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PEGylated liposomes for topical vaginal therapy improve delivery of interferon alpha

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Abstract

Recent studies regarding mucosal drug delivery indicate that nanosystems with surface-available polyethylene glycol (PEG) are able to penetrate mucus barrier, assure closer contact with the epithelium, and improve drug delivery to vagina. In the present work, we developed the mucus-penetrating PEGylated liposomes containing interferon alpha-2b (IFN α-2b), destined to provide localized therapy for human papilloma virus (HPV) vaginal infections. The PEGylated liposomes were of a mean size of 181 ± 8 nm, bearing a negative zeta potential of – 13 mV and an entrapment efficiency of 81 ± 10 %. *In vitro* release experiments on model membrane showed a nearly non-existent IFN α-2b release from both the control and liposomally-associated IFN α-2b. However, the *ex vivo* penetration studies performed on the vaginal tissue obtained from pregnant sheep, showed the clear elevated IFN α-2b penetration from PEGylated liposomes as compared to the control. Furthermore, mucin studies confirmed the absence of interaction between the PEG-modified liposomes and mucin, confirming their ability to penetrate mucus and reach the deeper epithelium. The system holds a promise in improving topical delivery of IFN α-2b through enhanced efficacy of local anti-viral therapy.

Key words: vaginal therapy; PEGylated liposomes; mucus-penetrating liposomes; interferon; human papilloma virus

Abbreviations:

HPV: human papilloma virus, IFN α-2b: interferon alpha-2b, PC: phosphatidylcholine, PEG: polyethylene glycol, STDs: sexually transmitted diseases, VFS: vaginal fluid simulant

1. Introduction

Human papilloma virus (HPV) infections, which are responsible for genital warts and transmitted via mucosal surfaces, are one of the most common sexually transmitted diseases (STDs) [1]. Although HPV infections are common in both genders, due to the physiology and anatomy of vagina, women are more prone to the infections and the efficacy of the treatment remains limited. It is estimated that 80 % of all sexually active women will acquire HPV infection by the age of 50 [2]. Some of the HPV infections may spontaneously resolve in younger women; however, the high-risk HPV infections are persistent among women over the age of 30 and often lead to cervical pre-cancerous lesions. Cervical cancer is the second most common cancer in women and the fifth most common cancer overall [1].

Currently available anti-viral therapies mainly target the visible lesions failing to eliminate the virus with the recurrence rate of up to 90 % [3]. For treatment of visible lesions, intralesional injections of interferon alpha-2b (IFN α-2b) have been an optional treatment; however, in this treatment option the patients suffer from the pain due to direct injections into each region and severe side effects due to systemic exposure. Moreover, only five visible lesions can be treated in a single session [1]. The treatment is not suitable for latent or subclinical infections and a more sophisticated non-invasive approach is desirable.

The potential of topical treatment of genital warts was one of the first studies reporting vaginal applications of liposomal drugs. In a preliminary clinical testing, topical treatment with liposomal IFN α-2b achieved complete resolution of cervical lesions in a female patient at the end of therapy [4]. PEGylation of IFN α-2b can provide a prolonged half-life and a shift of distribution towards infected tissues due to increased capillary permeability, thereby improving efficacy and reducing toxicity [5]. Additionally, incorporating IFN α-2b in liposomal formulations may

increase its stability and alter its pharmacokinetics, two issues which often limit the success of IFN therapy [6,7].

To achieve an efficient local delivery to mucosal tissue, the penetration into/through the mucus mesh, uniform distribution of drug into the underlying tissue and sufficiently high drug concentration are required. Mucus, a physical barrier in the form of an adhesive gel that stick to most particles, prevents most of the foreign particles from penetrating into the epithelium surface. Moreover, mucus exhibits the ability to form an unstirred layer of mucus adjacent to epithelial surfaces not affected by the shearing actions [8]. To penetrate this unstirred layer, nanosystems should be able to diffuse through it in a manner similar to viruses. Viruses can overcome this barrier and cause infection rather easily [9]. Therefore, biomimicking the viral properties might be a promising approach.

Polyethylene glycol (PEG) is an uncharged hydrophilic polymer widely applied in pharmaceutical formulations, including those for topical vaginal therapy. When used as a coating material, PEG enables nanoparticles to diffuse through vaginal mucus by eliminating the adhesive interactions between the nanoparticles and mucus [9,10], assuring a closer contact to the vaginal epithelium, and enabling improved drug effectiveness. The synergy between the properties of liposomes as a protective carrier for sensitive biologicals and the mucus-penetrating properties of PEG available on liposomal surface, enables the development of a vaginal drug delivery system providing the controlled drug release in a close proximity to the vaginal epithelium.

In the present study, we developed liposomal carriers containing IFN α-2b with surface-available low molecular weight PEG (MW of 2000) as a mucus-penetrating delivery system able to distribute IFN α-2b to vaginal mucosa assuring improved localized therapy.

2. Materials and Methods

2.1. Materials

Lipoid S 100 (PC, soybean lecithin, > 94 % phosphatidylcholine) was a gift from Lipoid GmbH, Ludwigshafen, Germany; methoxy poly (ethylene glycol)-modified lipids (mPEG 2000) was from the same manufacturer. IntronA® 50 MIU/mL injection fluid in multiple dose pen was the product of MSD AS, Drammen, Norway. Acetic acid, bovine serum albumin, calcium hydroxide, chitosan (low MW, Brookfield viscosity 20.000 cps, degree of deacetylation 92 %), cholesterol, fructose, glycerol, mucin from porcine stomach (type III, bound sialic acid 0.5 % - 1.5 %, partially purified), potassium phosphate monobasic, Sephadex® G-50, Triton® X-100 and zinc chloride were all purchased from Sigma-Aldrich Chemie GMbH, Steinheim, Germany. Di-sodium hydrogen phosphate, sodium dihydrogen phosphate monohydrate, potassium chloride and titriplex (ethylenedinitrilotetraacetic acid disodium salt dihydrate) were obtained from Merck KGaA, Darmstadt, Germany. Glucose, lactic acid, polysorbatum, potassium hydroxide, sodium citrate dihydrate and urea were the products of NMD, Oslo, Norway. Ammonium acetate, magnesium chloride and potassium chloride was the product of VWR International BHD Prolab, Leuven, Belgium. ELISA kit was purchased from Bio-Techne, Abingdon, UK.

2.2. Preparation of PEGylated liposomes

The PEGylated liposomes were prepared by the conventional film method as previously described [11]. Briefly, cholesterol (10 mg), mPEG 2000 (36.3 mg) and PC (200 mg) were dissolved in methanol and chloroform (1:1, v/v) in a round bottom flask. Solvents were removed through evaporation (Büchi rotavapor R-124 with vacuum controller B-721, Büchi Vac® V-500, Büchi Labortechnik, Flawil, Switzerland) for 2 hours at 50 mm Hg and 50 °C. The remaining film was flushed with nitrogen to assure no residual solvents. The lipid film was then re-suspended in 5 mL of IFN α-2b solution from IntronA® 50 million IU/mL (MIU/mL) injection fluid and Intron A buffer (pH 7.4; 7.5 g/L NaCl, 1.8 g/L NaH_2PO_4, 1.3 g/L Na_2HPO_4, 0.1 g/L EDTA and 0.1 g/L Polysorbate 80) resulting in a final IFN α-2b concentration of 2 MIU/mL. Similar procedure was applied in the preparation of empty liposomes; the lipid film was re-suspended in Intron A buffer free of IFN α-2b. Liposomal suspensions were kept in a refrigerator (4 - 8˚C) for at least 12 hours prior to further use.

2.3. Vesicle size reduction

Extrusion through polycarbonate membranes (Nuclepore Track-Etch Membran, Whatman House, Maidstone, UK) [12] was employed in the reduction of liposomal size. The extrusion was performed stepwise through 0.8, 0.4 and 0.2 µm pore size filters, respectively. Three extrusions were performed on each pore size filters. Extruded liposomes were kept in a refrigerator (4-8 °C) for at least 6 hours prior to characterization and further experiments.

2.4. Particle size analysis

The particle size distribution of liposomal samples was measured by photon correlation spectroscopy (Submicron particle sizer model 370, Nicomp, Santa Barbara, California, USA) according to Jøraholmen et al. [11]. Analyses were run in a vesicle mode and the particle intensity of approximately 200-350 kHz. The data were calculated as intensity weighted distribution from three measuring cycles (each with a run time of 10 min).

2.5. Zeta potential measurements

Zeta potential determinations were performed on a Malvern Zetasizer Nano ZS (Malvern, Oxford, UK). Prior to measurement, the measurement cells were properly cleaned with ethanol and filtrated water, respectively. To obtain a suitable count rate, the liposomal suspensions samples were diluted in filtrated water to adequate concentrations (typically 1:20, v/v) before loading the sample into the cells [12]. Three parallels were determined for each sample measurement.

2.6. IFN α-2b entrapment

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

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

2.7. In vitro mucin-binding

The binding of PEGylated liposomes to mucin was determined to confirm that the delivery system is not mucoadhesive. The test was performed as previously described [12]. Briefly, aliquots (1 mL) of empty PEGylated, non-coated and 0.1 % (w/v) chitosan-coated liposomes (prepared according to Jøraholmen et al. [11]) were added to an equal volume of mucin suspension (400 µg/mL) in phosphate buffer (pH 7.4) and acetate buffer (pH 4.6), respectively. Incubation at room temperature for 2 hours was followed by ultracentrifugation at 216 000 *g* for 1 hour, at 10 °C (Optima LE-80; Beckman Instruments, Palo Alto, California, USA). Free mucin and four aliquots (200 µL) from the supernatant were transferred to a microtitre plate (Costar® UV 96-well plate with UV transparent flat bottom, Acrylic, Costar®, Corning, New York, USA) and measured spectroscopically at 251 nm (Microtitre plate reader; Spectra Max 190 Microplate,

Spectrophotometer Molecular devices, Sunnyvale, California, USA). Mucin-binding capacity was calculated based on Naderkhani and colleagues [13]. The experiments were performed in triplicates.

2.8. *In vitro release*

The *in vitro* IFN α-2b release experiment was performed on the Franz cell manual diffusion system (Perme Gear Ink, Diffusion cells and Systems, Hellertown, USA) as previously described [11]. The system and accessories were thoroughly cleaned with methanol, demineralized water and distilled water, respectively, prior to experiment. The heating circulator (Julabo Laboratechnik, F12-ED, Seelback, Germany) was set to maintain a temperature of 37 °C. The acceptor chambers with a volume of 12.1 mL were completely filled with acetate buffer (pH 4.6; 77.1 g CH_3COONH_4, 70 mL glacial acetic acid and distilled water up to 1000 mL). Polyamide membrane (Sartorius polyamide membrane, 0.2 µm pore size, Sartorius AG, Gröttingen, Germany) was cut to a suitable size (diffusion area of 1.77 cm^2) and fixed between the donor and acceptor chambers. The IFN α-2b content in all included samples was determined (ELISA) prior to the experiment to prepare a control solution (IFN α-2b in Intron A buffer) containing similar drug concentration. Liposomal samples and controls (550 µL), as well as vaginal fluid simulant (VFS) (pH 4.6; 3.51 g/L NaCl, 1.40 g/L KOH, 0.222g/L $Ca(OH)_2$, 0.018 g/L bovine serum albumin; 2 g/L lactic acid, 1g/L acetic acid, 0.16 g/L glycerol, 0.4 g/L urea and 5 g/L glucose; 50 µL) [14] were added in the donor cells and the system was closely sealed. Sampling from acceptor chamber (500 µL) was done after 1, 2, 4, 6 and 8 hours and the samples withdrawn from the acceptor chamber were replaced by an equal volume of acetate buffer. IFN α-2b

concentrations in the withdrawn samples, remaining in the donor cells and retained in the polyamide membrane were determined with ELISA kit and UV spectrophotometry as described above. The experiments were performed in triplicates.

2.9. Preparation of vaginal tissue

Vaginal tissue from pregnant sheep was acquired from the Laboratory Animal Centre, University of Oulu, Finland. The experiments were performed according to the guidelines of the National Animal Experiment Board in Finland. The vaginal tissue was cautiously dissected and removed from the underlying tissue, cleaned and moistened with physiological solution (pH 7.4), then packed in a clinging film before the tissues were frozen (-20 °C). Prior to the experiments, the tissue was left to defrost in phosphate buffer (pH 7.4) at room temperature for at least 1 hour. We have earlier reported that no significant differences were observed in using fresh or snap-frozen vaginal tissue samples and that the barrier properties of the fresh vaginal tissue and frozen and thawed tissue are similar [11].

2.10. Ex vivo penetration

The experiment was performed on the Franz cell manual diffusion system as described above. Briefly, the acceptor chambers were filled up with phosphate buffer (pH 7.4, 8 g/L NaCl, 0.19 g/L KH_2PO_4, and 2.38 g/L Na_2HPO_4). Sheep vaginal tissue was defrosted, cut to appropriate size (1.77 cm^2) and fixed between donor cell and acceptor chamber. Samples and controls (550 µL), with similar amount of IFN α-2b, as well as VFS (50 µL) were added in the donor cells and the system was thoroughly sealed. Sampling from acceptor chamber (500 µL) was done after 1, 2, 4,

6 and 8 hours and withdrawn volumes replaced by an equal volume of phosphate buffer. Amount of IFN α-2b in the collected samples, remaining in the donor cells and retained in the vaginal tissue were determined with ELISA kit and UV spectrophotometry as described above. The experiments were performed in triplicates.

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

Human semen simulant was prepared according to Owen and Katz [15]. Briefly, the solution 2 (101 mg $CaCl_2$ x $2H_2O$, 15.13 mL H_2O), the solution 3 (92 mg $MgCl_2$ x $6H_2O$, 15.13 mL) and solution 4 (34.4 mg $ZnCl_2$, 15.13 mL H2O) were added into the solution 1 (5.6 mL 0.123 M NaH_2PO_4 x H_2O, 49.14 mL 0.123 M Na_2HPO_4, 813 mg sodium citrate dehydrate, 90.8 mg KCl, 88.1 mg KOH, 272 mg fructose, 102 mg glucose anhydrase, 62 mg lactic acid, 45 mg urea, 5.04 mg bovine serum albumin). The solutions were mixed and pH was adjusted to 7.7 with sodium hydroxide solution.

Aliquots (500 µL) of liposomal suspensions containing IFN α-2b (free of unentrapped IFN) were mixed with human semen simulant (500 µL) and VFS (50 µL), and incubated at room temperature for 2 hours. The leaked (free) IFN α-2b was separated from liposomally-associated IFN α-2b by size-exclusion gel chromatography (as described in *2.6*). Quantification of IFN α-2b in the fractions was performed with ELISA kit and UV spectrophotometry as described above.

2.12. Statistical analyses

For the comparison of two means, statistical significance was determined using the student's *t*-test. A *p*-value less than 0.05 was considered statistically significant.

3. Results and Discussion

Interferons are widely used clinically relevant biologicals. Their anti-viral, anti-proliferative and immunomodulatory effects have been confirmed and several subtypes are already clinically approved for various indications [16]. IFN exhibits anti-viral effect on HPV-infected cells, and is of a great interest in the search for therapeutic use in the treatment of HPV infections [1]. The short circulation time and unwanted effects on the non-target tissues following systemic administration limit its wider utilization as biological. Topical treatment option assures drug delivery directly to the site of infection and increased efficiency at lower doses, reduced adverse effects due to decreased systemic levels and a more adequate treatment even for non-visible lesions. Currently, the lack of suitable delivery systems is the main challenge to successful HPV infection treatment. With an optimized formulation, that enables an efficient local delivery to mucosal tissue and provides increased stability of IFN, these limitations can be overcome [17].

3.1. Liposomal characteristics

The effectiveness of local drug delivery at the vaginal site is determined by the physicochemical characteristics of the delivery system. The vesicle size affects the ability to fit within the mucin pores, while the particles surface charge and properties establish the potential attraction or repulsion towards mucus [18]. Thus, the main focus in the development of a delivery system

aiming for local therapy at the mucosal site is controlling the surface properties, particle size and surface charge [19]. The protective mucus layer traps the foreign particulates and acts on their removal [20]. Biomimetic approach in design of novel delivery systems for IFN can offer the necessary improvements in the efficacy of localized vaginal therapy. Polyethylene glycol (PEG) shields the nanoparticles from adhesive interactions with mucus [21]. Sufficient number of PEG molecules available on the vesicle surface effectively minimizes the adhesive interactions between vesicles and mucus, creating the mucus-penetrating particles that can efficiently penetrate the human mucus [10,22]. The optimal PEG content and density on the particle's surface were studied to optimize the penetration potential and system's stability in vaginal environment. Xu and colleagues [20] suggested that at least 5 % PEG (wt) was required to assure complete shielding and avoid mucoadhesion. The surface density and molecular weight of PEG directly affect the particle transport, and literature indicates that dense coating with low molecular weight PEG increases the transport rate through the mucus significantly [23]. We used targeted PEG content of 14 % (wt), which is expected to provide surface PEG content of over 7 % (wt), as reported by Hanes group [20] for biodegradable poly(lactic-co-glycolic acid) nanoparticles-bearing PEG. PEG in this concentration is expected to be in the dense brush conformation [20]. The same group [24] further examined the diffusion of liposome-based mucus-penetrating particles in human cervicovaginal mucus by magnetic resonance imaging and suggested that 7 % (molar) was an optimal coating concentration for theranostic purposes. By modifying liposomal surface with low molecular weight PEG we aimed to develop an advanced carrier for IFN α-2b for the local therapy at vaginal site.

Uniform distribution deep into the folded vaginal epithelium over the cervicovaginal mucosa is a prerequisite for efficient drug delivery [9].

Although there is no consensus regarding the effect of nanocarrier size on the mucosal targeting at vaginal site, it is known that small particles will easily be trapped in the mucus mesh while larger particles will be retained on the mucus surface. Viruses with diameters between 30 and 200 nm are able to reach and infect the vaginal mucosa efficiently [18]. However, Lai and colleagues [23] showed that larger particles (200-500 nm) densely coated with PEG are more rapidly transported through fresh undiluted human mucus than the corresponding particles of smaller size (100 nm). Based on the above, in our study, we aimed for vesicles in size range of around 200 nm. The vesicle size of our PEGylated liposomes was close to the targeted size range (Table 1). We reported earlier [12] that the extrusion as a size reduction method can be readily applied to obtain vesicles in desired size range with favourable polydispersity. Our liposomes exhibited rather narrow size distribution with low PI (Table 1).

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

Considering optimal mucus-penetration, a nearly neutral vesicle surface charge is desirable to assure mucus-penetrating properties [23]. However, it is also suggested that a negatively charged component present in the liposomal bilayer responsible for negative charge on the vesicle surface is beneficial for a stable association and will improve IFN α-2b entrapment and prevent drug leakage from the liposomes [6]. The optimized formulation should be a balance between the drug load and surface characteristics of the vesicles. Our PEGylated liposomes exhibited a slightly negative zeta potential (Table 1).

Even if the carrier system exhibits desired mucus-penetrating properties, it is very important that the drug load is sufficient to achieve desired therapeutic response. We were able to achieve rather high entrapment efficiency of 81 % (Table 1). This is in accordance with literature data [7,25-27]. Compared to other lipid-based delivery systems, such as the biphasic IFN-containing vesicles for topical therapy of HPV infections, the entrapment efficiency in our vesicles was in the same range [28]. However, both the vesicle size and size distribution of the biphasic vesicles were considerably larger (1000-1100 nm) compared to our PEGylated liposomes (181 ± 8 nm), suggesting that even vesicles of smaller sizes can carry sufficient drug load. In addition, it is known that by incorporating IFN α-2b in liposomal formulations, an increase in its stability and positive alteration of its pharmacokinetics can be obtained [6,7]. The size and size distributions of our vesicles, as well as the presence of PEG on vesicle surfaces indicate that the vesicles should remain stable in respect to both aggregation and potential loss of originally encapsulated IFN α-2b.

3.2. Mucus-penetrating properties

Mucus immobilizes particles by hydrophobic and electrostatic interactions as well as hydrogen bonding [29]. Mucin fibers in the healthy human vaginal mucus are negatively charged and reported to have a diameter of approximately 340 nm [10]. Mucus properties are affected by the environmental changes, such as the changes in pH due to vaginal infections and the viscosity of vaginal fluid. When exposed to bacterial vaginosis, a reduction in the viscosity of vaginal fluid causes the reduced barrier properties and increased risk of infection [30]. It is further suggested that mucoadhesive nanoparticles may disrupt the protective microstructure of mucus by

increasing the average pore size of mucin fibers. This can provide easier access for foreign particles to vaginal mucosa, including the pathogens and other potentially toxic nanomaterials, due to the impaired mucus barrier. However, the limited literature data indicate that the mucus-penetrating particles did not exhibit similar effect on the cervicovaginal mucus [21].

It was originally proposed that PEGylation of nanoparticles leads to increased mucoadhesiveness, and that PEG exhibits mucoadhesive properties that are expressed by the interpenetrating polymer network effects between PEG chains and the mucus mesh [31-33]. Recent studies, however, have demonstrated that PEG-surface modified nanoparticles minimize mucoadhesion and are able to effectively penetrate the mucus enabling closer contact between the nanosystem and the underlying epithelium [9,10,22,34]. To achieve the mucoinert surfaces a fine-tuning of the interactions between particles and mucus is required. Coating of particles with a dense layer of low molecular weight PEG effectively reduces the hydrophobic interactions, hydrogen bonding interpenetrating polymer network effects below the threshold required to actually slow and immobilize nanosystems [34]. In addition, incorporation of PEG into nanosystems also reduces the interactions of the systems with other proteins and biomacromolecules. It was suggested that the molecular weight of PEG is the determining factor whether PEG will exhibit mucoadhesive or mucus-penetrating characteristics and recent findings indicate that when densely coated with low molecular weight PEG, nanoparticles avoid adhesive interactions with mucus [9,18].

To confirm that PEG on the liposomal surface indeed exhibits a mucus-penetrating effect and contributes to a reduced association between liposomes and mucus, the binding between mucin

and liposomal preparations was determined. Commercially available pig mucin is commonly used as a mucus substitute due to its similarity in the structure and molecular weight to human mucus [35]. Based on the fact that the vaginal pH varies in the healthy and infected, as well as pre- and post-menopausal women, the experiments were performed both at the conditions of healthy vaginal environment (pH 4.6) and those expected to occur during the vaginal bacterial infection and post-menopause (pH 7.4). As expected, the PEGylated liposomes did not exhibit mucin-binding activity (Table 2). The results indicate a significantly reduced binding efficiency at the different pH conditions for the PEGylated liposomes compared to both the conventional non-coated liposomes and the 0.1 % (w/v) chitosan-coated liposomes ($p < 0.001$).

Table 2: Mucin-binding of PEGylated, non-coated and chitosan-coated liposomes (n=3).

The lack of mucin-binding activity coincides with the expected mucus-penetrating properties of PEG-modified liposomes [20]. The results indicate that the PEGylated liposomes might be able to penetrate the mucus and assure closer contact with the epithelium, thus, enhance mucosal delivery of incorporated drug and improve its localized therapeutic outcome.

3.3. In vitro IFN α-2b release

When evaluating *in vitro* drug release from topical formulations, including those intended for vaginal use, the Franz cell diffusion system is generally considered one of the most appropriate methods [36]. Liposomes are expected to provide controlled release of incorporated drug;

therefore we followed the release of liposomally-associated IFN α-2b over 8 hours and compared the release to the IFN α-2b release from a control solution. The experimental setup was designed to mimic the human conditions; the pH of acceptor medium mimicked the healthy human vaginal environment (4.6) and a temperature was set at 37 °C. Further, the experiment was performed in the presence of vaginal fluid simulant (VFS), since it is known that the flow, retention, drug delivery kinetics and bioactivity of vaginal formulations are influenced by the compounds present in the vaginal fluid [13]. The VFS is commonly used in *in vitro* evaluations of contraceptive and prophylactic vaginal drug delivery systems [37].

Figure 1: A) In vitro IFN α-2b release. Results are expressed as percentage mean ± SD (n = 3). B) IFN α-2b partitioning after 8 hours. Results are expressed as percentage mean ± SD (n = 3).

Both the PEGylated liposomes and control solution seem to sustain the release of IFN α-2b (Figure 1A). Even though the release of liposomally-associated IFN α-2b increased significantly ($p < 0.001$) compared to the control solution after 8 hours, the release was very limited. In a preliminary experiment, we have tested three types of membranes, namely cellophane, polyamide and cellulose acetate membrane. All three membranes allowed for very limited penetration of IFN (data not shown). The pore size of the membrane used in testing should be sufficiently large to allow the passage of IFN molecules through; however, it seems that IFN α-2b was mostly retained in the membrane, or stayed in the liposomes retained on the membrane surface (Figure 1B). The significant retention of IFN α-2b in the membrane could be attributed to possible surface adsorption; at least in the case of IFN α-2b solution. However, we used marketed IFN α-

2b solution (IntronA®) in the buffer containing polysorbate. Polysorbate is expected to reduce the protein adsorption onto the surfaces [38]. Moreover, the concentration of IFN α-2b applied onto the diffusion cells was well below the manufacturer's specification regarding protein adsorption onto the membrane. The sustained IFN α-2b release from liposomal formulation was in agreement with previously published literature [39]. The release profile observed for our system also corresponds to the IFN release profiles from other lipid-based delivery systems [40]. A prolonged release is of great importance considering vaginal administration, as reduced administration frequency is coherent with better patient compliance and the success of mucosal drug delivery is highly dependent on a suitable drug carrier able to remain at the vaginal site over prolonged period of time [19].

3.4. Ex vivo penetration

Franz cell diffusion system is also suitable for determination of *ex vivo* tissue penetration [41-43]. Sheep vaginal epithelium is a stratified squamous tissue, similar to that of human, and is used in *ex vivo* testing of vaginal formulations [11,44]. The tissue used in this experiment was from pregnant animals [11] and the thickness was 750 µm. In contrast to the strongly sustained release of IFN α-2b from liposomes through the artificial membrane, the IFN α-2b penetration through the sheep vaginal tissue was prominent, and a distinct increase in IFN α-2b release from the PEGylated liposomes was seen (Figure 2A). This demonstrates that the PEGylated liposomal formulation enables IFN α-2b to penetrate the vaginal mucus to a higher extent compared to the

solution formulation used as a control. This indicates the potential of PEGylated liposomal formulation of IFN α-2b in efficient local vaginal therapy of HPV-infected areas.

Figure 2: A) *Ex vivo* IFN α-2b penetration. Results are expressed as percentage mean ± SD (n = 3). B) IFN α-2b partitioning after 8 hours *ex vivo* penetration experiment. Results are expressed as percentage mean ± SD (n = 3).

Majority of IFN α-2b from control solution appeared to be retained within or on top of the tissue and only a minor amount of drug was able to penetrate through the tissue after 8 hours (Figure 2B). This limitation of the IFN α-2b in solution form, not in delivery system, is the reason that the current IFN α-2b therapy involves direct injections in the affected lesions and is limited by both the patient acceptance and efficacy [1].

3.5. Stability

Very early work on lipid-based formulations for interferons suggested that the formulations remain stable over a period of at least 1 month [45]. It is known that the physical and chemical properties of the environment and the presence of semen, might affect the stability and performance of the drug delivery system destined for the administration at the vaginal site [15]. The composition, volume, pH and rheological properties of vaginal fluids are affected by the age, the menstrual cycle or sexual arousal. Some studies suggest that on average 0.5 – 0.75 g of vaginal fluid is contemporary present in the vagina [14]. Moreover, the presence of semen will

increase the acidic pH (4-5) to levels closer to neutral range, which might also affect the stability and performance of some vaginal drug delivery systems. The average volume of human ejaculate is found to be 3.4 mL [15]; it is expected that the effect of the semen presence on the vaginal pH can last for several hours [46].

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

PEGylated liposomes were diluted 1:10 (v/v) with VFS and 1:1 (v/v) with semen fluid simulant to determine possible IFN α-2b leakage from the delivery system once exposed to the vaginal environment. An incubation time of 2 hours was selected as an appropriate challenge. Only a minor leakage of 5.1 % IFN α-2b was detected after 2 hours, indicating the stability of PEGylated liposomes in a simulated vaginal environment and in the presence of semen fluid simulant (Table 3). Considering the high drug load in PEGylated liposomes, the leakage can be considered negligible.

We have proven *ex vivo* that the mucus-penetrating liposomes can assure superior drug penetration. However, one has to consider that the retention time at vaginal site might be insufficient to assure that an adequate amount of drug can actually penetrate the mucus due to rather rapid clearance of foreign particles by the vaginal discharge. Therefore, it might be that both mucoadhesive and mucus-penetrating properties of vaginal delivery systems hold promise in improved vaginal drug delivery. Very recently, the approach based on combining both the mucoadhesive and mucus-penetrating properties within the single delivery system has been proposed [47]. To confirm that our mucus-penetrating delivery system can stay in a closer contact

with the vaginal epithelium and assure penetration of drug in the deeper epithelial layers, *in vivo* testing in infected animals would be required.

4. Conclusions

Our findings suggest a lack of interactions between mucin and PEGylated liposomes confirming the mucus-penetrating properties of PEG. The ability of the IFN α-2b entrapped in PEGylated liposomes to penetrate through the vaginal tissue was distinctively increased for the PEGylated liposomal formulations as compared to IFN α-2b in solution. This indicates that PEGylated liposomal formulation represents a promising approach to assure drug delivery in the close proximity to the vaginal epithelium for therapeutic use. Furthermore, the PEGylated liposomes were shown to be stable in the simulated vaginal environment.

Conflict of interest

The authors declare no conflict of interest.

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*Polydispersity index

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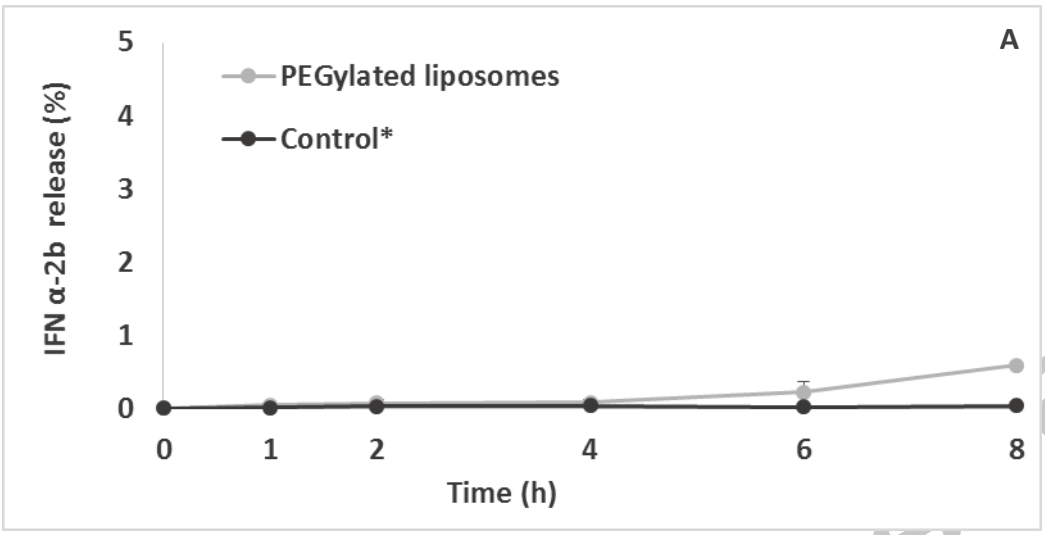
*Empty liposomes

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

* Polydispersity index, [a] Freshly made liposomal formulation, [b] After storage in a fridge (4 °C) for 1 month, [c] After exposure to simulated vaginal environment for 2 hours.



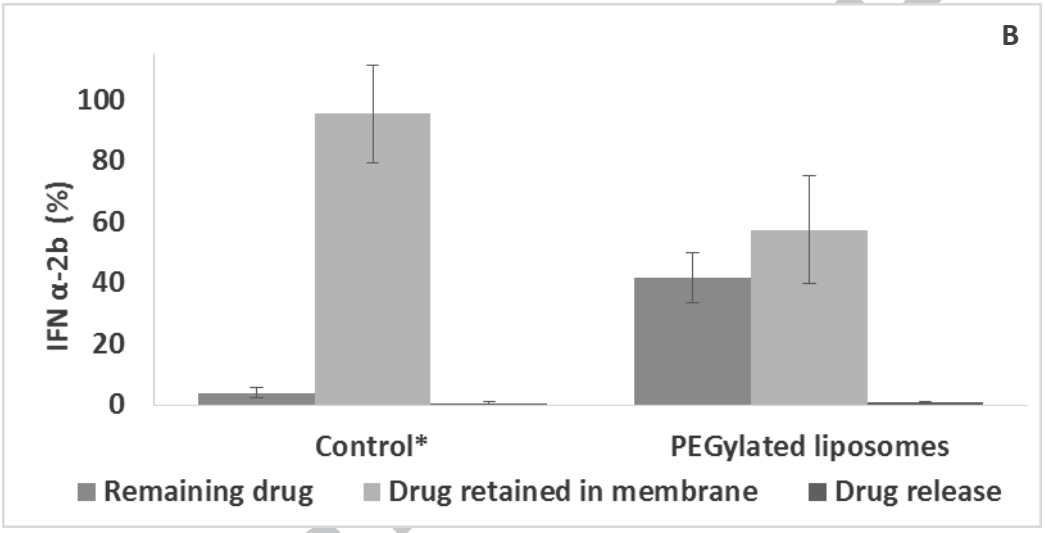


Figure 1: A) *In vitro* IFN α-2b release. Results are expressed as percentage mean ± SD (n = 3). B) IFN α-2b partitioning after 8 hours. Results are expressed as percentage mean ± SD (n = 3).

*IFN α-2b in Intron A buffer.

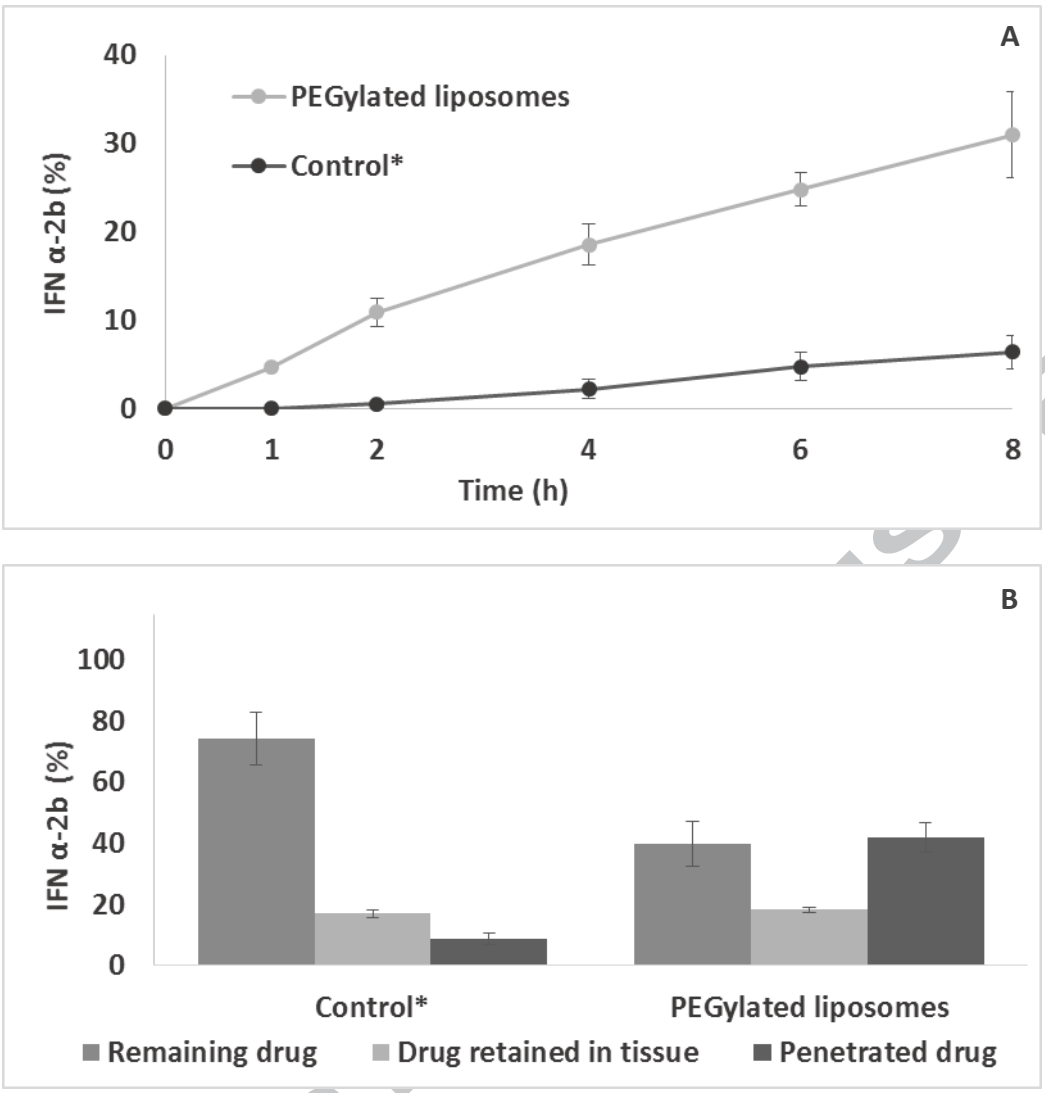


Figure 2: A) *Ex vivo* IFN α-2b penetration. Results are expressed as percentage mean ± SD (n = 3). B) IFN α-2b partitioning after 8 hours *ex vivo* penetration experiment. Results are expressed as percentage mean ± SD (n = 3).

*IFN α-2b in Intron A buffer

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

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

*Polydispersity index

Table 2: Mucin-binding of PEGylated, non-coated and chitosan-coated liposomes (n = 3).

Liposomal samples*	Mucin-binding (%)	
	pH 7.4	pH 4.6
PEGylated	3.8 ± 2.6	7.0 ± 14.6
Non-coated	22.1 ± 3.3	36.0 ± 3.2
0.1 % chitosan-coated	65.1 ± 0.1	64.5 ± 2.9

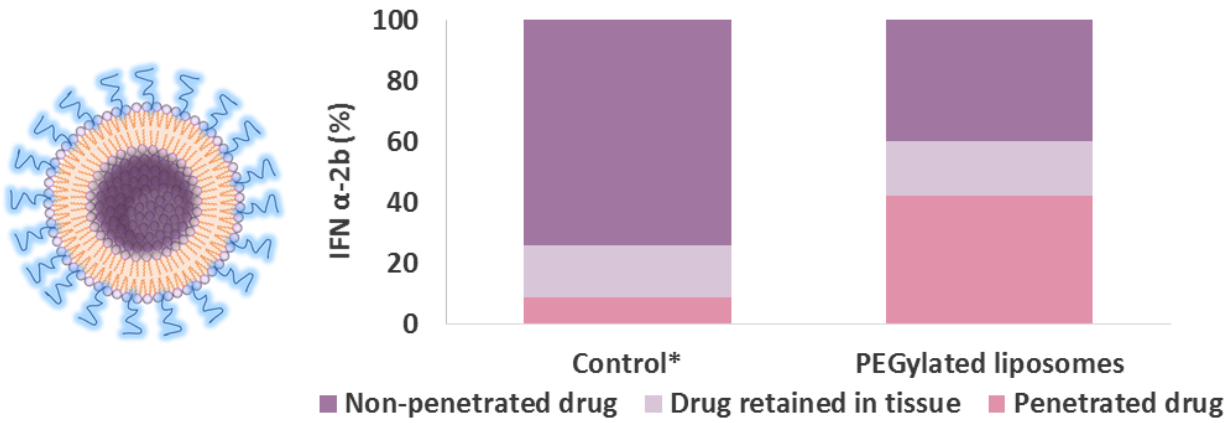
*Empty liposomes

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

Time of measurement	Size		Zeta potential	Entrapment
	(nm)	PI*	(mV)	(%)
Fresh [a]	181 ± 8	0.129	- 13.33 ± 0.81	81.0 ± 9.8
After storage [b]	182 ± 7	0.098	- 13.00 ± 0.45	-
After exposure [c]	184 ± 8	0.132	- 13.27 ± 0.37	75.9 ± 6.2

* Polydispersity index, [a] Freshly made liposomal formulation, [b] After storage in a fridge (4 °C) for 1 month, [c] After exposure to simulated vaginal environment for 2 hours.

Graphical abstract



Schematic drawing of PEGylated liposome and the IFN α-2b partitioning after 8 hours *ex vivo* penetration experiment.

*IFN α-2b in Intron A buffer