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Research paper

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.

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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

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

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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 h at 50 mmHg 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 h 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 h 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

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