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Freeze-dried mucoadhesive polymeric system containing pegylated lipoplexes: Towards a vaginal sustained released system for siRNA

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ABSTRACT

Topical vaginal sustained delivery of siRNA presents a significant challenge due to the short residence time of formulations. Therefore, a drug delivery system capable to adhere to the vaginal mucosa is desirable, as it could allow a prolonged delivery and increase the effectiveness of the therapy. The aim of this project is to develop a polymeric solid mucoadhesive system, loaded with lipoplexes, able to be progressively rehydrated by the vaginal fluids to form a hydrogel and to deliver siRNA to vaginal tissues.

To minimize adhesive interactions with vaginal mucus components, lipoplexes were coated with different derivatives of polyethylene glycol: DPSE-PEG_{2000}, DPSE-PEG_{750} and ceramide-PEG_{2000}. Based on stability and diffusion properties in simulated vaginal fluids, lipoplexes containing DSPE-PEG_{2000} were selected and incorporated in hydroxyethyl cellulose (HEC) hydrogels. Solid systems, called sponges, were then obtained by freeze-drying. Sponges meet acceptable mechanical characteristics and their hardness, deformability and mucoadhesive properties are not influenced by the presence of lipoplexes. Finally, mobility and stability of lipoplexes inside sponges rehydrated with vaginal mucus, mimicking *in situ* conditions, were evaluated by advanced fluorescence microscopy. The release rate was found to be influenced by the HEC concentration and consequently by the viscosity after rehydration.

This study demonstrates the feasibility of entrapping pegylated lipoplexes into a solid matrix system for a prolonged delivery of siRNA into the vagina.

1. Introduction

In the context of genital diseases, the vaginal route of administration has gained great attention for drug delivery and has been extensively studied for effective delivery of different drug molecules [1–3]. Advantages over other routes of drug administration include low drug doses, reduced risk of systemic immune activation, site-specific delivery, and most importantly, circumvention of first-pass hepatic clearance [4]. The ease of administration and low toxicity profile make the vaginal route an excellent site for the delivery of many drugs and particularly for siRNA delivery for the treatment and prevention of vaginal and/or cervical diseases [5–7]. However, naked siRNAs have difficulties in achieving efficient mucosal uptake if administered directly into the vagina due to degradation, poor cellular uptake, low mucus diffusion and high clearance. In order to overcome these obstacles, siRNA need to be encapsulated in a vector, such as liposomes.

Liposomes have been largely investigated as vaginal drug delivery system [8–11]. However, vaginal conditions are subject to changes because of numerous physiological and non-physiological factors that can lead to variations in the bioavailability of drugs. Moreover, a vaginal administration of lipoplexes (liposomes encapsulating siRNA) encounters important barriers such as the penetration through the mucus to reach the epithelial tissue and a short residence time. One strategy to

Abbreviations: Ceramide-PEG_{2000}, *N*-octanoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)2000]}; DSPE-PEG_{2000}, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000]; DSPE-PEG_{750}, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-750]; FCS, fluorescence correlation spectroscopy; fluo-siRNA, fluorescent small interfering RNA; fSPT, fluorescence single particle tracking; HEC, hydroxyethyl cellulose; PdI, polydispersity index; PEG, polyethylene glycol; PEG400, polyethylene glycol 400; SEM-EDX, Scanning Electron Microscopy and Energy Dispersive X-Ray Analysis; siRNA, small interfering RNA; SVF, simulated vaginal fluid; TA, Texture Analyzer; TAE, tris acetate EDTA buffer.

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improve the particle diffusion through the mucus and to create "mucopenetrating" lipoplexes is to densely coat their surface with polyethylene glycol (PEG) [1,12,13]. PEG is a neutral hydrophilic polymer that has been described to minimize adhesive interactions between nanoparticles and mucus components, allowing them to penetrate rapidly through viscoelastic human mucus secretions [14,15]. The size of the lipoplexes is also another important parameter to consider for the diffusion. It has been shown that particles with a diameter around 200–300 nm can diffuse more rapidly through undiluted human vaginal mucus, than smaller ones (100 nm) and bigger ones (>500 nm) [16,17].

Unfortunately, even if lipoplexes are mucopenetrating, they have a short residence time, which conducts them to be quickly eliminated. In order to improve the vaginal retention, lipoplexes should be incorporated in an appropriate depot system with a desirable viscosity and with mucoadhesive properties. For this purpose, a polymeric hydrogel can be a good solution [18]. Among the different mucoadhesive polymers used for vaginal administration and based on previous results [19–21], cellulosic derivatives and particularly hydroxyethyl cellulose (HEC) are attractive candidates. It has also been described that lipid vesicles are compatible with HEC hydrogels [22].

Finally, in order to avoid drug degradation and to obtain a solid and easy to handle system, the hydrogels containing pegylated lipoplexes should be freeze-dried. The obtained system, called sponge, has been previously described and characterized [19].

Taken together, increasing the residence time with the vaginal mucosa by introducing pegylated lipoplexes inside a mucoadhesive solid system can be crucial for efficient vaginal siRNA delivery. Combining mucoadhesion and prolonged drug delivery possesses the advantages to improve patient's compliance and to reduce the frequency of application.

Here, we develop a novel solid matrix system able to adhere to the vaginal mucosa, to be *in situ* rehydrated by the vaginal fluids to form a hydrogel and to deliver in a sustained manner mucopenetrating pegylated lipoplexes and consequently siRNA to vaginal tissues under pathological conditions.

2. Material and methods

2.1. Material

1,2-Dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), cholesterol, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG_{2000}), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-750] (ammonium salt) (DSPE-PEG_{750}) and *N*-octanoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)2000]} (ceramide-PEG_{2000}) were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA). Scramble siRNA (siRNA) and fluorescent scramble siRNA with alexa Fluor® 647 (fluo-siRNA) were provided by Eurogentec® (Eurogentec SA, Liège, Belgium) with the following sequence: sense strand: 5′-AGAGUUCAAAAGCCCUUCdTdT-3′ and antisense strand: 5′-GAAGGGCUUUUGAACUCUdTdT-3′ (alexa Fluor 647 in position 5′). TAE buffer (50× pH 8.0) was obtained from VWR (Leuven, Belgium). *D*-(+)-trehalose dehydrate (from *Saccharomyces cerevisiae*, ≥99%) was purchased from Sigma-Aldrich (Schnelldorf, Germany). Hydroxyethyl cellulose 250 M (HEC) was purchased from Ashland (Covington, USA) and polyethylene glycol 400 (PEG400) was purchased from Fagron (Waregem, Belgium). All the components used to prepare synthetic vaginal mucus were purchased from Sigma-Aldrich (Schnelldorf, Germany).

2.2. Lipoplexes formulations

2.2.1. Preparation of lipoplexes

Liposomes were prepared from a mixture of DOTAP, cholesterol and DOPE at the molar ratio 1/0.75/0.5, by the hydration of lipid film method, as described previously [23]. Briefly, lipids were dissolved in chloroform at a total concentration of 5.6 mM. The organic solvent was removed using a rotary evaporator. The resulting thin lipid film was hydrated with 2 mL of RNAse free water and vigorously vortexed. Finally, the suspension was repeatedly extruded through polycarbonate membranes with 200 nm pore size.

Lipoplexes were obtained in RNAse free water by electrostatic interaction between liposomes and siRNA at the N/P ratio of 2.5 [19].

Lipoplexes were pegylated by addition of 30% of DSPE-PEG_{2000}, DSPE-PEG_{750} or ceramide-PEG_{2000} (%mol/DOTAP) by the post-insertion technique. In brief, the PEG in RNAse free water (1 mM) were added to preformed lipoplexes and the resulting suspension was vortexed and maintained 1 h at 37 °C under continuous stirring.

2.2.2. Freeze-drying of lipoplexes

Samples were prepared at 300 nM siRNA concentration in a final volume of 1 mL. Different amounts of trehalose were added (from 1 to 10% m/v) to the lipoplexes. Lipoplexes were then freeze-dried using a vacuum freeze-dryer (Heto-Holten DW 8030, Vacuubrand RZ8 pump) with a freeze-drying cycle previously described [19].

2.2.3. Particles characterization

The physicochemical characteristics of the lipoplexes were evaluated before and after freeze-drying. Freeze-dried lipoplexes were rehydrated with 1 mL of RNAse free water and stirred for 30 min at room temperature.

a. Particle size, polydispersity, zeta potential

The mean diameter (nm) and the polydispersity index (PdI) of the lipoplexes (100 nM siRNA, 1 mL) were determined by Dynamic Light Scattering method. The charge density was evaluated by examining the zeta potential (mV). Both measures were made at 25 °C, using a Malvern Zetasizer® (Nano ZS, Malvern Instruments, UK) [23].

b. Complexation efficiency

The level of siRNA complexation was evaluated by agarose (4%) gel electrophoresis. In brief, lipoplexes (300 nM, 30 μL) were loaded onto the agarose gel in TAE buffer and the electrophoresis was performed at 100 V for 1 h in a Horizon 11.14 horizontal gel electrophoresis apparatus (Biometra, Goettingen, Germany). Gel was visualized by exposure to UV-illumination by a Molecular Imager Gel Doc XR System (Bio-Rad, Hercules, CA, USA).

c. Stability

Freeze-dried lipoplexes were stored in closed glass vials at 4 °C for 25 days. The integrity of complexed siRNA was assessed by agarose gel electrophoresis. Triton X-100 (0.5% w/v) was used to break vesicles and release the complexed siRNA [23]. Gel retardation assay was performed in the same conditions as described in Section 2.2.3.b.

The mean diameter, the PdI and the zeta potential of the freeze-dried lipoplexes were also measured, as described in Section 2.2.3.a.

2.3. Sponges formulations

2.3.1. Preparation of placebo sponges

Hydrogels (6 g) were prepared by gradual dispersion in water of HEC polymer (0.83% or 1.67%) and PEG400 (0.41%), at room temperature and under magnetic stirring. Once homogeneous aqueous dispersions were obtained, the hydrogels were then freeze-dried to form sponges [19].