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Mucus-penetrating drug carriers for vaginal drug delivery

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Sammendrag

Vaginal legemiddeladministrering er utfordrende grunnet kroppens naturlige forsvarsmekanismer og krever en spesiell tilnærming i utvikling av legemidler. Likevel er topikal tilførsel foretrukket fremfor systemisk behandling der det er mulig. Fordeler med lokal applikasjon er at man unngår nedbrytning i fordøyelsessystemet samt førstepassasjeeffekt. Dessuten kan lokal noninvasiv anvendelse befri pasienter fra potensielt ubehagelige prosedyrer.

Målet med dette prosjektet var å utvikle og optimalisere mukuspenetrerende liposomer for vaginal behandling av human papilloma virus (HPV). Et naturlig forekommende protein, interferon α -2b (IFN α -2b), brukes blant annet i behandling av vaginale infeksjoner. Mukoadhesive nanovesikler har vist utilstrekkelig vaginal oppholdstid på grunn av fornyelse av vaginale sekresjoner. Med dette utgangspunktet var det et ønske å forbedre terapeutisk legemiddeleffekt ved å designe nye, mukuspenetrerende partikler. For å oppnå gjennomtrenging av mukusbarrieren ble partiklens overflate modifisert med polyethylene glykol (PEG), en lav molekylvekt polymer.

PEGylerte liposomer med IFN α -2b ble tilberedt ved hjelp av den såkalte “thin film hydration” metode. Vesikkelstørrelse ble redusert ved hjelp av ekstrudering gjennom polykarbonatmembraner. Størrelse, polydispersitet, zetapotensialet og grad av legemiddelinkorporering for liposomene ble karakterisert. En velegnet størrelse (185 ± 3 nm) ble målt og lav polydispersitet (PI 0.09) indikerte uniform størrelses distribusjon. Zetapotensialet var negativt (-12.2 ± 1.4 mV). Fritt legemiddel ble separert fra inkorporert legemiddel ved hjelp av gel kolonne kromatografi, og inkorporeringsgrad (88%) var bestemt ved hjelp av IFN α ELISA kit. Det nylig utviklede systemet for lokal IFN α -2b levering innehar potensialet til å behandle HPV infeksjoner.

Nøkkelord: Mukuspenetrerende liposomer, PEG, vaginal tilførsel av legemidler, IFN α -2b

Abstract

Vaginal drug administration is a challenging approach due to the body's natural defense mechanisms and specificity in formulation design. However, where applicable, topical drug delivery is preferable to systemic therapy. Firstly, it allows averting hepatic first pass effect and degradation by GI enzymes. Secondly, non-invasive application provides closer and direct contact with the affected area and relieves user from an unpleasant procedure.

The aim of this project was development and optimization of mucus-penetrating liposomes for vaginal treatment of human papilloma virus (HPV). The naturally occurring protein interferon α -2b (IFN α -2b) is commonly used in treatment of vaginal infections. Due to continuous vaginal fluid renewal the residence time of mucoadhesive nanoparticles is shown to be insufficient. Treatment efficacy can be increased by designing novel, mucus-penetrating particles. To overcome the mucosal barrier, surface modification with the low molecular weight polymer polyethylene glycol (PEG), was applied.

PEGylated liposomes containing IFN α -2b were prepared by thin film hydration method. Vesicle size was reduced by extrusion through polycarbonate membranes. Liposomal size, polydispersity, surface charge and IFN α -2b entrapment were determined. An adequate vesicle size (185 ± 3 nm) was obtained and a low polydispersity (PI 0.09) indicated a monodisperse size distribution. Net surface charge was measured to be -12.2 ± 1.4 mV. Free drug was separated from liposomally encapsulated IFN α -2b by gel column chromatography, and entrapment efficiency (88%) was determined using human IFN α ELISA kit. The newly developed system for local IFN α -2b delivery has a potential to treat HPV infections.

Keywords: Mucus-penetrating liposomes, PEG, vaginal drug delivery, IFN α -2b

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Abbreviations

C₂H₄O₂	Glacial acetic acid
CH₃COONH₄	Ammonium acetate
CP	Conventional particles
EDTA	Ethylenedinitrilo tetraacetic acid, disodium salt dehydrate
ELISA	Enzyme-linked immunoassay
HPV	Human papilloma virus
HRP	Horse reddish peroxide
IFN α-2b	Interferon α -2b
IU	International units
LMV	Large multilamellar vesicles
LUV	Large unilamellar vesicles
MPP	Mucus-penetrating particles
Na₂HPO₄ × 2H₂O	di-Sodium hydrogen phosphate dehydrate
NaCl	Sodium chloride
NaH₂PO₄ × H₂O	Sodium hydrogen phosphate monohydrate
PCS	Photon correlation spectroscopy
PEG	Polyethylene glycol
PI	Polydispersity index
PVA	Polyvinyl alcohol
STD	Sexually transmitted disease
SUV	Small unilamellar vesicles
TMB	Tetramethylbenzidine

1. General introduction

Conventional routes of administration (oral and topical) are used to obtain either systemic or local effect respectively. Other application routes were not widely practiced since health care providers had responsibility for drug administration which left almost no room for discreet and private treatment (Alexander *et al.*, 2004). In spite of the extended knowledge on vaginal physiology and the potential for local drug delivery, this route of administration has not been extensively explored (Hussain and Ahsan, 2005). However, there is growing interest in evolving drug delivery systems for vaginal administration (Hussain and Ahsan, 2005).

A central aim in designing drug delivery systems for local administration is the close proximity to an affected area and more target-oriented treatment. Topical administration of the controlled release treatment formulations, on the other hand, will allow lower dosing and influence intake regimen. Painless and discreet self-administration makes it easy to use, and increases compliance. The additional advantage of local treatment is minimizing interference with other orally taken drugs (Alexander *et al.*, 2004).

The scope of conventional vaginal dosage forms is diverse (tablets, creams, foams, gels and suppositories) (Khan and Saha, 2015), however they have certain limitations. Itching and local irritation, messiness during application and low residence time due to the self-cleansing action of vaginal tract are the most common (Robinson and Bologna, 1994, Vermani and Garg, 2000, Khan and Saha, 2015). In order to improve vaginal drug delivery the attention was turned to developing novel delivery systems that should be able to meet both pharmaceutical and patient requirements. Such systems include controlled/sustained release vaginal tablets, vaginal ring, vaginal microspheres and nanoparticles (Khan and Saha, 2015).

It was mentioned earlier that residence time of a drug in vaginal tract should be prolonged to increase bioavailability (Robinson and Bologna, 1994). However, increased residence time will not necessarily better distribution because the risk of being entrapped by vaginal secretions is high (Ensign *et al.*, 2012a, Ensign *et al.*, 2012b). Instead, avoiding vaginal mucus entrapment and reaching epithelial cell lining might improve bioavailability profile (Lai *et al.*, 2007).

For the last decade or so, a significant amount of work has been done in order to find better and more effective approaches of drug application (Tong *et al.*, 2014). The field of nano-scale

materials gives an opportunity to slightly open a door to a new world of nanomedicine. Design and medical applications of “smart” therapeutics provides with the opportunity to achieve enhanced efficacy, reduced toxicity, and to revolutionize treatment (Vanić and Škalko-Basnet, 2013). Liposomes are an example of such “smart” therapeutics. They are composed of phospholipids with bilayer membrane structure and possess a wide application list as pharmaceutical carriers for drugs and genes (Sawant and Torchilin, 2012). Liposomes vary in size; from nanometers to microns and can be loaded with a variety of drugs (Lasic, 1998). The advantageous properties of liposomes such as biocompatibility, biodegradability, low toxicity and a capacity to modify the pharmacokinetic profile of the loaded drug are valuable in drug delivery purposes (Sawant and Torchilin, 2012).

Most of the liposomal formulations that are on the market or in clinical trials nowadays are administered intravenously, although the application range is wide (Bozzuto and Molinari, 2015). Nonetheless, there are abundant amount of liposomal formulations also for topical treatment under development.

The prevalence of sexually transmitted diseases (STDs) is increasing rapidly across the world and infections do not have age or race limits (Nardis *et al.*, 2013). STDs are no longer restricted to third world countries, but occur frequently in industrial and developed megalopolises. The prevalence of HPV constitute 11-12 % worldwide and approximately 1 of 10 sexually active individuals is a carrier at some point during their lifetime (Forman *et al.*, 2012). HPV is an infectious disease, which infects a wide variety of organisms including humans. Current way of contamination is skin-to-skin intimate contact which makes it one of the most common sexually transmitted diseases in both genders (Mohammad and Zargar, 2014). Physico-clinical manifestations of this disease are anogenital warts that vary in size and complexity (King *et al.*, 2013). The numbers are large and intimidating, that is why painless, easy to access treatment that does not intervene with daily routines is in demand, not only as a cure but also as a preventative measure.

IFN α -2b is used to treat various diseases, including vaginal viral infections and new applications for vaginal treatment are on their way to the market (Foldvari and Kumar, 2012).

2. Introduction

2.1 Vagina

The curved form of the vagina consists of two distinct portions: a lower convex portion and a wider upper portion (Alexander *et al.*, 2004) and is 6-10 cm long (Khan and Saha, 2015). It is extensively supplied with blood through a vast vascular network (**Figure 1**) that encompasses the vagina from various sources (Alexander *et al.*, 2004). Vaginal epithelium presents an uneven and extensively folded lining (rugae) that is able to stretch when undergoing either external or internal strains (for example during childbirth or coitus) (Ensign *et al.*, 2012b).

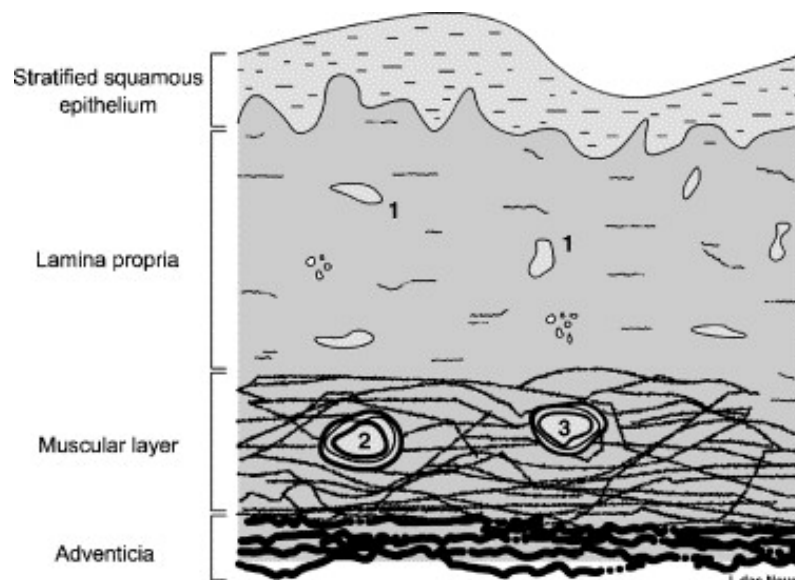


Figure 1: Schematic drawing of the vaginal mucosa. 1: capillary vessels; 2: artery; 3: vein (das Neves and Bahia, 2006)

Various factors, such as level of pH, age, hormone status and pregnancy influence vaginal physiology. Normal pH level in healthy and premenopausal women varies between 3.5-4.5 (Hussain and Ahsan, 2005, Valenta, 2005), and may rise close to 7.0 in postmenopausal women (Robinson and Bologna, 1994). *Lactobacillus* bacteria mostly dominate healthy bacterial flora and generate among other, hydrogen peroxide scavenging enzymes (for example catalase) making the environment less hospitable to other microorganisms (Alexander *et al.*, 2004). Additionally, vaginal slightly acidic environment is caused by fermentation of lactic acid under anaerobic conditions (Lai *et al.*, 2009). Menstruation blood collected by tampon, on the other hand, have an alkalizing effect, leading to insufficient

protective properties of *Lactobacillus* (Alexander *et al.*, 2004). The following induces pathogen bacterial colonization, thus increasing the vaginal pH. Also, the presence of semen (pH 7.0 – 8.0) turns slightly acidic vaginal environment to somewhat basic by raising normal pH level (Vermani and Garg, 2000, Alexander *et al.*, 2004). Maintenance of natural pH is important to avoid microbial growth and vaginal infections.

Female reproductive hormone (estrogen) controls the thickness of the vaginal epithelium (Alexander *et al.*, 2004). Small amount of estrogen leads to dryness and vaginal atrophy, while constant level of the hormone keeps the thickness of epithelium lining stable. The level of estrogen declines with increasing age (for example in post-menopausal women), which commonly leads to discomfort and unpleasant nuisances (Khan and Saha, 2015). However, the thickness of vaginal epithelium increases during puberty, reaches a plateau, followed by a decline during menopause (Justin-temu *et al.*, 2004).

Vaginal epithelium appears to be the primary physical barrier with a protective function. Its stratified construction (25 layers thick with estrogen present) makes it hard for toxins and small organism to invade the basement of membrane (Alexander *et al.*, 2004).

2.1.1 Vaginal mucus

The vaginal mucus is a heterogeneous mesh network of mucin fibers of a gel-like appearance (Lai *et al.*, 2010). The mucus is essentially composed of 90-95% water, 1-2% mucin, and other low-content constituents such as cells, bacteria, lipids, salts, proteins and macromolecules (Lai *et al.*, 2009, das Neves *et al.*, 2011b).

A single mucin is a long fiber, 5-10 nm in diameter, flexible and highly glycosylated protein (Lai *et al.*, 2010), however several mucin fibers self-condense into network and the diameter of formed mesh-spaces is estimated at 20-200 nm (das Neves *et al.*, 2011b) and is able to increase up to 340 nm (Lai *et al.*, 2010). Mucin fibers also have short hydrophobic domains (lipid-coated, non-glycosylated and cysteine-rich domains) interspersed between long glycosylated regions. The negatively charged glycosylated domains likely repel each other, but the hydrophobic domains may cause mucins to self-condense and/or bundle together thus creating a network with bigger pore sizes (Lai *et al.*, 2010). In this manner, mucins that are small constituents of mucus lining present an excellent line of defense, as it was mentioned earlier. Mucus is continuously secreted which induces shedding of foreign particles and limiting their residence time on the surface. Being aware and being able to predict mucus

clearance time, presents an opportunity that might be exploited in developing nanoparticles for vaginal administration, where one might be able to penetrate the first line of defense at rates faster than mucus renewal (Ensign *et al.*, 2012a).

In addition to shedding, mucus gel traps molecules by forming polyvalent adhesive interactions. Hydrophobic interactions between large particles and lipophilic parts of mucin contribute to bundling of mucin strands into thick cables resulting in immobilization of foreign particles (Lai *et al.*, 2009).

2.1.2 Vagina as a site for drug delivery

The conventional route of administration is preferred, however, under certain conditions local treatment is chosen. For example, in treatment of vaginal microbial, fungal and viral infections local drug delivery route will be preferential due to the proximity to site of action and ability to escape systemic drug effect. In addition, such application averts hepatic first pass metabolism that allows administration of a safer lower therapeutic dose. Drugs that are poorly absorbed after oral administration can be delivered via vaginal route of administration as well (Hussain and Ahsan, 2005). Easily accessible local application may enhance compliance regimen by increasing the intervals between the doses (Alexander *et al.*, 2004). Furthermore, large surface area due to folded rugae presents a promising site for vaginal drug delivery (das Neves and Bahia, 2006).

However, vaginal drug delivery route encounters for some limitations. Firstly, such treatment is gender specific, and secondly, vaginal permeability is strongly influenced by estrogen concentrations (Alexander *et al.*, 2004). Changes in environment arise certain challenges in development of delivery systems for local application. Examples of factors that may affect vaginal drug delivery (das Neves *et al.*, 2011a):

1. Menstrual cycle; Escalated shedding of vaginal fluids during menstruation may hinder residence time of a drug formulation and make it hard to apply. In addition, menstrual cycle has an effect on vaginal pH (increases) and epithelial layer (thickening) (Valenta, 2005). In post-menopausal women, for example, decrease in epithelial thickness will change the drug absorption rates
2. Intravaginal practices; Daily and excessive douching can for example disrupt the effect of intended prolonged release

3. Health; Reoccurring vaginal infections not only disturb natural microflora but also affect normal vaginal pH gradient which plays significant role in drug absorption thus important for drug delivery systems
4. Sexual activity; Increased sexual activity predisposes to specific cautions during treatment, for example regulation of drug administration timeframe (hours, minutes, before or after coitus). During penile penetration the formed friction may disturb for example mucus-entangled particles and lead to shedding of the latter. Compared to the non-stimulated state where level of secretion is regular, lubrication efficiency increases during sexual arousal

Another important fact to consider while developing formulations for vaginal therapy is consumer's preferences. It should be odorless and colorless, non-leaking and avoid causing the feeling of messiness and fullness (Vermani and Garg, 2000). Most of all, the product and its metabolites should be non-toxic, biodegradable, not cause local irritation, burning, itching or swelling and not interfere with normal immune functions. The convenience of application and dosage regimen plays an essential role in development.

The search for modified and improved treatment using vaginal delivery route is in progress and constantly new approaches are being developed.

2.2 Mucoadhesion vs. mucus-penetration

Adherence to the surface and penetration through the biological barrier to the underlying epithelial layer (the site of action) is the aim when developing nanosystems for mucosal surface (das Neves *et al.*, 2011b, das Neves *et al.*, 2012). The significant advantage is the prolonged residual time that can benefit the total drug payload to the surface and underlying layers (das Neves *et al.*, 2011b). On the other hand, the prolonged retention time and nanosize may contribute to the uptake by off-target epithelial cells or other cell types present at the mucosal surface, or even cross the mucosal barrier and continue its migration through the surrounding tissue (das Neves *et al.*, 2011b).

2.2.1 Mucoadhesion

Mucoadhesion is described as a phenomenon that occurs in two steps: close contact between a material and mucosal tissue, and the establishment of intermolecular interactions between the two (**Figure 2**) (Shaikh *et al.*, 2011).

Mucoadhesive polymers in drug delivery purposes are used in vesicle surface modifications in order to establish polymer-mucus interactions which are complex in their nature (Andrews *et al.*, 2009). The variety of mucoadhesive polymers is diverse and the most commonly used are chitosan, polyethylene glycol (PEG) and polyvinyl alcohol (PVA) (Yoncheva *et al.*, 2005). The choice of polymers intended for nanoparticle coating (an intelligent surface design/modification) is based on desired property characteristics, for example mucoadhesion or mucus-penetration. The polymers intended for mucoadhesion should possess following characteristics: be non-toxic and not cause irritation/inflammation, form rapid and strong non-covalent bond between mucosal tissue and a material, allow easy drug incorporation and minimum (preferably none) hindrance during drug release and avoid decomposing throughout storage (Ahuja *et al.*, 1988, Shaikh *et al.*, 2011).

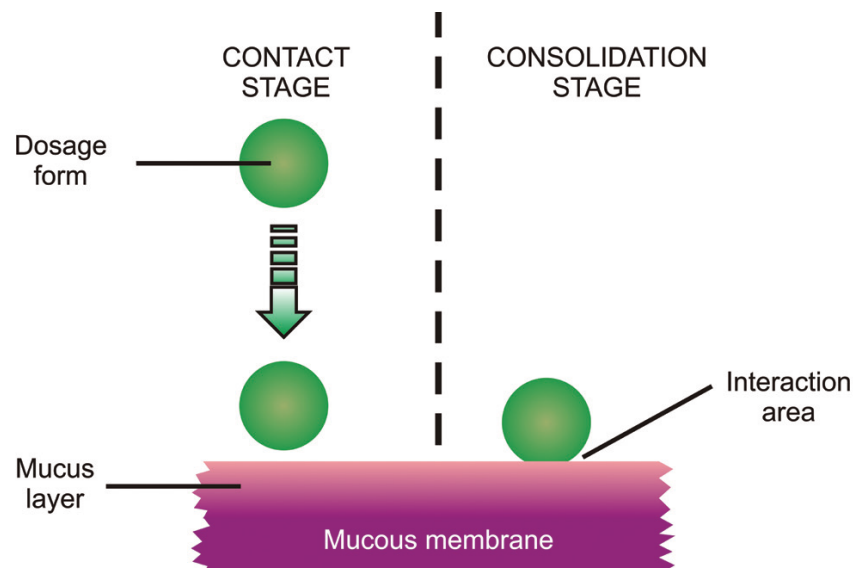


Figure 2: Nanoparticle adhesion to mucus. Two steps of the process (Carvalho *et al.*, 2010)

The considerable advantage of the mucoadhesive application for vaginal drug administration is the prolonged residence time and more direct approach. As a result, novel mucoadhesive formulation would be able to contribute to stable and effective drug concentration at the active site (Carvalho *et al.*, 2010). On the other hand, taking into consideration that primary

vaginal defense mechanism is mucus clearance mucoadhesive, formulations will simply lack time to discharge therapeutic agents and provide the optimal therapeutic effect (Knowles and Boucher, 2002, Ensign *et al.*, 2012a).

2.2.2 Mucus penetration

It was considered that mucus gels sterically exclude pathogens and other particles that are larger than the estimated pore sizes in the mesh network. However, it was observed that even small pathogens (50 nm) can be “captured” by adhesive interactions with mucin. Due to disturbed hydrophobic interactions, followed by pore size reduction, particle free diffusion slows down significantly (Lai *et al.*, 2009).

Small polymeric nanoparticles (around 100 nm) have shown to be less diffusive than the larger ones (200-500 nm) (Lai *et al.*, 2007, Ensign *et al.*, 2012a). This paradox can be explained by turning attention to mucus structure. Small molecules pass easily through narrow channels but are retained in small pockets of mucin network (das Neves *et al.*, 2012). Analogically, large particles will diffuse easily through wide channels with reduced viscosity. However, it was observed that small viruses could get fast and efficiently through the first line of defense and thereby infect underlying epithelial cells (Lai *et al.*, 2010). It was also noted that viruses, which were able to rapidly penetrate mucus lining, were densely coated with both positive and negative charges, thus creating a hydrophilic and net-neutral shell that minimizes mucoadhesive interactions (Lai *et al.*, 2007). The physicochemical characteristics that govern the rapid transport of specific viruses allow them to avoid mucoadhesion. Applying the knowledge on essential properties of a virus to the development of nanoparticles for drug delivery, may improve local treatment of vaginal infections.

Considering uneven vaginal epithelium (rugae), much of the folded lining can be left untreated and unprotected. Mucuoadhesive particles are excluded from the rugae because they are trapped in the upper mucosal layer (Ensign *et al.*, 2012b). Mucus-penetrating particles have shown to provide more homogenous distribution than the conventional mucoadhesive nanoparticles (**Figure 3**) thus leading to increased local drug delivery (Ensign *et al.*, 2012b). However, uniform distribution of a system is not yet enough to boost drug bioavailability. Avoiding mucus entrapment and rapidly penetrating first line of defense is beneficial in reaching underlying epithelial cells (Lai *et al.*, 2007). Manipulating formulation

characteristics may prevent nanocarriers from being shed, thus escalating drug bioavailability (Wang *et al.*, 2008).

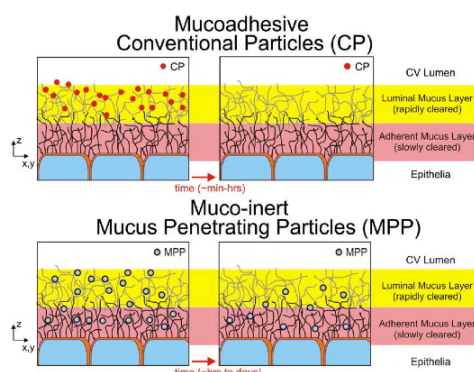


Figure 3: Schematic illustration of the fate of CP and MPP administered to mucosal surface (Lai *et al.*, 2009)

PEG (**Figure 4**) is one of the most well-known polymers that possesses distinguished physicochemical and biological properties, and is frequently used in nanoparticle surface modification for drug delivery purposes. It is hydrophilic, non-ionic and nontoxic in nature and do not possess such strong mucoadhesive properties like chitosan (Ge *et al.*, 2002, Yoncheva *et al.*, 2005). PEG is used to prolong the systemic circulation time of nanoparticles by “camouflaging” them from the body defense mechanisms.

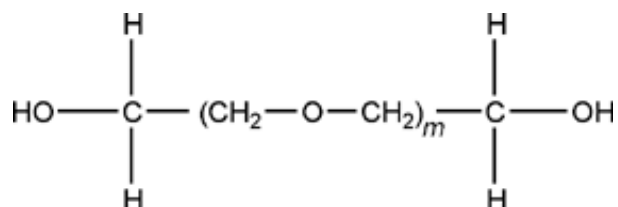


Figure 4: Structural formula of polyethylene glycol (Medicines Complete)

The density and molecular weight determines whether PEG will act as a mucoadhesive or a penetrating agent. Nanoparticles densely coated with low molecular weight PEG have nearly neutral surface charge and minimize mucoadhesion by reducing hydrophobic or electrostatic interactions (Ensign *et al.*, 2012a). High molecular weight PEG, on the other hand, exhibits increased mucoadhesion due to greater number of intermolecular interactions (Ensign *et al.*, 2012a).

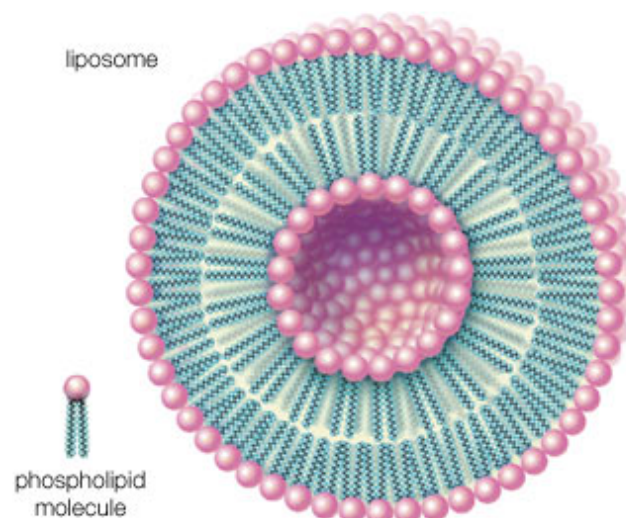
Modulating nanoparticles (coating) with short-chain PEG will be able to create a hydrophilic shell that prevents hydrophobic and electrostatic interactions with mucin, thus reducing particle diffusion hindrance (Wang *et al.*, 2008, das Neves *et al.*, 2012). Additionally, PEG

may enhance the stability of nanoparticles in mucus and large PEGylated particles may afford higher drug encapsulation with a possibility of sustained release (Lai *et al.*, 2009). Nevertheless, dense PEGylation may increase producing costs, thus making nanopharmaceuticals expensive and less affordable (das Neves *et al.*, 2012).

2.3 Liposomes

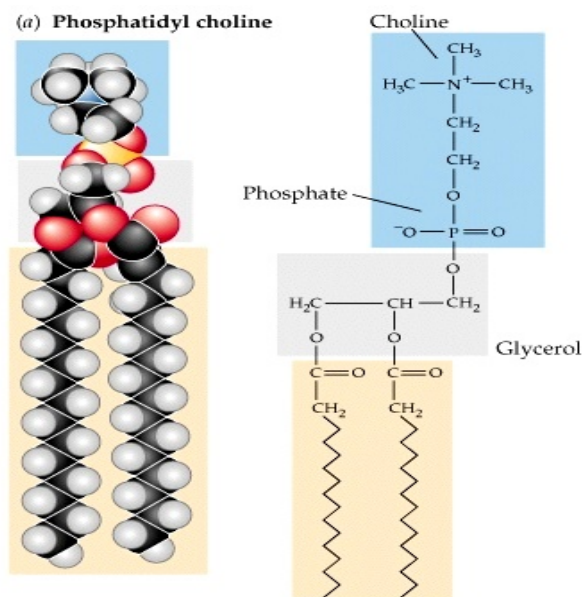
Liposomes are small spherical lipid vesicles, composed mainly of phospholipids (amphiphilic molecules). The most common phospholipids are assembled from phosphatidyl choline molecules. These amphiphilic molecules have hydrophilic “head” and lipophilic “tail” (**Figure 5**). A “glycerol bridge” holds the two entities together (**Figure 6**). In aqueous media they have a strong tendency to form membranes where polar heads face hydrophilic environment and tails cluster together and form lipid layers (Bozzuto and Molinari, 2015). The formation of liposomes is spontaneous and gives rise to vesicles that may differ in size: from few nanometers (nm) to tens of microns in diameter (New, 1990).

Number of bilayers (lamellae), size and method of preparation gives rise to a liposome classification (Barratt, 2000, Bozzuto and Molinari, 2015). Liposomes can be classified as large multilamellar vesicles (LMV), small unilamellar vesicles (SUV) or large unilamellar vesicles (LUV).



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Figure 5: General structure of liposomes (Encyclopædia Britannica)



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Figure 6: Structure of a phosphatidyl choline molecule (Electronic Journal of Biomedicine)

The lipid bilayer is fluid and flexible which can compromise the stability of liposomes because the molecule may suddenly “burst”. In order to avoid this and to design more stable liposomes, cholesterol is incorporated in the membrane. Cholesterol is a naturally occurring molecule (**Figure 7**) and an important component in most membranes (New, 1990). It is inserted into membrane with its hydroxyl group oriented towards the aqueous surface, and the aliphatic chain aligned parallel to the acyl chains in the center of the bilayer. The incorporation of a cholesterol molecule will result in structural and chemical changes, making the bilayer rigid and less permeable. These features can be exploited in development of drug delivery systems.

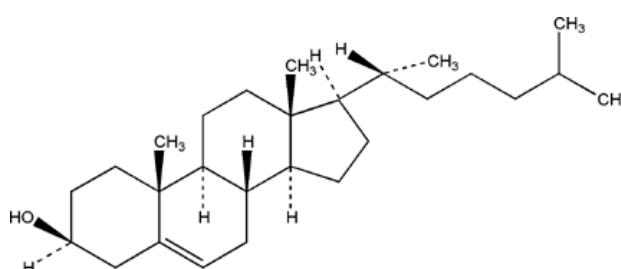


Figure 7: Molecular structure of cholesterol (Medicines Complete)

2.3.1 Liposomes as drug delivery system

Liposomes were first proposed as biological carriers in 1971 (Gregoriadis *et al.*, 1971). The ability of liposomes to function as drug carriers depends on factors such as physiochemical membrane properties, the nature of compositional elements, size, surface charge and lipid organization (Bozzuto and Molinari, 2015). The greatest value of liposomes is that they are composed of natural constituents and can nearly be tailor-made in order to achieve the desired properties, both chemically and structurally (Singh and Lillard Jr, 2009). Liposomes can be designed to be target-specific and release its content only under favorable conditions (for example specific pH value or temperature). The release timeframe may be prolonged if needed to establish sustained drug discharge over a period of hours or even days at the site of action (Singh and Lillard Jr, 2009). The latter can be achieved with surface modifications and/or using biodegradable materials. Taking into consideration that certain amount (compared to local treatment) of an active ingredient taken orally is required to achieve and thereafter maintain therapeutic effect, the development of “smart” pharmaceuticals predisposes to dose reduction and improving of bioavailability.

Liposome properties as variation in size and composition provide a unique opportunity to incorporate active ingredients both on the outer membrane, inside the phospholipid bilayer and within the aqueous core. Liposomes can be constructed in such a manner that will ensure the best encapsulation and targeted delivery of a therapeutic agent.

As a result of their properties, liposomes have already been used as drug delivery systems in treatment and prevention of vaginal viral infections and cervical cancer, though the need for new formulations is persistent (Vanić and Škalko-Basnet, 2013).

2.3.2 Preparation of liposomes

Liposomes can be prepared using several methods: mechanical method, methods based on replacement of organic solvent(s) by aqueous media and methods based on detergent removal (Wagner and Vorauer-Uhl, 2011). The thin film method, a type of mechanical method, is a widely used technique that produces heterogeneous population of multilamellar liposomes, where vesicle size is influenced by the lipid charge (Wagner and Vorauer-Uhl, 2011, Bozzuto and Molinari, 2015). A convenience of this method is that it can be applied for various lipid compositions. Further it is easy to perform and high encapsulation of both lipid and aqueous

soluble substances can be achieved, since the molecules are amphiphilic in nature and high lipid concentration may be used (Wagner and Vorauer-Uhl, 2011). However, the scale of production is limited and not well suited for industrial manufacturing (Wagner and Vorauer-Uhl, 2011).

2.4 Vaginal infection

2.4.1 General

The human body presents extraordinary machinery that is capable of self-inspection and control. Physical barriers (skin) as well as biological barriers (pH) shelter our body from external exposure. Symbiosis with microorganisms (microbiota) that reside in the body of a host is favorable for both the recipient and microorganisms (Reid *et al.*, 2011). During healthy state, vagina is colonized with microbiota, for example *Lactobacilli* that make intravaginal pH (3.5 - 4.5) slightly acidic due to lactic fermentation (Petrova *et al.*, 2013). These relations are valuable (symbiosis) and benefit both the host and organisms.

On entering the cervicovaginal tract, viruses compromise the acidic pH, epithelial barrier, mucus lining and innate immune system. This activates an immune response. The latter consists of four general steps (Kumamoto and Iwasaki, 2012):

1. Recognition of virus by innate immune system, thus leading to activation of defense mechanisms, for example secretion of cytokines
2. Processing and presentation of the virus antigens by adaptive immunity
3. Elimination of a pathogen
4. Establishing long-term memory

Vaginal infections are not limited to viruses (for example HIV and HPV), but can also be caused by pathogenic bacteria (*E.coli*) and yeast (*Candidas*) with their own disease progression.

2.4.2 Human Papillomavirus (HPV)

HPV is an infectious disease, which infects a wide variety of organisms including humans. Current way of contamination is close skin-to-skin intimate contact which makes it the most common sexually transmitted diseases in both genders (Mohammad and Zargar, 2014). Physico-clinical manifestations are anogenital warts which vary in size and complexity (King *et al.*, 2013).

Papillomaviruses (**Figure 8**) are defined as a group of small, nonenveloped, double-stranded DNA viruses belonging to the family Papovaviridae (Mohammad and Zargar, 2014). The two important constituents are the major capsid protein, L1, and the minor capsid protein L2. The infection that is caused by this type of virus is restricted to epithelial cells with preference for either cutaneous or mucosal surfaces (Groves and Coleman, 2015). Thus presentation of viral antigens to the host immune system is limited (Dillner *et al.*, 2007).

HPV can be divided into two types: low-risk and high-risk subtypes based on the oncogenic potential. Low-risk subtypes, for example HPV 6 and HPV 11 are associated with benign anogenital warts, whereas high-risk subtypes (HPV 16, 18, 31 etc) have a strong predisposition to anogenital cancer (Groves and Coleman, 2015). Despite the fact of clinical manifestations, most infections are unapparent and cleared by host immune system in short time (Groves and Coleman, 2015).

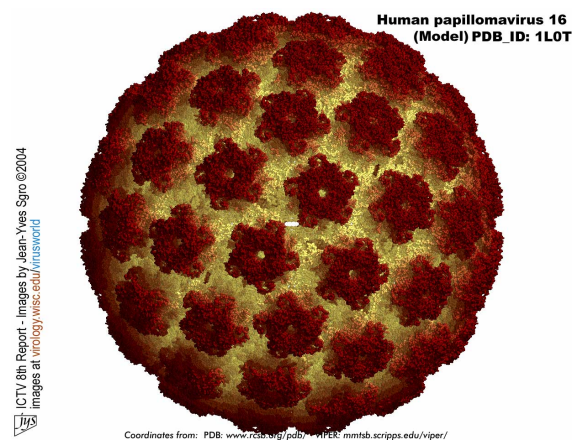


Figure 8: A model of Human Papillomavirus (Virusworld)

2.4.3 Pathogenesis of HPV

In the interest of developing not only targeted but also effective treatment we need to take a look at virus life cycle (**Figure 9**). Small papillomavirus targets basal epithelial cells through entry mechanisms where it initiates proliferation of new daughter cells.

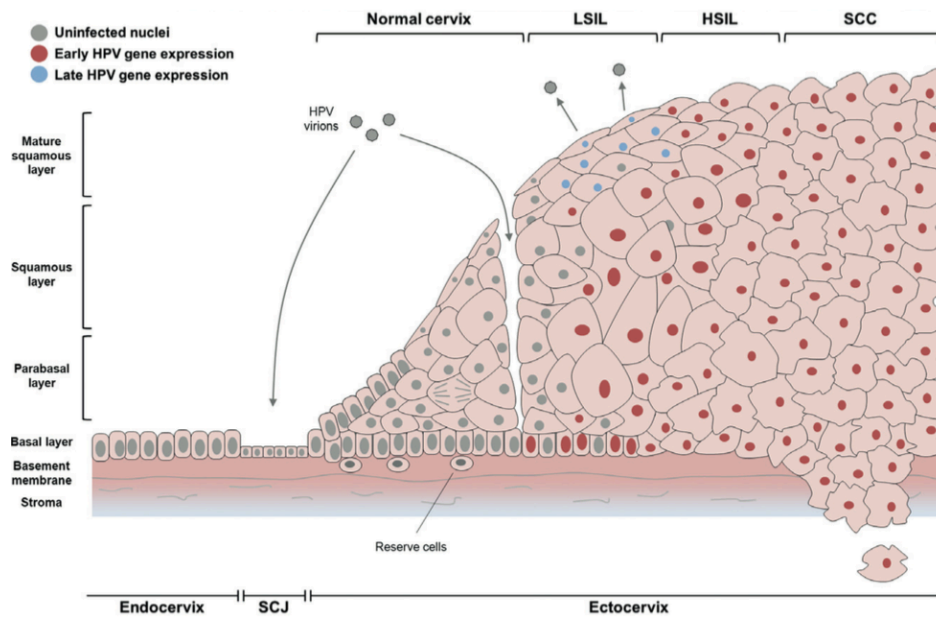


Figure 9: Illustration of HPV pathogenesis (Groves and Coleman, 2015)

After a while, new replicates traverse to a parabasal layer continuing expressing virus proteins. The number of migrated cells increases while virus continues climbing upper layers. Expression of structural proteins L1 and L2 in the latest stages allows encapsidation of infectious virions, which in the end are shed from the cornified surface (Groves and Coleman, 2015).

2.4.4 Current treatment of HPV infections

HPV infections are difficult to cure, because of the high re-occurrence rate (King *et al.*, 2013). Current treatment of human papillomavirus includes:

1. Vaccination as a preventative measure, composed of virus-like particles (Gardasil[®] and Cervarix[®]) (Dillner *et al.*, 2007)
2. Electrocoagulation; the use of intense heat generated by electric current
3. Cryotherapy; use of extreme cold and
4. Laser ablation or local surgery.

Alternative topical treatment is Veregene[®] ointment (podophyllotoxin, sinecatechin), 5-fluorouracil and trichloroacetic acid that are applied only on lesions thus avoiding healthy tissue (Foldvari and Kumar, 2012). Interferon alpha is a currently approved treatment of anogenital warts and can be administered as intramuscular (for treating exophytic, visible lesions) or intralesional injection (Foldvari and Kumar, 2012). Zyclara[™] and Aldara[™] that contain Imiquimod, an IFN inducing agent, are also used for treatment of external and perianal warts (Foldvari and Kumar, 2012).

Nonetheless, these treatments (except vaccination) are effective as long as the disease is localized (Mohammad and Zargar, 2014). The largest limitation of injection include pain and systemic exposure thus elevating the possibility of side effect occurrence (King *et al.*, 2013). Topical treatment on the other hand will contribute to better compliance from the patient side, avoid first metabolic passage and thereafter degradation by liver enzymes, and make localized approach more accessible.

There have been made attempts to expand the possibility of topical application of IFNs using such conventional formulations as creams and gels with an active pharmaceutical ingredient (Foldvari, 2010, Foldvari and Kumar, 2012). Unfortunately, the results were neither conclusive nor consistent. This demonstrates a persistent need for improving existing drug delivery systems.

2.5 Interferon α -2b (IFN α -2b)

2.5.1 General

IFNs are a group of naturally occurring proteins and the first prototypes to developing other types of cytokines (**Figure 10**). Time has shown that IFNs possess important immunomodulatory, antiviral (any stage in viral replication seems to be susceptible to IFNs), antiangiogenic, antiproliferative, and antitumor properties that can be exploited for medical purposes (Killion *et al.*, 1994, Stark *et al.*, 1998, Parmar and Plataniias, 2003).



Figure 10: IFN α 2-b protein structure (Drug Bank)

IFN α -2b was discovered as antiviral agent during studies on virus interference (Parkin and Cohen, 2001) and provide an early (hours or even days) line of defense against viral infections in our body.

2.5.2 Classification

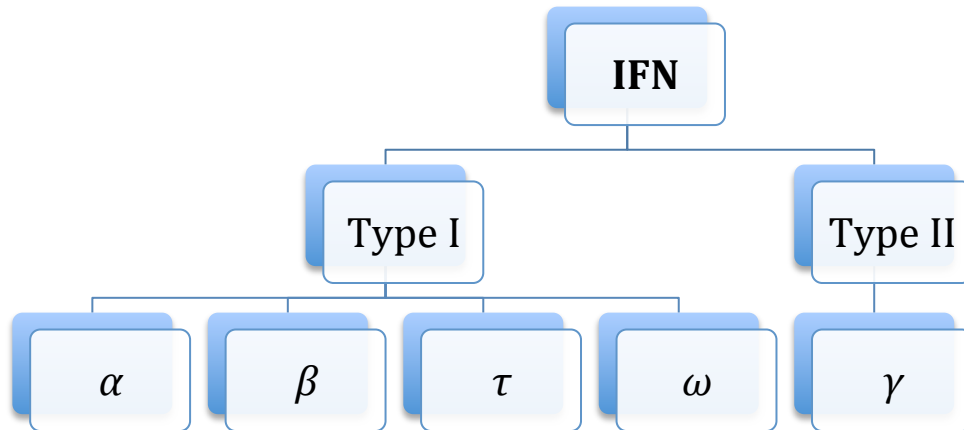


Figure 11: Classification of IFNs

In the beginning, classification of IFNs was based on their separation profiles in HPLC, later on it was discovered that function of IFN molecules is defined by the genesis. For example, IFN α is produced by leukocytes, β by fibroblasts and γ by immune cells (Parmar and Plataniias, 2003).

IFNs are divided into 2 classes: type I and type II (**Figure 11**). IFN subtypes α , β , τ and ω fall under type I, while type II is subdivided only to γ . All subclasses in their turn are divided as well.

IFN α 2-b is a clear, colourless or slightly yellowish liquid with the average protein weight of 19271.0000 Da (Drug Bank). It is produced by a method based on recombinant DNA technology using bacteria as host cells (Med. Complete).

2.5.3 Application

IFNs have a wide range of application. They target several viral diseases such as chronic hepatitis B and C, HPV and show promising anticancer activities (Hamidi *et al.*, 2007, Med. Complete).

On the other hand, IFNs have certain limitations as short circulation time and unwanted effects of non-targeted tissues (Hamidi *et al.*, 2007). It is worth noticing that interferons are not absorbed from the gastrointestinal tract, and when attached to large molecules, reduce the rate of excretion and increase the plasma concentration (Med. Complete).

Improving existing formulations, by for example using “smart” technologies the application range widens. That will give an additional freedom in designing and choosing the appropriate treatment. IFN-containing liposomes have been evolving and developing for many years and the application range is still growing (Hamidi *et al.*, 2007).

3. Aims of the study

The aim of this study was the development and characterization of mucus-penetrating liposomes as a model for antiviral drugs in localized vaginal delivery. Surface modified vesicles (liposomes) were expected to encapsulate sufficient drug amount and enhance penetrating properties at a vaginal site.

The aims were divided as following:

- Development of mucus-penetrating liposomes by modification of liposomal surface
- Characterization of liposomes in respect to size, polydispersity, surface charge and entrapment efficiency
- *In vitro* drug release testing

4. Materials and Methods

4.1 Materials

- Acetic acid (glacial) anhydrous GR for analysis, Merck KGaA, Darmstadt, Germany
- Ammonium acetate, VWR International, Leuven, Belgium
- Chloroform, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany
- Cholesterol (from Lanolin) for GC, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany
- Di-Sodium hydrogen phosphate dehydrate GR for analysis, Merck KGaA, Darmstadt, Germany
- Distilled water
- Ethanol 96% (v/v), Sigma-Aldrich, Chemie GmbH, Steinheim, Germany
- (Ethylenedinitrilo)tetraacetic acid, disodium salt dehydrate (EDTA dinatriumsalt), Merck KGaA, Darmstadt, Germany
- IntronA[®] 50 million IU/ml injection fluid in multiple dose pen (active pharmaceutical ingredient Interferon alpha-2b), MSD AS, Drammen, Norway
- Lipoid S 100 (soyabean lecithin, >94% phosphatidylcholine), Lipoid GMBH, Ludwigshafen, Germany
- Methanol CHROMASOLV[®] for HPLC, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany
- N-(Carbonyl-methoxypolyethylene glycol-2000)-1,2-distearyl-sn-glycero-3-phosphoethanolamine, (sodium salt), Lipoid GMBH, Ludwigshafen, Germany
- Polysorbatum 80, Norsk Medisinal Depot, Harstad, Norway
- Sephadex[®] G-25, for molecular biology, DNA grade superfine, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany
- Sodium chloride for AT, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany
- Sodium dihydrogen phosphate monohydrate, Merck KGaA, Darmstadt, Germany
- Triton[®] X-100, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany
- VeriKine[™] Human IFN α Multi-Subtype ELISA Kit, Pestka Biomedical Laboratories Inc, PBL Assay Science, Piscataway, USA

4.2 Computer programs

- Nicomp Particle Sizing System, CW 388 version 1.68
- Soft Max Pro[®], Molecular Devices Corporation
- UV-Visible Chem Station Software, Agilent Technologies
- Zetasizer Software

4.3 Instruments

- Agilent 8453 UV-visible spectroscopy system, Agilent technologies, Santa Clara, USA
- Branson 1510, Bath sonicator, Branson Ultrasonics, Danbury, USA
- Büchi rotavapor R-124, Büchi, Flawil, Switzerland
- Büchi Vacuum Controller B-721, Büchi, Flawil, Switzerland
- Büchi Vac[®] V-500 vacuum pump, Büchi, Flawil, Switzerland
- Büchi waterbath B-480, Büchi, Flawil, Switzerland
- Julabo Refrigerated/Heating circulator F12-ED, Julabo Labortechnik GmbH, Seelbach, Germany
- Metrohm 744 pH Meter, Metrohm AG, Herisau, Switzerland
- Microplate spectrophotometer SpectraMAX 190, Molecular Devices, Sunnyvale, USA
- PermeGea Ink, Diffusion cells and Systems, Hellertown, USA
- Sartorius BP2HD, Analytical Scale, VWR International, Oslo, Norway
- Submicron Particle Sizer, model 370, Nicomp, Santa Barbara, USA
- Vortex Genie 2[™], Bender & Hobein AG, Zurich, Switzerland
- Zetasizer Malvern, Malvern Zetasizer Nano L, Oxford, UK

4.4 Equipment

- Cellophane, Bringmann, Wendelstein, Germany
- Filter 0.22 μm non-sterile syringe filters, Pall Life Sciences, Acrodisc[®], Cornwall, UK
- Glass wool superfine, Assistent[®], Kebolab, Darmstadt, Germany
- Microtubes, 6×50 mm, Borosilicate Glass, Disposable Culture tubes, Kimble Chase, Vineland, USA

- Nuclepore® Track-etched Membranes, 0.2 μm , 0.4 μm , 0.8 μm , Nuclepore® Polycarbonate (PC), Whatman International Ltd, Whatman House, Kent, UK
- Round bottom flask, 50 ml, NS 29/32, Boro 3.3, VWR, Darmstadt, Germany

4.5 Methods

4.5.1 Preparation of IFN α -2b buffer

IFN α -2b buffer was prepared by dissolving NaCl (15 g), $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$ (3.6 g), $\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$ (2.6 g), Polysorbatum 80 (0.2 g) and EDTA (0.2 g) in distilled water, and the volume was adjusted to 2 L. Measured pH was 6.77.

4.5.2 Preparation of IFN α -2b solution

IFN α -2b solution (10 million IU) was transferred from dose pen to a 5 ml volumetric flask and diluted with buffer prepared for IFN α -2b. The concentration of the IFN α -2b solution was calculated to be 2 million IU/ml.

4.5.3 Preparation of empty liposomes

Liposomes were prepared by thin film method (New, 1990). In brief, Lipoid S 100 (200 mg), PEG-2000 (36.3 mg) and Cholesterol (10 mg) were weighed in the round bottom flask and dissolved in methanol and chloroform solution (1:1). Using rotoevaporator, for at least 90 min at 50 mm Hg and 51°C, the solvent composition was evaporated and thin lipid layer observed. Lipid composition in the round bottom flask was flushed with nitrogen for 1 min to make sure that all solvent was evaporated. The remaining film was re-suspended with buffer solution prepared for IFN α -2b and shaken vigorously in order to dislodge all the film. If necessary, vortex was used. Liposomal suspension was stored in the refrigerator (4-8°C) overnight prior to further use.

4.5.4 Preparation of liposomes with IFN α -2b

Liposomes containing IFN α -2b were prepared using the same method described above, only liposomal film was re-suspended in 5 ml of IFN α -2b solution. Liposomal suspension with IFN α -2b was stored in the refrigerator (4-8°C) overnight prior to further use.

4.5.5 Vesicle size reduction

Liposomal suspension was extruded through 0.8 μ m, 0.4 μ m and 0.2 μ m polycarbonate filters. Extrusion was performed 5 times on each filter. Extruded liposomes were stored in the refrigerator (4-8°C) overnight prior to further use.

4.5.6 Particle size analysis

The analysis of liposomal particle size was performed by photon correlation spectroscopy (Nicomp model 370). In order to avoid interference, microtubes that were used in particle size analysis were sonicated for 10 min in ultrasonic bath and then rinsed twice with distilled, filtered water (0.2 μ m pore size syringe filter) prior to further use. Small amounts of the liposome dispersions were diluted with freshly filtered distilled water to achieve the intensity of approximately 250-350 kHz (Ingebrigtsen and Brandl, 2002). All preparations were done in a laminar airflow bench. Each sample was analyzed for 3 cycles with time duration 10 min each. Gaussian and NICOMP distribution analysis were used accordingly.

4.5.7 Zeta potential determination

The zetasizer capillary cells were rinsed with 96% ethanol (one time) and filtered water (3 times) prior to experiment conduction. The liposome samples were diluted 1:19 with filtered water. Zeta potential was measured for 3 cycles with a voltage of 4 mV.

4.5.8 Gel column preparation

Gel column was prepared by blending Sephadex G-25* (15 g) with 120 ml IFN α -2b buffer (Gel Filtration Bok). The components were gently stirred in a beaker and placed for swelling overnight at 4°C prior to further use. Before packing, the mixture of Sephadex was brought to room temperature (23-24°C) and the opening on the bottom of burette was covered with an adequate amount of glass wool. The viscous mixture was transferred to a burette in a continuous speed to avoid formation of air bubbles. The column was equilibrated with 100 ml of a buffer solution and stored in room temperature prior to further use.

4.5.9 Separation of a free drug

Before applying the sample, the top of the column was freed from buffer to avoid further dilution of the active ingredient. Liposomal IFN α -2b solution was applied evenly on the top of the column and thereafter was pulled further into the column by gravitational force. After gel separation, fractions containing liposomes were determined by UV-spectrophotometer. The wavelength was set to 205 nm.

4.5.10 Preparation of 0.3 % Triton buffer

In this experiment, Triton buffer (Yang *et al.*, 2006) is needed for lysing liposomes for further analysis. It was prepared from 300 mg Triton X-100 solved in buffer solution for IFN α -2b in a volumetric flask. The volume was adjusted to 100 ml and stored at a room temperature prior to further use.

4.5.11 Enzyme-linked immunoassay (ELISA)

Preparation of samples includes merging and dilution of liposomal fractions. IFN α -2b standards (10 000 pg/ml) (ELISA) were diluted to appropriate concentrations with IFN α -2b buffer.

Wash solution concentrate (50 ml) (ELISA) was diluted with distilled water up to 1 L in a volumetric flask and stored at a room temperature prior to further use. Diluted HRP solution was in its turn prepared by blending HRP concentrate (80 μ L) (ELISA) and concentrate

diluent (12 ml) (ELISA). Diluted antibody solution was prepared by merging antibody concentrate (120 μ L) (ELISA) and dilution buffer (12 ml) (ELISA).

- **Step 1**

Samples, standards and blank (100 μ l) were applied in wells, thereafter covered with plate sealer and incubated for 1 h. After 1 h the content was emptied and wells washed once with diluted wash buffer.

- **Step 2**

Diluted antibody solution (100 μ L) was added to each well, covered with plate sealer and incubated for 1 h. After 1 h the content was emptied and wells washed 3 times with diluted wash buffer.

- **Step 3**

Diluted HRP (100 μ L) solution was added to each well, covered with plate and incubated for 1 h. During this hour TMB substrate solution was brought to room temperature. After 1 h the wells were emptied and washed 4 times with diluted wash buffer.

- **Step 4**

TMB substrate solution (100 μ L) was added to each well. The plate was covered with aluminium foil and incubated in dark for 15 min.

- **Step 5**

After 15 min, 100 μ L of stop solution was added. The drug content of samples was determined spectrophotometrically at 450 nm by microplate reader.

4.5.12 Preparation of acetate buffer

Acetate buffer was prepared by dissolving 38.55 g of ammonium acetate ($\text{CH}_3\text{COONH}_4$) in distilled water, afterwards 35 ml of glacial acetic acid ($\text{C}_2\text{H}_4\text{O}_2$) was added and the volume adjusted to 1L with distilled water (Ph.Eur). Measured pH 4.51.

4.5.13 *In vitro* drug release study

Before use, Franz Diffusion cells were washed once with methanol (30 min) and twice with distilled water (30 min). The acceptor chambers were 12.0 and 12.1 ml. The temperature was set to 37°C and cellophane membrane was soaked in acetate buffer for at least 30 min prior to use. The reception chamber was filled with acetate buffer and covered with pre-soaked cellophane membrane. Samples (600 μ l) were applied in the donor cells and the system was

completely sealed. Samples (500 μ l) were collected after 1, 2, 3, 4, 5, 6, 7 and 8 h. An equal amount of buffer was added to replace extracted sample. Drug amount was assessed by ELISA kit.

5. Results and discussion

5.1 Liposome characterization

5.1.1 Liposomal size

The most common methods for size reduction are sonication, extrusion and high-pressure homogenization (Bozzuto and Molinari, 2015). Extrusion is characterized as size reduction by passing through a membrane with a defined pore size. Additionally, going from multimodal to unimodal distribution allows correct size estimation and evaluation. Berger *et.al* has shown that small variations in extrusion method (for example continuous or discontinuous extrusion) gave rise to populations that deviated from the target size (Berger *et al.*, 2001). Their finding indicated that the choice of extrusion method might influence the outcome. In this project membrane extrusion was used with pore sizes 800, 400 and 200 nm. The choice of size was based on vaginal mucus physiology, where the diameter of mesh spaces is estimated to be between 200 and 340 nm (Lai *et al.*, 2010, das Neves *et al.*, 2011a). Current reduction method was also used by Karau *et.al* and Li *et.al* to yield reproducible 200 nm IFN α -2b liposomes (Karau *et al.*, 1996, Li *et al.*, 2011). Thus the obtained vesicle size (**Table 3**) was considered to be well suited for vaginal drug delivery.

Vesicle size was estimated by photon correlation spectroscopy (PCS) using Gaussian and NICOMP distribution analysis accordingly. Liposomes re-suspended in distilled water (L1), IFN buffer (L2) and buffer containing IFN α -2b (L3) were analyzed and liposomal size distribution was determined before extrusion (**Table 1**) and after extrusion through 400 nm (**Table 2**) and 200 nm (**Table 3**) pore size membranes.

Table 1: The size of pre-extruded liposomes (n=4)

Sample	Peak 1 (nm)	Intensity (%)	Peak 2 (nm)	Intensity (%)	Peak 3 (nm)	Intensity (%)	PI
L1	35 ± 25	0.9	181 ± 58	16.4	890 ± 46	82.3	0.40
L2	71 ± 4	8.6	107 ± 1	12.2	905 ± 0.1	81.0	0.67
L3	90 ± 37	9.5	146 ± 37	16.0	845 ± 202	78.0	0.61

L1: liposomes re-suspended in distilled water, L2: liposomes re-suspended in IFN buffer and L3: liposomes containing IFN α -2b.

PI - polydispersity index. The values denote the average of three cycles \pm SD

Table 2: The size of liposomes after extrusion through 400 nm membrane (n=4)

Sample	Peak 1 (nm)	Intensity (%)	Peak 2 (nm)	Intensity (%)	Peak 3 (nm)	Intensity (%)	PI
L1	94 ± 49	19.0	287 ± 70	81.5	-	-	0.17
L2	85 ± 41	9.8	256 ± 84	50.6	706 ± 251	42.1	0.33
L3	110 ± 35	17.0	357 ± 72	82.1	-	-	0.23

L1: liposomes re-suspended in distilled water, L2: liposomes re-suspended in IFN buffer and L3: liposomes containing IFN α -2b.

PI - polydispersity index. The values denote the average of three cycles \pm SD

Table 3: The size of liposomes after extrusion through 200 nm membrane (n=4)

Sample	Mean diameter (nm)	PI
L1	200 ± 11	0.08
L2	195 ± 13	0.10
L3	185 ± 3	0.09

L1: liposomes re-suspended in distilled water, L2: liposomes re-suspended in IFN buffer and L3: liposomes containing IFN α -2b.

PI - polydispersity index. The values denote the average of three cycles \pm SD

Pre-extruded liposomes (**Table 1**) gave rise to 3 distinct distribution peaks with diverse intensity. According to the results, pre-extruded suspensions (L1 – L3) contain large liposomes (890, 905 and 845 nm) and display high PI (0.40 – 0.67). An acceptable PI value should not exceed 0.7, since the results at this value are not reliable. Large size suggests the presence of multilammellar liposomes, and high PI value indicates the presence of both large and small vesicles in suspension.

Liposomes were first extruded through 800 nm membrane as an additive measure in order to minimize vesicle resistance and avoid spillage in further extrusion steps. Particle size was not measured during this step.

We could clearly observe that the average vesicle size and PI was reduced after extrusion through 400 nm membrane for liposomes re-suspended in water (L1) and liposomes after extrusion through 200 nm membrane (L3), but not for liposomes re-suspended in IFN buffer (L2) (**Table 1** and **Table 2**). However there are still some smaller vesicles in formulation with the size range between 85 and 110 nm (**Table 2**). Liposomes containing only IFN buffer (**Table 2**) showed an additional third peak (706 nm) with a relatively high intensity (42.1 %) and there is no clear size reduction observed. Particular liposomal behavior is unexpected, especially after extrusion through 400 nm membrane and might indicate liposomal agglomeration as a result of inability to form stable vesicles in this size range. Nevertheless, the desired liposome size has not been reached.

After extrusion through 200 nm membrane, the IFN α -2b containing liposomes display values close to the desired size and uniformity (**Table 3**). Experimentally received diameter (185 nm) slightly deviates from the desired (200 nm) but is still in the accepted size range. **Tables 1 - 3** illustrate the effectiveness of extrusion as size reduction method and uniformity of vesicles in a suspension. Liposomal size in formulations L1 and L3 decreases and displays a more uniform size distribution. We can observe that the size reduces with every extrusion step and PI value decreases. Uniformity, described by PI, is an important tool in drug delivery due to possible prediction of entrapment and even drug distribution.

5.1.2 Liposomal zeta potential

Zeta potential presents “the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle” (Honary and Zahir, 2013) and is very important factor in targeting drug delivery. Charged lipids form smaller liposomes with less lamellae (Wagner and Vorauer-Uhl, 2011) and vice versa. It has earlier been discussed that charged drug loaded nanocarriers are expected to interact with mucus layer by forming electrostatic interactions (Honary and Zahir, 2013), thereby nanoparticles with net charge close to neutral may aid in achieving mucus-penetrating properties by avoiding interactions with mucin (Cu and Saltzman, 2008).

Table 4: Zeta potential values of liposomes (n=4)

Zeta potential (mV)			
Sample	L1	L2	L3
Pre-extruded	-20.5 ± 0.8	-10.7 ± 2.3	-11.4 ± 1.4
400 nm	-17.4 ± 1.4	-10.5 ± 0.9	-12.2 ± 1.4
200 nm	-15.1 ± 1.3	-11.1 ± 1.4	-12.2 ± 1.4

L1: are liposomes re-suspended in distilled water, L2: liposomes re-suspended in IFN buffer and L3: liposomes containing IFN α -2b. The values denote the average of three cycles \pm SD

The net charge of pre-extruded non-drug loaded liposomes (L1) presented in **Table 4** is negative (-20.5 ± 0.8 mV), however, the charge decreases alongside vesicle size (-15.1 ± 1.3 mV). Zeta potential of liposomes re-dispersed in IFN buffer (L2), on the other hand, remains approximately the same in pre-extruded (-10.7 ± 2.3 mV) and in extruded suspensions (-11 ± 1.4) (**Table 4**). The outcome (-12.2 ± 1.4 mV) deviates from the expected (close to neutral) and might influence mucus-penetrating properties of IFN α -2b-loaded liposomes, because negative charge can establish electrostatic interactions with the mucus layer. There is no difference in surface charge between L2 and L3 after extrusion through 200 nm membrane. It indicates that the charge remains the same despite the addition of IFN α -2b.

5.1.3 IFN α -2b entrapment

Separation of liposome encapsulated and free IFN α -2b is an important step in vesicle characterization, since the outcome allows estimating drug-loading capacity. This knowledge will aid in formulation design and may propose lower drug amount for an improved therapeutic effect, for example vaginal administration route.

As described in section 4.5.8, liposomal formulation containing IFN α -2b was separated through gel column. Separation principle is based on size exclusion, where large molecules are expected to elute first, and small molecules (free drug) last. Drifting through gel pores, small molecules use more time on eluting, large particles, on the other hand, evade tiny “pockets” and appear earlier. **Figure 12** and **Figure 13** demonstrate gel separation of nanoparticle suspension where we can observe liposomes appearing already in fraction 20. The peak is reached after 23 fractions (**Figure 12**). Majority of liposomes are eluted in a few fractions, confirming size uniformity. However, a short elongation after liposome elution was

seen. The vesicle size distribution of IFN α -2b liposomes (**Table 3**) indicate that this is not due to presence of smaller liposomes, thus elongation might be caused by free drug. Further, such elongation pattern might also be explained by retention of liposomes at the site of elution. Based on these findings, we cannot be certain of a good separation despite the fact that given column material is able to separate molecules with high molecular weight (IFN α -2b is 19 kDa). Possibly another type of column material (for example Sephadex G-50) would achieve clearer separation. A similar approach (Sepharose CL-4B column) was used by Karau and co-workers to isolate drug-loaded liposomes (200 nm) from free IFN α -2b and their method demonstrated good outcome (Karau *et al.*, 1996). In case of poor separation, there will be difficulties in establishing precise amount of encapsulated drug, which in its turn will affect entrapment efficiency.

Figure 13 depicts elution pattern of free IFN α -2b, suggesting drug appearance in patches (fractions 56-59 and 83-94), and not as a collected uniform mass. It is difficult to observe from the figure, so the conclusion was based on values that are not presented here. Such elution pattern implies uneven and slow drug passage throughout the column, because, as it was mentioned earlier, the appearance of small liposomes is excluded. Obvious plateau between liposome and free IFN α -2b peaks confirm separation to some extent.

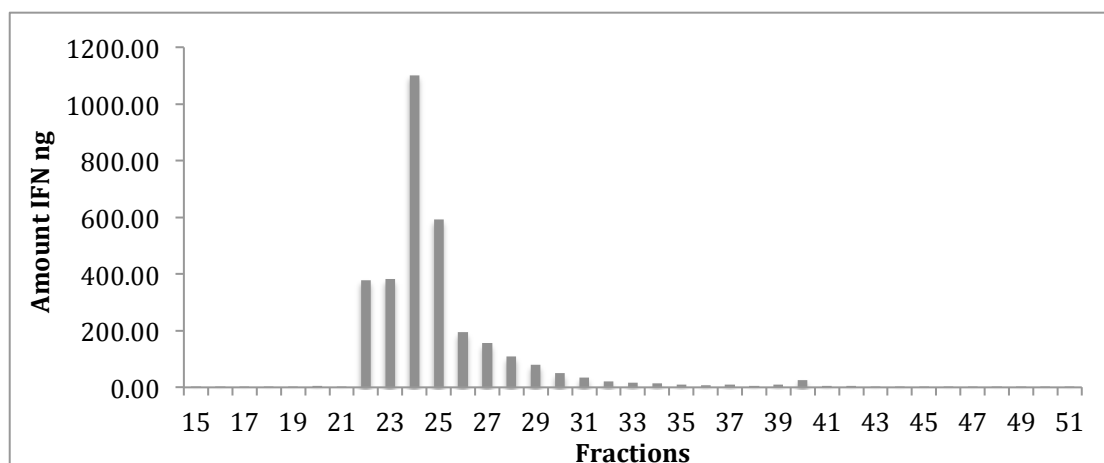


Figure 12: Gel column separation of liposomes. Fractions 15-51

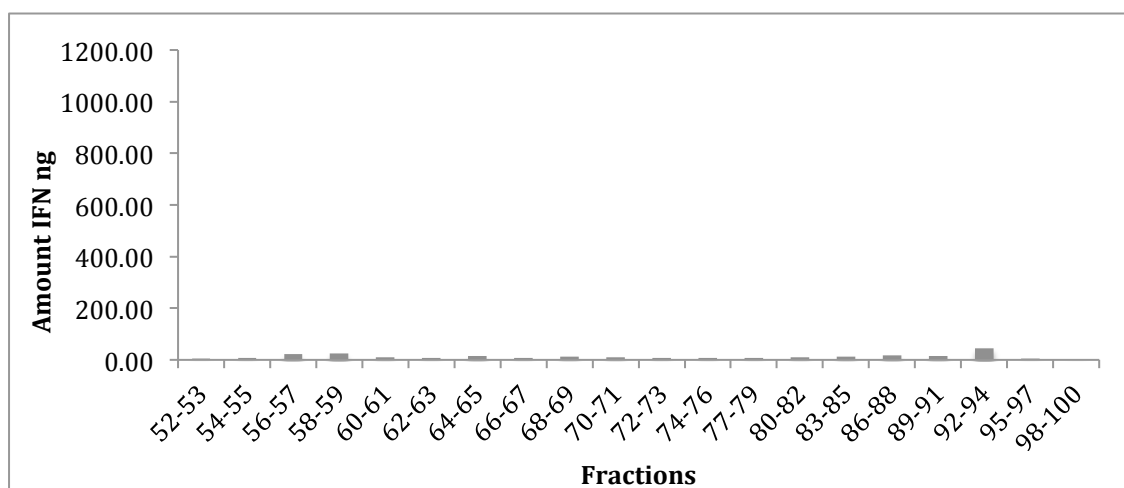


Figure 13: Gel column separation of liposomes. Fractions 52-100

Succeeding in drug encapsulation will allow avoidance of inherent drug limitations such as short circulation lifespan and adverse effects on non-targeted tissues (Hamidi *et al.*, 2007, Foldvari *et al.*, 2010). Entrapment efficiency and recovery values in this project were obtained with the help of ELISA kit. Regression equation from standard curve (repeated for each measurement) was used to determine the IFN α -2b entrapment for each liposomal batch (n=4).

The first three batches yielded entrapment efficiency of 43 %, 72 % and 54 % respectively. These values were calculated against theoretically established amount of IFN α -2b in samples, and loss of drug during experimental steps was not taken into consideration. Thus, entrapment efficiency and drug recovery is affected by calculation approach. In our case, experimentally obtained entrapment and especially recovery values are lower compared to the values we

would have received if correct total values were used. Moreover, the measurements seem to be quite variable while more uniform results are expected. This is an unwelcome feature in developing new formulations since it will be impossible to predict amount of incorporated active ingredient. It is useful to mention that variation (43 %, 72 % and 54 %) might be questionable, because we are not confident it would be present if we have used correct value of total drug amount. Consequently, the need of corroboration of entrapment efficiency variation is required.

We ran an additional experiment where actual drug amount in sample was considered. The entrapment efficiency was found to be 88 % with a drug recovery of 97 %. Unfortunately, due to the fact that this is based on a single experiment (n=1), the reliability is questionable; nevertheless we could have recommended such approach for further investigation. However, our findings are in accordance with results presented by Yang and co-workers who prepared reproducible liposomes with entrapment efficiency over 80 % (Yang *et al.*, 2006). Although, they have used different preparation method (multiple step hydration-dilution technique), homogenization to reduce the size and ultracentrifugation to separate drug-loaded liposomes from free IFN α -2b. Karau *et.al*, have investigated the effect of lipid composition (das Neves *et al.*) and size reduction method (homogenization vs. extrusion) of liposomes on IFN α -2b entrapment efficiency (Karau *et al.*, 1996). They have found that liposomes with negative charge resulted in increased IFN α -2b entrapment compared to neutral liposomal composition. In our case, negative charge is not optional because of the potential interactions with mucin fibers during vaginal mucus penetration. Karau *et.al* have also shown that less drug was lost throughout extrusion than during homogenization (Karau *et al.*, 1996).. Foldvari *et.al*, on the other hand, have demonstrated that multilammellar liposomes (50-200 nm) prepared by modified solvent evaporation method yielded vesicles with high IFN α -2b incorporation degree ($91.7 \pm 2.2\%$) (Foldvari and Moreland, 1997). Despite the differences in preparation methods, our outcome seems to be comparable with the results demonstrated by Foldvari *et.al*, indicating the effectiveness and potential of using liposomes for IFN α -2b entrapment.

To confirm experimentally received high encapsulation efficiency (88%) and reproducibility, the experiment must be repeated. By all means, such high entrapment percentage is a desirable result, since drug amount needed to establish good therapeutic effect, decrease application frequency and extend drug release time will be reduced.

5.2 *In vitro* release of IFN α -2b

Over the years, Franz diffusion cell system has become one of the most widely used methods for measuring *in vitro* drug release. It provides some insight in relationship between drug, formulation and the barrier, thus being useful for designing novel formulations (Ng *et al.*, 2010). Against this background, Franz diffusion cell system was chosen to establish efficiency of IFN α -2b release from liposomes *in vitro*.

Acetate buffer (pH 4.6) was chosen as a receptor medium based on its preferential pH value, since healthy vaginal pH varies between 3.5 - 4.5 (Hussain and Ahsan, 2005, Valenta, 2005). The temperature was set to 37 °C to imitate natural value. The sample was drawn from the acceptor cell every hour for in total 8 hours. The timeframe of the experiment was set due to the mucosal physiological properties such as vaginal shedding (das Neves *et al.*, 2011a). The experiment was run in 3 parallels (data not shown). However collected results were both inconsistent and unpredictable. Possible source of error are mistakes in the experimental performance. Seemingly, little has been done on establishing diffusion degree of IFN α -2b encapsulated liposome molecules through mucus lining. On this basis it is difficult to discuss the anticipated results and draw a conclusion regarding possible origin of experimental mistakes.

6. Conclusion

Surface modified (PEGylated) liposomes containing IFN α -2b prepared by thin film method showed to yield high drug entrapment. The method is easy to perform and produces highly reproducible uniform liposomes. Vesicle size reduction by extrusion method proved to be suitable both in regard to size distribution and in maintaining sufficient entrapment efficiency. PEGylated liposomes appear to be well suited as IFN α -2b carriers.

Designing mucus-penetrating drug carriers for vaginal delivery will allow more direct approach by bringing the active ingredient closer to the sight of action. This approach presents an alternative to painful and unpleasant invasive current treatment and opens up to treatment of non-visible lesions.

7. Perspectives

PEGylated IFN α -2b containing liposomes prepared during this project will be further analyzed in regard to characterization and properties as a drug delivery system. IFN α -2b entrapment efficiency and reproducibility of the method needs to be confirmed as well as *in vitro* drug release testing should be repeated. Furthermore, this experiment should be extended to *ex vivo* testing, and the stability of the drug delivery system in the presence of vaginal fluid simulant and the *in vivo* safety should be determined before animal studies.

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