were the smallest after size reduction and the plain liposomes were the largest. Interestingly vesicles containing FITC-dextran 20, the larger model-substance, were smaller than the same formulations containing FITC-dextran 4. The smaller size of liposomes formulated with FITC-dextran 20 can be seen as a contributing factor to the smaller degree of entrapment of this model substance; smaller vesicles have less available aqueous compartment space to accommodate the hydrophilic model substance. One unexpected result is that the size of the plain liposomes was greater than the coated liposomes. There are, however, similar findings where polymer-coated liposomes were smaller than non-coated liposomes (Naderkhani *et al.*, 2014; Tan and Misran, 2012). The reason behind this observation could be that chitosan is known to form a cage-like steric barrier that protects liposomes

from aggregation, whereas in the case of non-coated liposomes the agglomeration can occur (Tan and Misran, 2012).

Table 5.3: size and polydispersity index of the liposomes (n=3).

Type of Liposomes	Peak 1		Peak 2		PI
	Size (nm)	%	Size (nm)	%	
FITC-dextran 4					
Chitosan-containing	76	20	287	79	0.302
Chitosan-coated	48	69	197	21	0.348
Plain	56	16	337	85	0.359
FITC-dextran 20					
Chitosan-containing	49	29	206	64	0.333
Chitosan-coated	27	26	99	74	0.342
Plain	50	39	218	54	0.371

The size and size distribution of chitosan-containing liposomes and plain liposomes were compared to Carbopol-containing liposomes (Table 5.4). The Carbopol-containing liposomes were of a larger size compared to the other two formulations, 500 nm as opposed to around 200 nm. Therefore the Carbopol-containing liposomes were subjected to a second minute of sonication before the size was measured again. The size was reduced to 400 nm. Still not as much as the other formulations, but further size reduction was not attempted as the released studies indicated that the metronidazole was very loosely associated with the polymer-containing liposomes at this degree of sonication (see section 5.5.1.1), and further sonication was believed to give reduced entrapment efficiency due to the strain. Still Carbopol appeared to have a stabilizing effect of the polymer-containing liposomes similar to the pectin with a 50 % DE.

Table 5.4: size and polydispersity index of the liposomal formulations (n=3).

Type of liposomes (sonication time)	Peak 1*		Peak 2*		PI
	Size (nm)	%	Size (nm)	%	
Chitosan-containing (1 min)	44.9	35	188.7	59	0.357
Carbopol-containing (1 min)	90.9	15	508.6	83	0.456
Carbopol-containing (2 min)	72.0	15	401.6	85	0.517
Plain (1 min)	41.4	13	224.5	86	0.368

5.3.2 Zeta potential

Initially, the pH and the zeta potential was used to indicate that the polymer used in the making of polymer-containing liposomes was found both on the outside of the vesicle as a polymer coating and inside the vesicle, in the aqueous compartments. The pH could be seen to change towards more neutral from the polymer solution to the corresponding polymer-containing liposome suspension. Where the pectin solutions had pH values of 2.93 (35 % DE) and 3.05 (50 % DE), the pH of the pectin-containing liposomes was 3.31 (35 % DE) and 3.42 (50 % DE); similarly the chitosan solution has pH values of 3.43 (77 % DD) and 3.78 (95 % DD), the chitosan-containing liposomes had pH values of 3.91 (77 % DD) and 4.26 (95 % DD). The pH of the plain liposomes was at 5.5. The increase of pH from the polymer solution to the polymer-containing suspension towards a more neutral pH was believed to indicate the camouflaging of some of the ionized polymer, indicating that some of the polymer was indeed embedded within the vesicles and not just surface available.

The zeta potential of the liposomal vesicles was measured for all the liposomal formulations. In Figure 5.4 a compilation of the zeta potential of all formulations after one minute of sonication in presented. The pectin-containing liposomes (35 % DE and 50 % DE) exhibit a clearly negative zeta potential, while the chitosan-containing liposomes exhibit a slightly positive zeta potential (95 % DD and 77 % DD), the chitosan-coated liposomes (77 % DD) exhibit a more highly positive zeta potential, the Carbopol-containing

liposomes exhibit a slightly negative zeta potential. As expected the plain liposomes have a neutral zeta potential. The zeta potential is dependent on the amount of the polymer on the outside of the liposomes and the degree of ionization of this polymer. As discussed in section 5.2.1 the pK_a of the different polymers indicates that the polymers will have different degree of charge density at the pH in the liposomal suspension, and at the pH in the dilution media for the zeta potential measurement, filtered water.

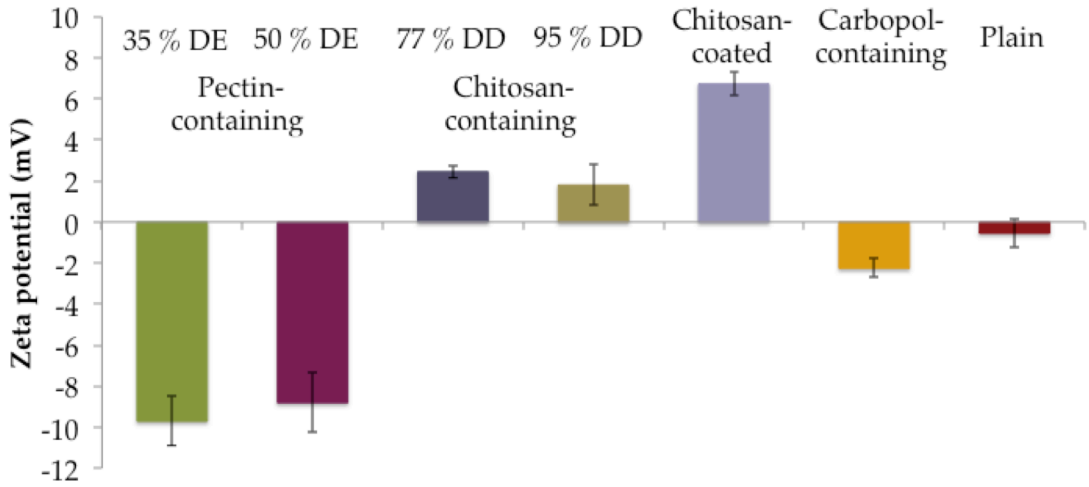


Figure 5.4: Zeta potential of different liposomal formulations after 1 minute of sonication time. All values represent the mean ± SD (n=3).

5.3.3 Determination of surface-available chitosan

Two different formulations with chitosan were compared, chitosan-containing liposomes and chitosan-coated liposomes. Although the zeta potential indicated the presence of chitosan on the surface of the liposomes for both formulations, we wanted to confirm the availability of chitosan that could interact with mucin, and insure the adhesiveness of the system, and quantify the amount of surface-available chitosan. As the two different formulations are different in when the chitosan was added, a difference was expected. As chitosan was present during formation of the liposomes in the case of chitosan-containing liposomes, it is expected that a portion of the chitosan is lodged in the aqueous compartments between the lipid bilayers of the vesicle. Figure 5.5 shows that the plain liposomes did not exhibit any, or a negligible amount, of surface available-chitosan. The small amount seen can be contributed to variation in the test. The chitosan-containing and the

chitosan-coated liposomes exhibited a high degree of surface-available chitosan; the chitosan-coated contained significantly more ($p < 0.05$) at 80 % than the chitosan coated (at approx. 65%). This finding was as expected, because with chitosan-coated liposomes all, or most of the chitosan, is expected to be surface-available. The 35 % of the original chitosan that is not surface-available in the chitosan-containing liposomes strongly indicate that this fraction is embedded within the liposomal structure, thus proving our initial hypothesis. This is also in agreement with the findings that the measured zeta potential for chitosan-coated liposomes is higher than for the chitosan-containing liposomes, indicating a higher density of charge at the surface of the liposomes.

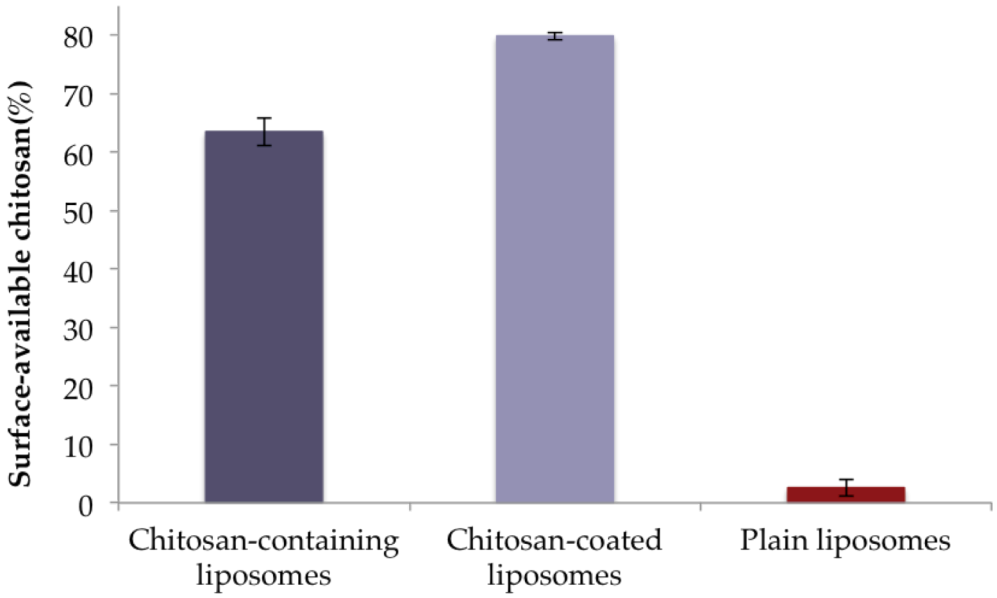


Figure 5.5: Percentage of surface-available chitosan determined in chitosan-containing, chitosan-coated and plain liposomes. All values represent the mean ± SD (n=3).

5.3.4 Mucin-binding test as an indicator of mucoadhesiveness

Following the confirmation that both chitosan-containing and chitosan-coated liposomes have a high degree of surface-available chitosan that can interact with mucin and form mucoadhesive bonds, the mucin binding efficiency was tested *in vitro*. The binding of the liposomal formulations to the model pig mucin was used to estimate the mucoadhesive characteristics. As seen in

Figure 5.6 the chitosan-coated liposomes exhibited the highest pig mucin binding capacity followed by the chitosan-containing liposomes, the plain liposomes exhibited lower mucoadhesiveness, as would be expected. These results where in direct agreement with the results for the surface-available chitosan. The difference between mucin-binding capacity for the chitosan-coated liposomes and the chitosan-containing liposomes were not as pronounced as for the surface-availability of chitosan, however, the difference was significant ($p < 0.05$). Plain liposomes showed an unexpectedly high degree of mucin-binding capacity, at around 50 %, this is significantly less than the other two formulations. It can be argued that plain liposomes should have a negligible mucin-binding capacity; however the method used to separate free mucin from the liposomes and the liposome-bound mucin, centrifugation, could contribute to a physical interaction between the liposomes and a proportion of the mucin. Hydrophobic interaction between the liposomes and mucin is also possible as a contributing part in the apparent degree of mucin-binding capacity of plain liposomes seen in Figure 5.6. This result is also comparable to the findings reported earlier for similar sonicated plain liposomes (Naderkhani *et al.*, 2014).

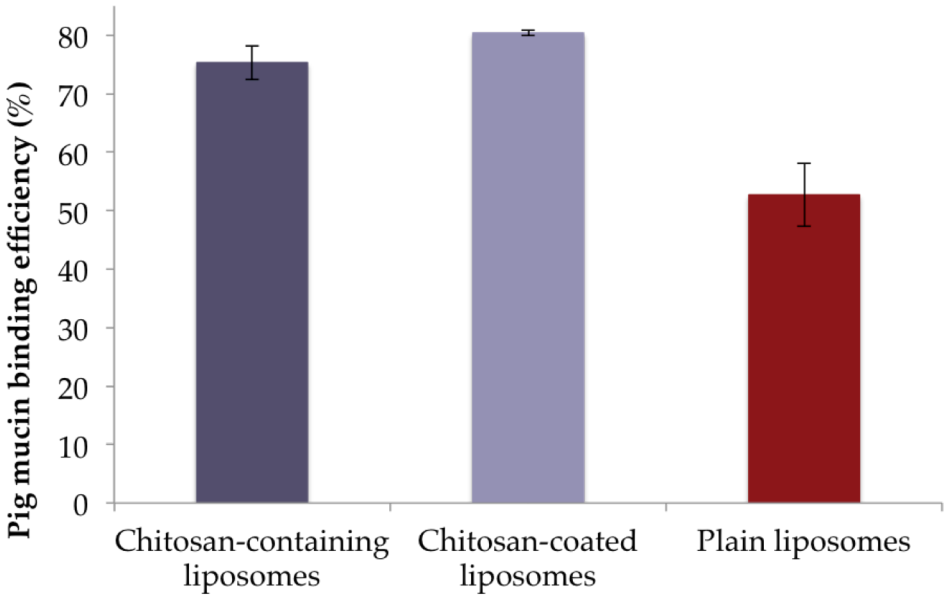


Figure 5.6: Pig mucin binding efficiency of the liposomes to porcine mucin. All values represent the mean ± SD (n=3).

5.4 Stability testing

To test the vesicles ability to retain the entrapped metronidazole during storage the liposomal formulations, chitosan-containing liposomes and pectin-containing liposomes, were subjected again to dialysis after being stored for one month of storage at 4 °C. Figure 5.7 represent the amount of drug (µg/mg lipid) that remained after the storage, the vesicles are corresponding to the entrapment values in Figure 5.1. Although the polymer-containing liposomes show a tendency towards improved metronidazole retention the differences were not significant.

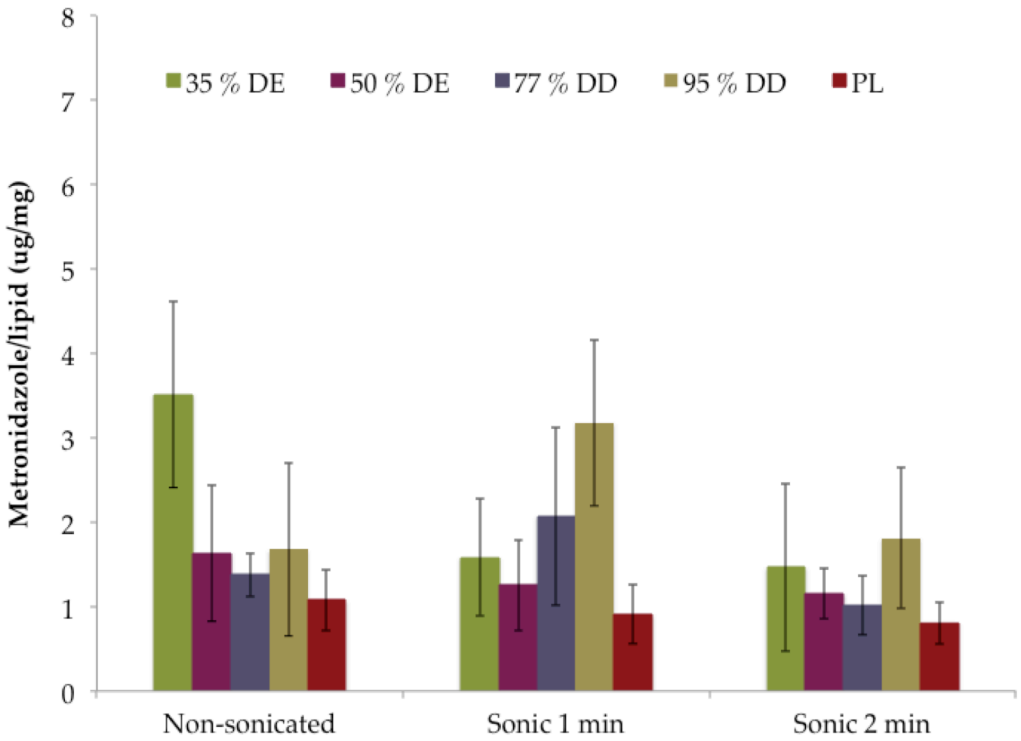


Figure 5.7: Liposomally retained metronidazole after storage for one month at 4 °C. All values represent the mean ± SD (n=3).

The size and size distribution of the vesicles were also tested as a part of their stability upon storage (Table 5.5); these vesicles are corresponding to the vesicles in Table 5.2. Stored vesicles appear to be smaller than the freshly prepared liposomes. Although this can appear to be contradictory, similar behavior has been observed with curcumin-containing vesicles (Basnet *et al.*, 2012). This could be explained by the stabilization of vesicles during storage at cold temperatures and the possibility that the measurements of freshly

prepared vesicles are actually overestimates and are including aggregates rather than mean diameters. The chitosan-containing liposomes that had been sonicated for 2 minutes appear to form agglomerates and exhibit larger means than the freshly prepared liposomes of the same type (Table 5.5), which may be contributed to a loss of the polymer layer on the vesicle surface during sonication.

Table 5.5: Size and polydispersity index (PI) of the polymer-containing liposomes (n=3).

Type of liposomes	Sonication time	Peak 1		Peak 2		PI
		Size (nm)	%	Size (nm)	%	
Pectin-containing (35% DE)	1 min	115	14	497	85	0.324
	2 min	69	14	265	85	0.275
Pectin-containing (50% DE)	1 min	113	10	508	90	0.390
	2 min	126	16	473	83	0.347
Chitosan-containing (77% DD)	1 min	68	23	310	75	0.360
	2 min	122	29	410	69	0.320
Chitsan-containing (95% DD)	1 min	115	6	625	93	0.525
	2 min	108	3	774	98	0.217
Plain	1 min	69	15	316	85	0.367
	2 min	47	7	222	93	0.454

5.5 Activity and release studies

5.5.1 *In vitro* release studies

In this study we tested the *in vitro* release of the model drug, metronidazole, and the model substances, FITC-dextran 4 and 20, on Franz diffusion cells.

5.5.1.1 In vitro release of metronidazole from liposomes

Figure 5.8 shows the cumulative release of metronidazole from the different liposomal formulations, using metronidazole in solution as a control. All the polymer-containing liposomes that had been sonicated for 1 minute showed a sustained release of the drug, compared with metronidazole in solution. The Carbopol-containing liposomes that had been sonicated for 2 minutes showed a release that did not differ from that of the control, reaching about 90 % release between 1-2 hours. The entrapment for this formulation did not differ

from that of the Carbopol-containing liposomes sonicated for 1 minute, even with a reduced size, which was unexpected. Together with the more rapid release of the drug this gives an indication that the drug is more loosely associated with the vesicles in the polymer coating, possibly even available on the liposomal surface, after the strain of 2 minutes of sonication. Carbopol is a more branched polymer compared to the other studied and could be more able to physically entrap the drug at the surface. This type of entrapment would be expected to have a more rapid release than drug entrapped in the lipid bilayer or aqueous compartments of the liposomes.

Conversely the Carbopol-containing liposomes sonicated for 1 minute showed the highest degree of sustained release of the drug, together with plain liposomes. They reached a plateau of 50 % released drug after about 1-2 hours. Chitosan-containing liposomes had a higher release of metronidazole, reaching 70 % released after 2 h before stabilizing at a plateau.

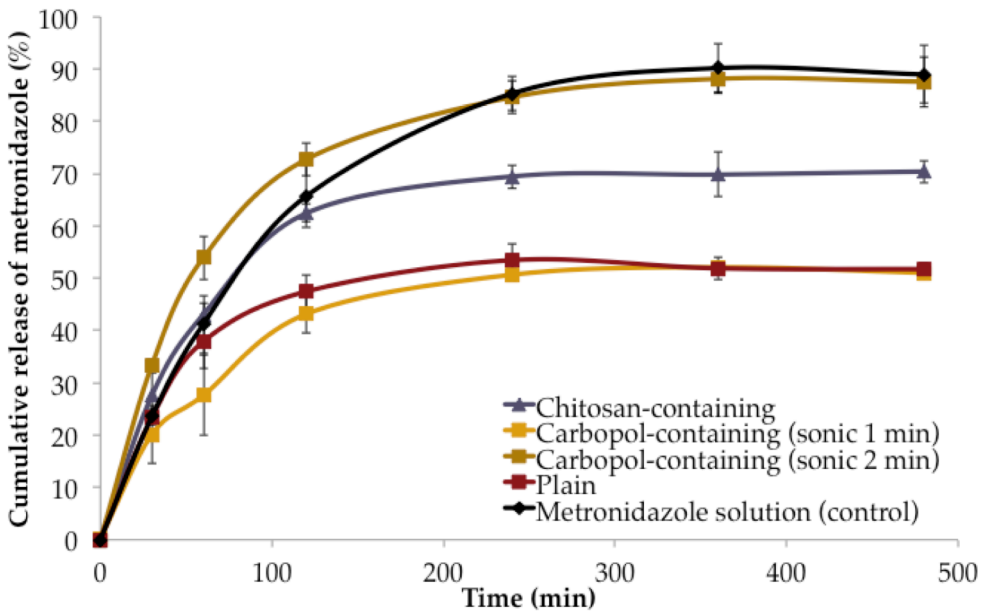


Figure 5.8: Cumulative release of metronidazole from chitosan-containing liposomes, Carbopol-containing liposomes (sonicated for 1 and 2 minutes), and plain liposomes. Metronidazole in solutions is used as a control. All values represent the mean ± SD (n=3).

5.5.1.2 In vitro release of FITC-dextrans from liposomes

In order to demonstrate the ability of the novel polymer-containing liposomes to entrap and release larger drug molecules FITC-dextran 4 and 20 was uses as model substances. The cumulative release of these from the three formulations tested is shown in Figures 5.9 and 5.10 respectively. All three types of delivery systems released the FITC-dextran 4 (Figure 5.9) in a manner comparable to the model substance in solution. The chitosan-containing liposomes demonstrated a sustained release of FITC-dextran 4 to a greater extent than the chitosan-coated liposomes, however after 2 h the release was slower from the chitosan-coated compared to the chitosan-containing liposomes, although not at a significant level.

The release of the higher molecular weigh FITC-dextran (Figure 5.10) was more rapid across the board compared to the FITC-dextran 4. Here again the control (FITC-dextran 20 solution) exhibited the highest release; however in this case the chitosan-containing liposomes sustained the greatest amount of FITC-dextran among the tested formulations. Interestingly the chitosan-coated liposomes released more FITC-dextran than the plain liposomes, which is exactly the opposite behavior as found in the low molecular weight FITC-dextran 4. Another interesting observation was the fast initial release of FITC-dextran from all liposomal formulations. It seems that the chitosan-coated liposomes provided an initial burst release of the high molecular weight fluorescent marker. One possible explanation can be that the rather larger molecule for FITC-dextran 20 (20 kDa) was not only entrapped but also embedded between the vesicle bilayers close to the outer bilayer and was released by a rapid diffusion at the start of the release study. In addition the smaller liposomal size, and thus the larger total surface area of liposomes containing FITC-dextran 20 could facilitate the faster release as compared to the release of FITC-dextran 4.

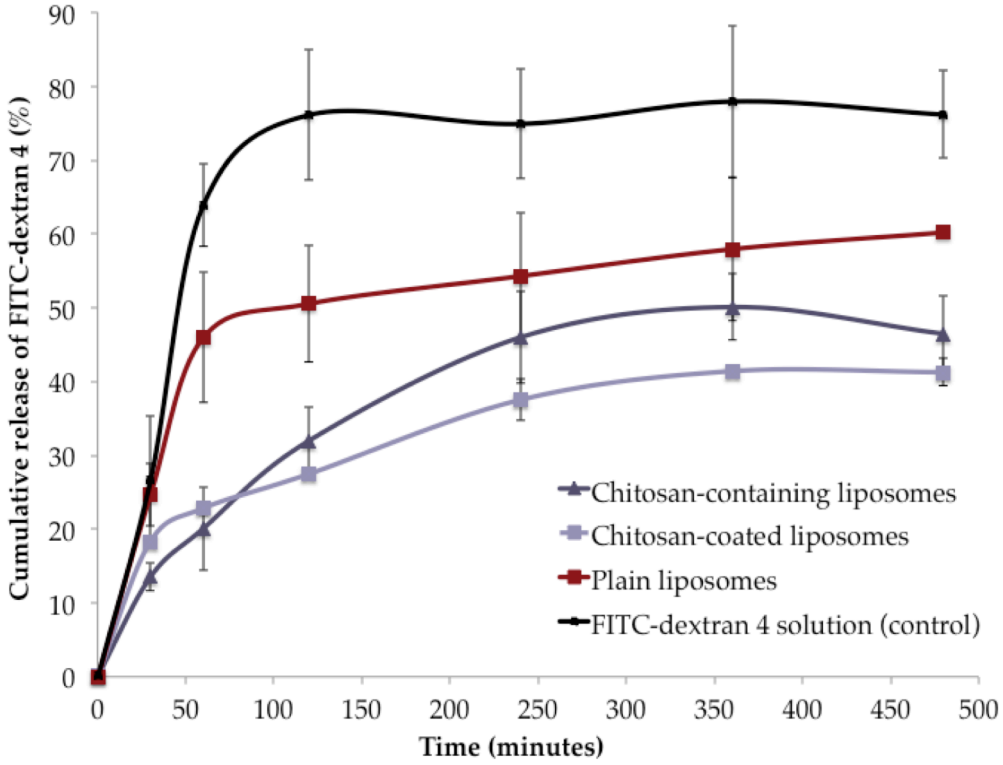


Figure 5.9: Cumulative release of FITC-dextran 4 from chitosan containing liposomes, chitosan-coated liposomes, and plain liposomes. All values represent the mean ± SD (n=3).

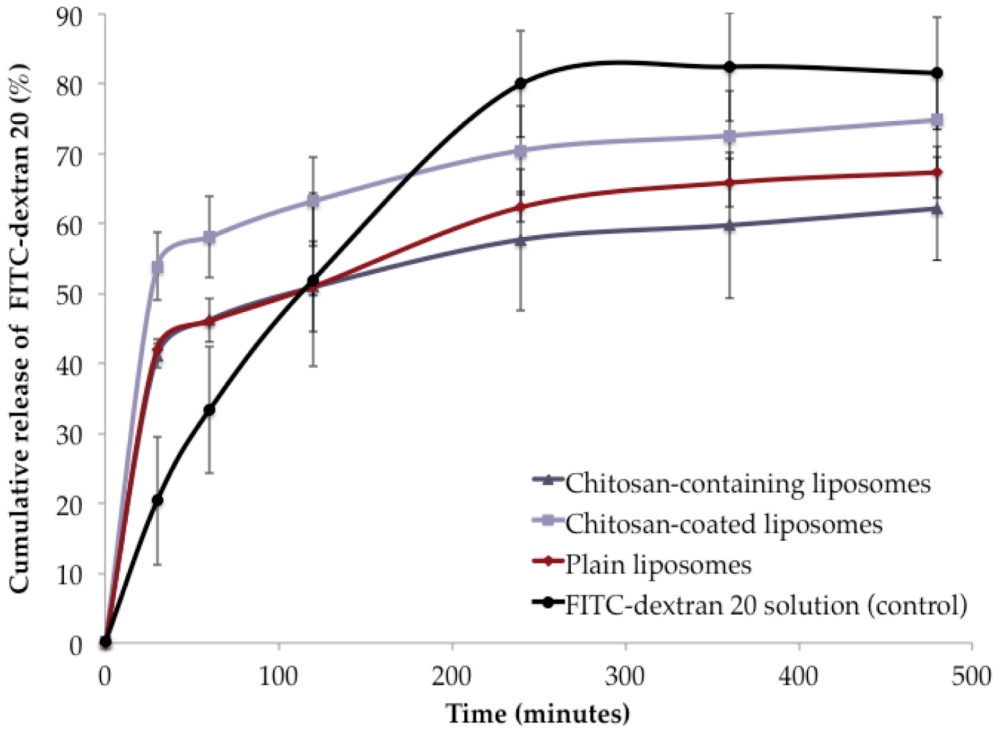


Figure 5.10: Cumulative release of FITC-dextran 20 from chitosan containing liposomes, chitosan-coated liposomes, and plain liposomes. All values represent the mean ± SD (n=3).

5.5.2 Antifungal activity assay

Antifungal activity of the polymer-containing liposomes was tested by growing *C. albicans* in the presence of the liposomal formulations for 24 hours. An aliquot of the polymer-containing liposomes and the fungi were subsequently incubated on agar plates for 24 hours to check if growth had been inhibited. Inhibition of *C. albicans* was defined as the concentration where there was no growth on the agar plate observed after 24 hours. The chitosan-containing liposomes, both loaded with the model drug, metronidazole, and empty liposomes, showed a potent ability to inhibit the growth of *C. albicans*. The lowest concentration in the two-fold dilution sequence that exhibited antifungal activity varied between the 4th and the 5th dilution as seen in Figure 5.11 A and B. This correlates to a concentration of 0.22 and 0.11 mg/mL respectively as seen in Table 5.6. Chitosan has previously been demonstrated to have high activity against *C. albicans* and in the prevention and disruption of biofilms of *C. albicans* (Pu *et al.*, 2014; Martinez *et al.*, 2010; Tayel *et al.*, 2010; Park *et al.*, 2008).

Table 5.6: Antifungal activity of the different liposomal formulations. The content of metronidazole in the drug-containing samples of chitosan-containing liposomes that showed inhibition were in the range of 18-36 µg/mL. MTZ: metronidazole.

Sample	*C. albicans* inhibition
	Chitosan (mg/mL)
Chitosan-containing (MTZ)	0.11 - 0.22
Chitosan-containing (no drug)	0.11 - 0.22
Carbopol-containing (MTZ)	No inhibition
Plain (MTZ)	No inhibition
Metronidazole in solution (control)	No inhibition

It has also been shown that *C. albicans* biofilms is resistant to commonly used antifungal agents, such as fluconazole (Mukherjee *et al.*, 2003; Hawser and Douglas, 1995), so the susceptibility of the *C. albicans* to chitosan-containing liposomes is very promising. The corresponding concentration of metronidazole in the drug-containing sample in the *C. albicans* inhibiting

dilutions is 36 and 18 μg/mL, respectively. The presence of metronidazole does not seem to alter or improve the *C. albicans* inhibition compared to the non-drug containing liposomes.

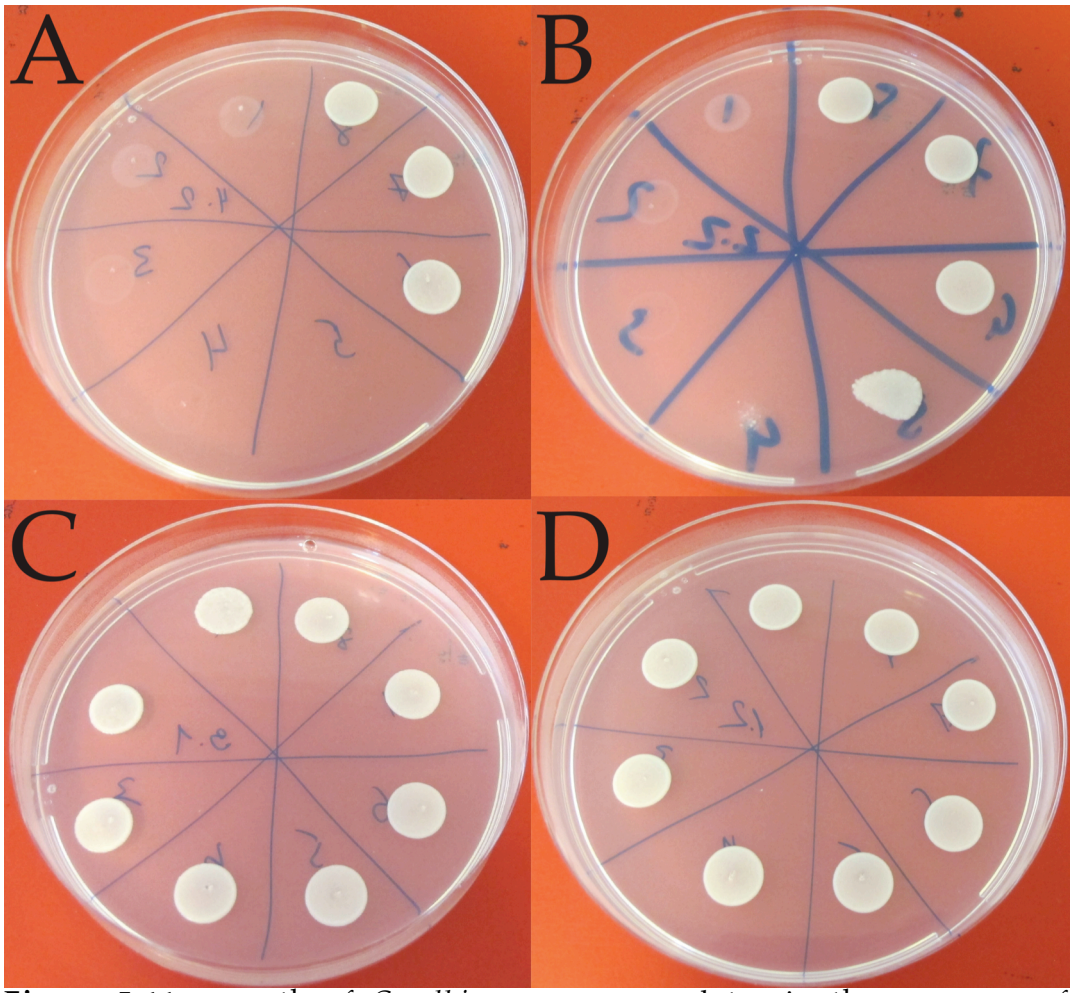


Figure 5.11: growth of *C. albicans* on agar plates in the presence of different liposomal formulations; A: chitosan-containing liposomes (no drug), B: chitosan-containing liposomes (MTZ), C: carbopol-containing liposomes (MTZ), D: plain liposomes (MTZ).

The Carbopol-containing liposomes, the plain liposomes (Figure 5.11 C and D respectively) and the metronidazole in solution showed no inhibition of growth. This is expected because neither metronidazole nor Carbopol has demonstrated any activity against *C. albicans*. However, vaginal infections often are complicated with multiple pathogens, both bacterial and fungal, either simultaneously or successively, which may contribute to a high degree of treatment failure and recurrence in this type of infection (Pirotta *et al.*, 2006; Donders *et al.*, 2011). The addition of an antibacterial agent, like

metronidazole, to a drug delivery system that is inherently able to inhibit fungal growth would be greatly beneficial to overall treatment and prevention of recurrence. In addition chitosan has demonstrated the ability to disrupt biofilms of the bacteria responsible for bacterial vaginosis (Kandimalla *et al.,* 2013). This makes chitosan an ideal component in our vaginal drug delivery system, because the system is then able to disrupt the biofilm and the antibacterial agent would be more able to reach the bacteria that would otherwise be shielded by the biofilm.

6 Conclusions

We have developed novel polymer-containing liposomes for vaginal delivery prepared by a new one-pot preparation method. The liposomes contained polymer both as an outer coating as well as embedded within the liposomes. The method was applicable for various mucoadhesive polymers. Moreover, it provided an improved ability to entrap the model drugs and model substances of various molecular sizes, in a superior manner to plain liposomes and conventionally coated liposomes.

We have further demonstrated the polymer-containing liposomes ability to release the entrapped substances in a sustained manner, as well as its polymer surface-availability and mucoadhesiveness.

Finally, we confirmed the antifungal activity of the chitosan-containing liposomes as being inherent to the system rather than dependent on the entrapped drug by testing the antifungal activity of chitosan. We propose that newly developed chitosan-based system can provide synergistic effect in treatment of complicated vaginal infections; the entrapped antibacterial drug, such as metronidazole in combination with the chitosan-containing carrier acting on *Candida* could lead to improved treatment.

7 Future perspectives

Further testing of the biological activity of the chitosan-containing liposomes against other vaginal pathogens, such as bacteria involved in bacterial vaginosis, would be the first step in follow up research.

Testing of the delivery system effect against biofilm forming pathogens involved in vaginal infections would represent the final antimicrobial testing prior to animal challenge.

Developing a vehicle/delivery device for the new type of liposomes that is suitable for vaginal administration is important to be able to administer the delivery system vaginally.

Further testing of the stability of the chitosan-containing liposomes at different storage conditions and vaginal environment is necessary to ensure that our system is stable at relevant conditions for the necessary time period.

Evaluation of the system's safety in healthy and infected animals is of course highly interesting.

Scaling up of the manufacturing process will be an important step to bring the system closer to being a candidate for further development.

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