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

Tania Furst[1]*, George R. Dakwar[2], Elisa Zagato[2], Anna Lechanteur[1], Katrien Remaut[2], Brigitte Evrard[1], Kevin Braeckmans[2], Geraldine Piel[1]

[1] Laboratory of Pharmaceutical Technology and Biopharmacy, CIRM, University of Liege (4000), Belgium

[2] Laboratory of General Biochemistry and Physical Pharmacy, Ghent Research Group on Nanomedicines, Ghent University (9000), Belgium

Correspondence:

Tania Furst*

PhD student, University of Liege, Laboratory of Pharmaceutical Technology and Biopharmacy, CHU Bat. B36, Tour 4, Quartier de l'hôpital

Avenue Hippocrate, 15

4000 Liège

Belgium

E-mail: tania.furst@ulg.ac.be

Phone number: +32 4 366 43 07

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.

Keywords:

Vaginal drug delivery; freeze-drying; mucoadhesion; lipoplexes; diffusion; stability.

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 analyser

TAE: Tris Acetate EDTA buffer

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 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 (50X 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 250M (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].

2.3.2. Preparation of sponges loaded with lipoplexes

HEC (0.83% or 1.67%), PEG400 (0.41%) and trehalose (1%) were gradually dispersed in lipoplexes (300 nM, 6 mL) suspension at room temperature and under magnetic stirring. The obtained hydrogels containing lipoplexes were then freeze-dried as described above.

2.3.3. Preparation of artificial vaginal mucus

Simulated vaginal mucus (SVF) was prepared with NaCl (0.351 g), KOH (0.140 g), $Ca(OH)_2$ (0.022 g), bovine serum albumin (0.002 g), lactic acid (0.200 g), acetic acid (0.100 g), glycerol (0.016 g), urea (0.040 g), glucose (0.500 g) and dried porcine gastric mucin (type 3) (1.5% w/v) mixed to 90 mL of milliQ water [24, 25]. SVF was stirred until complete dispersion of the components. The pH was adjusted to 6 using HCl (0.1 M) and the final volume was adjusted to 100 mL with milliQ water.

2.3.4. Characterization

a. Mucoadhesion

The mucoadhesive strength (N) was determined using a Texture Analyzer (TA) (Lloyd Instruments, Ametek Company) in compression mode and with hydrated mucin disc, as described previously [19, 22]. Briefly, the sponges were attached with both side adhesive tape on the table of the TA. The mucin disc fixed to the probe (13 mm diameter) was brought into contact with the sponge with a preload of 0.1 N and was maintained for 60s to ensure intimate contact. The probe was then elevated and the mucoadhesive strength was determined from the detachment force between the disc and the sponge. The mucoadhesion of different commercialized pharmaceutical products for vaginal administration (Gynodaktarin®, Lubrilan®, Mithra Intim gel®, Gynoxin®, Lubexxx®, Canestene®, Crinone®, Preventex®) was also measured in the same condition in order to compare them to prepared sponges.

b. Hardness and deformability

The hardness (N) and the deformability (%) of the sponges were determined with a TA in compression and cyclic mode. The sponges were attached with both side adhesive tape on the table of the TA. A cylindrical probe (25 mm diameter) was compressed four times into each sample with a preload of 0.5 N, at a defined rate (1 mm/s) and to a defined depth (0.2 mm). The hardness is the force measured after the first compression. The deformability is the ratio of the force obtained after the first compression and the force measured after each cycle.

c. Scanning Electron Microscopy and Energy Dispersive X-Ray Analysis (SEM-EDX)

Scanning electron microscopy (SEM) was performed on sponges loaded with lipoplexes using a Field Emission Environmental microscope (Philips, model XL 30) after metallization with platinum (30 nm). Elemental detection was also performed with this microscope without preparation of the samples. The morphology of the lipoplexes was analysed. Lipoplexes were identified by the phosphorus atom (P) of siRNA molecules.

2.4. Diffusion and colloidal stability of lipoplexes

Lipoplexes were prepared with fluo-siRNA (300 nM) and the sponges were loaded with fluorescent lipoplexes, as described in sections 2.2.1. and 2.3.2. Sponges were previously rehydrated with 2 mL of SVF at 37°C in order to mimic *in situ* vaginal conditions before measuring the diffusion of lipoplexes.

2.4.1. Fluorescence Single Particle Tracking (fSPT)

For the analysis of lipoplexes inside mucus, 10 µL of lipoplexes were added to 40 µL of SVF in a 8-well plate and for the analysis of lipoplexes loaded into rehydrated sponges, 50 µL were sampled. Moreover, 10 µL of lipoplexes were added in 40 µL of RNAse free water, as a control condition. Each sample was allowed to equilibrate for 15 min at 37°C before being placed on the swept-field microscope (Nikon, Brussels, Belgium) equipped with a 60x oil immersion lens (Nikon) and with a stage top incubator kept at 37°C. Movies were recorded with NIS Elements software (Nikon) driving the Andor ixon ultra 897 camera (Belfast, UK). Analysis of the videos was performed using an house-developed particle tracking software [26].

2.4.2. Fluorescence Correlation Spectroscopy (FCS)

The samples were prepared in the same conditions as above (2.4.1.). They were placed in a glass-bottom 96-well plate (Greiner bio-one, Frickenhausen, Germany) and the fluorescent signal was measured respectively after 0 h, 2 h and 4 h of incubation at 37°C. FCS measurements were performed on a C1si laser scanning confocal microscope (Nikon), equipped with a time correlated single photon counting data acquisition module (Picoquant, Berlin, Germany). The laser beam was held stationary and was focused through an oil immersion objective lens (Plan Apo 60x, NA 1.2, collar rim correction, Nikon). The 647 nm laser beam of krypton-argon laser (Bio-Rad, Cheshire, UK) was used and the red fluorescence intensity fluctuations were recorded using Sympho-time (Picoquant, Berlin, Germany) for at least 60 s.

2.5. Release study

The release rate of fluorescent lipoplexes from the sponges was monitored over time and determined by fSPT technique. The sponges loaded with fluorescent lipoplexes were placed on ThinCert® with 0.4 µm pores diameter (PET membrane, Greiner bio-one) in a 6-well TC plate (Cellstar, Greiner bio-one) and were rehydrated with 2 mL of SVF (figure 1). The acceptor compartment was filled with SVF (2.5 mL) and all the system was incubated at 37°C. Every hour during 6 h, 100 µL were collected and fSPT movies were recorded, as described in section 2.4.1. in order to demonstrate the presence of lipoplexes in the acceptor compartment filled with SVF.

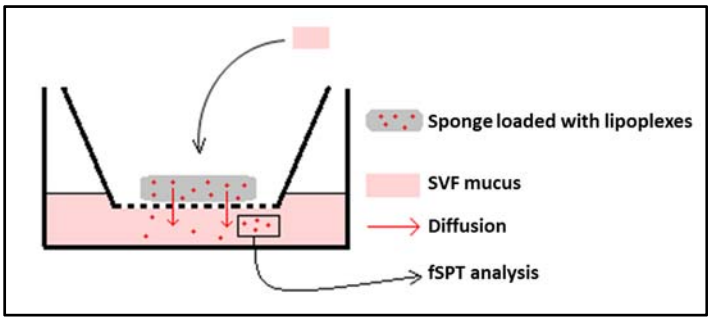


Figure 1: Schematic illustration of monitoring lipoplexes release from rehydrated sponges and diffusion through the SVF mucus into the acceptor compartment by fSPT.

2.6. Statistical analysis

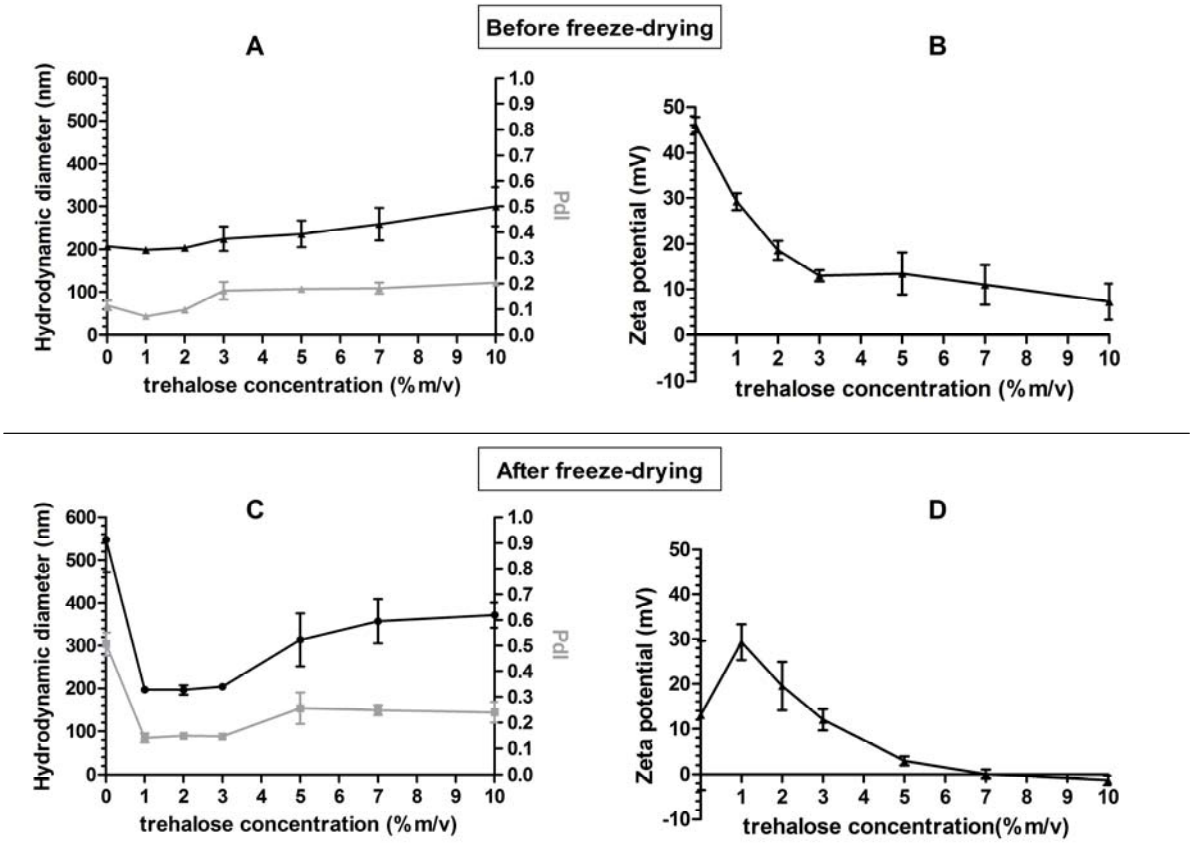
All values are expressed as the mean ± SEM. Statistical analyses were performed using GraphPad Prism® software. A p value < 0.05 was considered significant (*).

3. Results and Discussion

3.1. Preparation, characterization and freeze-drying of lipoplexes

3.1.1. Unpegylated lipoplexes

As shown in figure 2 , the mean size of the lipoplexes before freeze-drying (A and B) is around 200 nm in diameter with a low PdI (< 0.2) and a zeta potential around +50 mV. After freeze-drying and rehydration with water (figure 2 C and D), the physicochemical characteristics of these lipoplexes are completely different: the size and PdI increase (> 500 nm and > 0.5 respectively) and the zeta potential decreases (up to +10 mV). In order to prevent these variations, different percentages of trehalose were added. Indeed, as described by Chen et al. [27], the addition of a lyoprotectant, such as trehalose, is necessary to protect the membrane integrity of the lipoplexes during freeze-drying. It avoids a phase transition and can also improve drug retention by reducing the damages by ice crystals and inhibiting vesicles aggregation and/or fusion. It also favours the reversibility of nanoparticles rehydration after freeze-drying and the encapsulated drug protection [28-31]. Addition of increased amounts of trehalose (1 - 10%) only slightly modifies the size (~ 300 nm with 10% trehalose) and the PdI (~ 0.2) of lipoplexes (figure 2.A). However, the zeta potential decreases with increased concentration of trehalose (figure 2.B). After freeze-drying with trehalose and rehydration, lipoplexes recover their original size (~ 200 nm), particularly when small concentrations of trehalose were used (1 - 3%) (figure 2.C). Higher trehalose concentrations seem to slightly increase the size. Concerning the zeta potential, figure 2.D depicts that the surface charge decreases up to neutrality in the presence of 10% trehalose. In order to keep as close as possible the initial physicochemical characteristics of the lipoplexes, 1% of trehalose was selected for following experiments.



225

226 **Figure 2:** Hydrodynamic diameter (nm), PdI **(A) (C)** and zeta potential (mV) **(B) (D)** of
227 lipoplexes with increased percentages of trehalose (1 to 10 % m/v) before and after freeze-
228 drying (n=4).

229

230 **3.1.2. Pegylated lipoplexes**

231 Lipoplexes were then pegylated by addition of 30% of DSPE-PEG_{2000}, DSPE-PEG_{750} or
232 ceramide-PEG_{2000}. Indeed, it is well known that particles can easily diffuse through a mucosal
233 vaginal layer given that they are coated with PEG [2, 32, 33]. For this reason, three different
234 PEG derivatives were evaluated and compared. As highlights figure 3.A, lipoplexes containing
235 DSPE-PEG_{2000} or ceramide-PEG_{2000} have the same size before (1) and after (2) freeze-drying
236 (~ 200 nm). The PdI slightly increases but is still around 0.2. They also recover their zeta
237 potential once rehydrated (around -10 and +20 mV respectively). Concerning the lipoplexes
238 with DSPE-PEG_{750}, their size increases significantly after freeze-drying (> 600 nm). The PdI
239 also increases up to 0.4, giving rise to an increased heterogeneity of the system. However, the

zeta potential does not vary before and after freeze-drying (~ -12 mV) (figure 3.B). These results show that lipoplexes containing DSPE-PEG$_{750}$ are less stable than other lipoplexes, which can be a problem for further incorporation in a prolonged release system.

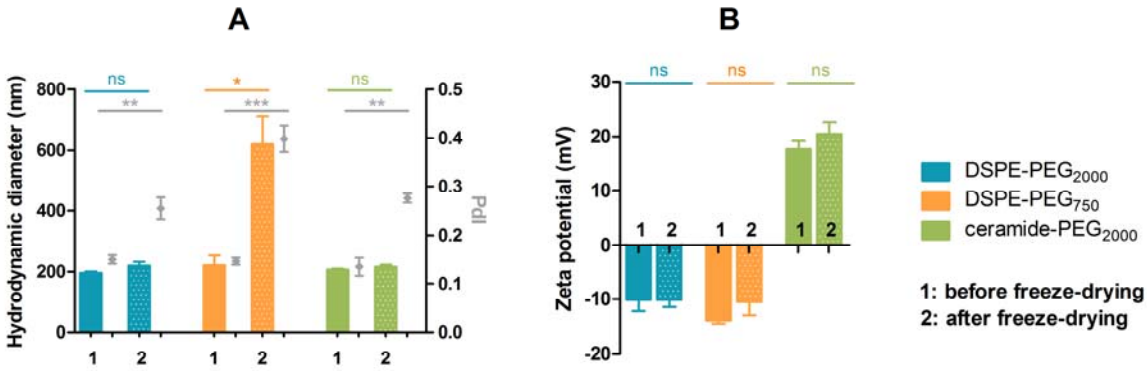


Figure 3: Hydrodynamic diameter (nm), PdI **(A)** and zeta potential (mV) **(B)** of lipoplexes with DSPE-PEG$_{2000}$, DSPE-PEG$_{750}$ or ceramide-PEG$_{2000}$ and with 1% of trehalose before **(1)** and after **(2)** freeze-drying. A paired Student's t-test is used to compare lipoplexes with DSPE-PEG$_{2000}$ **(1)-(2)**, DSPE-PEG$_{750}$ **(1)-(2)** and ceramide-PEG$_{2000}$ **(1)-(2)** (n=6).

Table 1 summarizes the physicochemical characteristics of lipoplexes before and after freeze-drying.

Lipoplexes	Freeze-drying	Diameter (nm)	PdI	Zeta potential (mV)
Without PEG	before after	198,8 ± 6,1 197,3 ± 7,4	0,07 ± 0,01 0,14 ± 0,02	29,3 ± 1,9 29,3 ± 3,9
DSPE-PEG$_{2000}$	before after	196,1 ± 5,8 220,6 ± 12,1	0,15 ± 0,01 0,25 ± 0,02	-10,1 ± 2,1 -10,2 ± 1,3
DSPE-PEG$_{750}$	before after	222,0 ± 32,0 620,9 ± 90,7	0,15 ± 0,01 0,40 ± 0,03	-13,9 ± 0,6 -10,5 ± 2,5
Ceramide-PEG$_{2000}$	before after	207,8 ± 3,2 216,9 ± 6,7	0,14 ± 0,02 0,27 ± 0,01	17,7 ± 1,5 20,5 ± 2,2

Table 1. Physicochemical characteristics of lipoplexes with 1% trehalose before and after freeze-drying. Values represent mean ± SEM (n=4).

3.1.3. Stability of freeze-dried lipoplexes

The stability of the freeze-dried pegylated lipoplexes was evaluated after storage at 4°C in closed glass vials for 1 month. The mean size, the PdI and the zeta potential were measured. It appears that the size of the lipoplexes does not vary significantly and ranged between 200 to 300 nm for lipoplexes with DSPE-PEG_{2000} and with ceramide-PEG_{2000}. Concerning the PdI, it is generally close to 0.2 and the zeta potential remains also constant for both types of pegylated lipoplexes (data not shown).

The complexation efficiency and the integrity of lipoplexes were assessed by agarose gel electrophoresis (figure 4). As shown in figure 4.A, the first two spots correspond to free siRNA (control). The three next correspond to lipoplexes with DSPE-PEG_{2000}. Before freeze-drying, no free-siRNA is detectable. The siRNA is complexed by liposomes (1: no visible spot) confirming our previous complexation results (more than 95% of complexation [23]). The addition of trehalose does not destabilize the particles and does not release the siRNA. After freeze-drying and rehydration (spot 2), no free siRNA is visible showing that the particles form again spontaneously to almost 100%. A positive control with Triton X-100 shows that this surfactant releases all the siRNA from the lipoplexes (spot 2+Trit.), no broken and no smearing bands are observed on the gel confirming that the siRNA is stable and protected by the lipoplexes during the freeze-drying process. Same observations were done for lipoplexes with DSPE-PEG_{750} and with ceramide-PEG_{2000}.

The same results were obtained after 25 days of storage at 4°C (figure 4.B). This experiment confirms that the storage at 4°C during 25 days has no deleterious effect on the siRNA. Moreover, in another study, we have shown with active siRNA that the freeze-drying process allows to keep the gene-silencing properties of siRNA (results not shown). The storage stability is one of the key challenges for a safe translation to the clinic and all these results indicate that pegylated lipoplexes freeze-dried with 1% of trehalose keep their characteristics during at least 25 days.

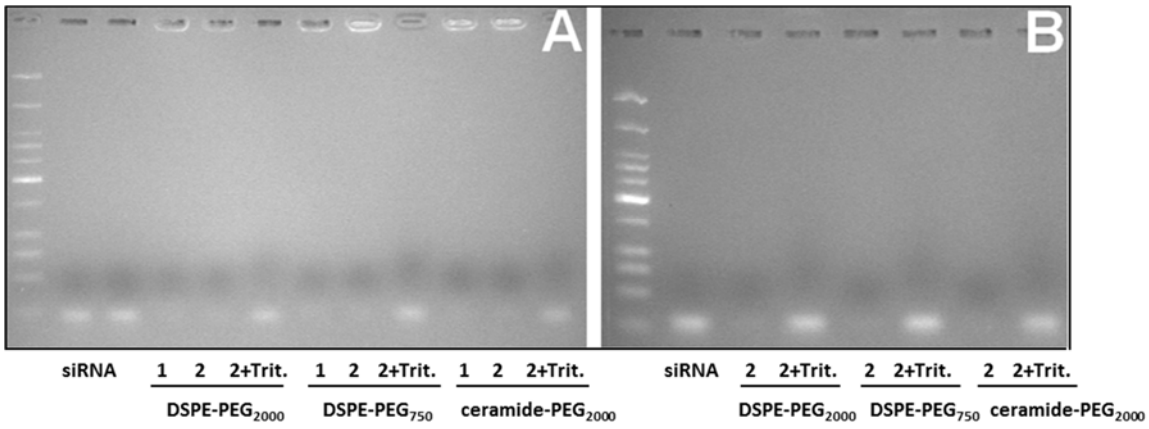


Figure 4: Complexation efficiency of pegylated lipoplexes evaluated by agarose gel electrophoresis. **(A)** Day 0. **siRNA**: control with free siRNA. **1**: lipoplexes before freeze-drying. **2**: lipoplexes after freeze-drying. **2+Trit.**: lipoplexes after freeze-drying and with 0.5% w/v of Triton X-100. Conditions **1**, **2** and **2+Trit.** were performed on lipoplexes with DSPE-PEG_{2000}, DSPE-PEG_{750} and ceramide-PEG_{2000}. **(B)** Same conditions after 25 days of storage at 4°C.

3.2. Behaviour of lipoplexes in artificial vaginal mucus

3.2.1. Diffusion and size of the lipoplexes

To reach the epithelial tissue, lipoplexes have to diffuse through the vaginal mucus. fSPT was used to estimate the mobility of lipoplexes in undiluted artificial vaginal fluids and to monitor their aggregation. fSPT technique makes use of videos of diffusing fluorescently labelled particles to analyse their individual motion trajectory in complex biological media and calculates their individual diffusion coefficient (D, $\mu m^2/s$). In case of freely diffusing particles, the D distribution so obtained is converted into a size distribution (nm) by using the Stokes-Einstein equation, as previously described [26, 34, 35].

Fluorescently labelled lipoplexes were incubated in RNAse free water, as a control, and in undiluted simulated vaginal fluids (SVF), both at 37°C. The movement of all individual lipoplexe was tracked and registered. From the analysis of the recorded trajectories, the diffusion coefficients ($\mu m^2/s$) were calculated in order to compare the diffusion ability of the different types of lipoplexes inside vaginal mucus.

Due to the complex ethical and practical procedures to obtain human vaginal fluids (limited quantity, stability and storage), SVF has been used as a model instead of natural mucus. It has similar viscosity, pH and osmolality to that of physiological fluids and mucus and thus should to a high extent resemble the human vaginal mucus [24].

As demonstrates in figure 5.A, the lipoplexes were able to freely diffuse in RNAse free water. The peak values of the diffusion distributions varied from 0.7 $\mu m^2/s$ for the lipoplexes with DSPE-PEG_{750} to 1.3 $\mu m^2/s$ for unpegylated lipoplexes. In SVF (figure 5.B), the lipoplexes are still able to diffuse, but slower than in water. This difference of diffusion is highly likely ascribed to the viscosity of SVF (~ 3 mPa.s) and its complex composition. In SVF, lipoplexes have to pass through the different components of mucus and particularly through the crosslinked mucin fibres, which form a highly heterogeneous mesh. These results underline also the necessity to measure the diffusion directly in the relevant biofluids rather than in diluted fluids. Despite the mucus barrier, lipoplexes are still capable to diffuse. Concerning the influence of the type of PEG on the diffusion, figure 5.B shows that lipoplexes with DPSE-PEG_{2000} and ceramide-PEG_{2000} are able to diffuse faster than those without PEG and with DSPE-PEG_{750}. This small difference could be due to the difference of the PEG length. Coating lipoplexes with PEG_{2000} could further minimize adhesive interactions between nanoparticles and mucus constituents, compared to PEG_{750} and without PEG, decreasing aggregation phenomenon and slightly increasing the diffusion.

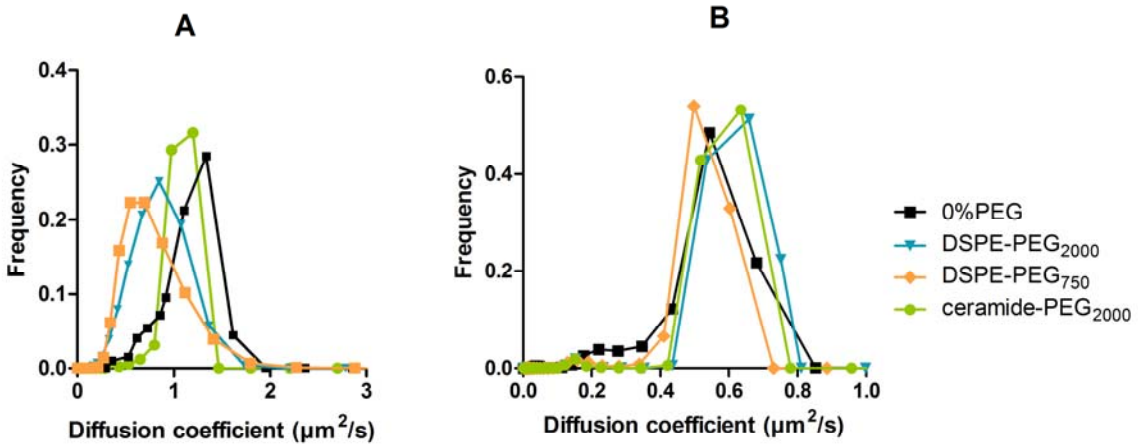


Figure 5: Diffusion distributions of lipoplexes without PEG and with DSPE-PEG_{2000}, DSPE-PEG_{750} or ceramide-PEG_{2000} following incubation at 37°C in RNAse free water **(A)** and in SVF **(B)**, determined by fSPT analysis.

In order to verify this hypothesis, the size of the lipoplexes was estimated in SVF compared to water. Although the Dynamic Light Scattering (DLS) is the most common technique for measuring particle size in aqueous media, it is difficult to directly measure the size in undiluted biological fluids by this technique. Therefore, fSPT was used and size distributions of the lipoplexes in SVF were obtained. In water (figure 6.A), mean values between 170 - 230 nm were observed for all the lipoplexes, PEG or not. These results are in good agreement with those obtained previously by DLS (section 3.1.2.). In SVF (figure 6.B), only the lipoplexes with DSPE-PEG_{2000} remain stable and keep their initial size close to 200 nm with a narrow distribution. The lipoplexes with ceramide-PEG_{2000} show a slight aggregation represented by a shift of the distribution compared to the size distribution in water. For the lipoplexes with DSPE-PEG_{750} and without PEG, the aggregation was more pronounced as particles with a diameter ranging from 300 to 500 nm were measured.

The size distributions outcomes confirm our hypothesis: coating lipoplexes with PEG_{2000} can minimize adhesive interactions between nanoparticles and mucus constituents, compared to

PEG_{750} and without PEG, decreasing aggregation phenomenon and slightly increasing the diffusion. The data stand in line with another previous study by J. das Neves *et al* [25]. Moreover, it is well known that the size is a major requirement for an optimal vaginal diffusion and it has been demonstrated that particles with a diameter around 200 to 300 nm show the best diffusive property contrarily to particles with a diameter higher than 500 nm [27, 30].

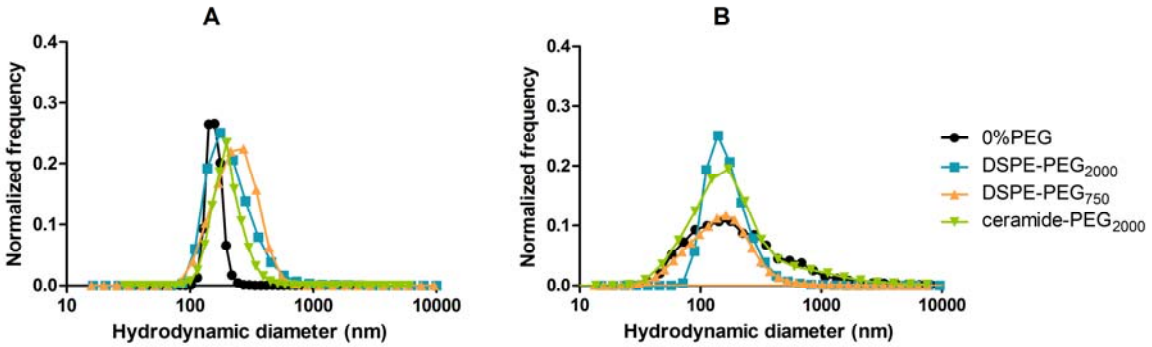


Figure 6: fSPT sizing of lipoplexes without PEG and with DSPE-PEG_{2000}, DSPE-PEG_{750} or ceramide-PEG_{2000} following incubation at 37°C in RNAse free water **(A)** and in SVF **(B)**.

3.2.2. Release of the siRNA

The release of siRNA from lipoplexes was evaluated using Fluorescence Correlation Spectroscopy (FCS). FCS is a technique used to calculate the percentage of complexed fluo-siRNA by the lipoplexes and to follow its release as a function of time, as described previously [36-39]. This technique monitors the fluorescence intensity fluctuations of molecules diffusing in and out the focal volume of a confocal microscope. When free siRNA is present in the focal volume, a fluorescence signal (baseline) proportional to the siRNA concentration is obtained. Contrariwise, when the siRNA is complexed within the nanoparticles, the concentration of free siRNA decreases (the baseline decreases also) and peaks with high fluorescence intensity appear each time a particle passes in the detection volume. Conversely, when the siRNA is dissociated from the lipoplexes, the concentration of free siRNA increases resulting in an increase of the baseline [37].

In RNAse free water (figure 7.A), the initial percentage of incorporation was high (more than 95%) for all the lipoplexes. These results are in accordance with those obtained previously, by another quantification technique (Quant-iT™ RiboGreen® RNA assay) [23]. After 4 hours, the overall siRNA released was limited to maximally 10%. In SVF (figure 7.B), no further release was observed and a very slight difference can be noticed between the studied lipoplexes, those with DSPE-PEG_{2000} retained the totality of complexed siRNA even after 4 hours in SVF. To reach the cytoplasm of targeted cells, the siRNA must be kept intact in the lipoplexes. The lipoplexes have to protect it from the mucus components to avoid its degradation. They have to diffuse into the mucus to reach the targeted cells and release their content only once in the cytoplasm of these cells reached. The low release of siRNA observed by FCS indicates that lipoplexes pegylated or not, are stable for at least 4 hours in SVF at 37°C.

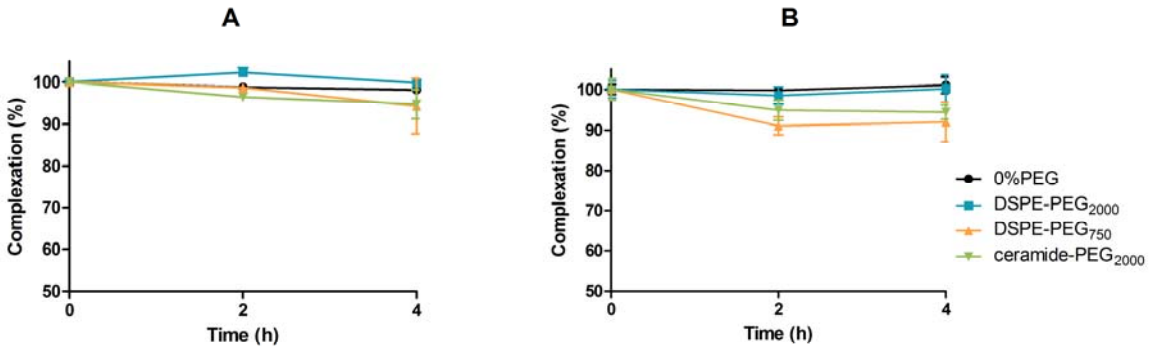


Figure 7: Percentage of complexed siRNA into lipoplexes according to the time in RNAse free water **(A)** and in SVF **(B)**, determined by FCS analysis (n=3).

In view of the colloidal stability results in both water and SVF (3.1. and 3.2. sections), lipoplexes grafted with DSPE-PEG_{2000} seem the most colloidally stable from the all formulations, and hence were selected for further hydrogel/sponge formulation.

3.3. Lipoplexes effect on the characteristics of the sponges

Placebo sponges, with different amounts of polymer (HEC) and plasticizer (PEG) were previously characterized [19]. As the polymer concentration directly influences the viscosity and probably the diffusion of lipoplexes, two concentrations of HEC were tested (0.83% and 1.67%). Moreover, the effect of the lipoplexes on the sponge's characteristics (mucoadhesion, hardness, deformability and morphology) was also investigated.

3.3.1. Mucoadhesion

HEC polymer has been chosen to prepare the sponges for its well-described mucoadhesive properties [22]. It has the possibility to anchor the formulation in the administration site and allows a prolonged delivery of the incorporated material, thereby maximizing the clinical performance [40]. Moreover, this polymer is considered as a non-toxic and non-irritating material. Thanks to its biocompatible property, it has been employed in several commercialized products intended for a vaginal use [4, 18, 41].

In this section, the ability of the sponges to adhere to a partially hydrated mucin disc, mimicking vaginal conditions, was studied. The mucoadhesive strength (N) was determined by the force required to separate the disc from the sponge. Figure 8 shows the mucoadhesion of the placebo sponges, of the sponges containing 1% trehalose and of the sponges containing both 1% trehalose and lipoplexes, in comparison with different vaginal commercialized products. All the selected commercialized forms are gels (Lubrilan®, Mithra Intim gel®, Lubexxx®, Crinone®), creams (Gynodaktarin®, Gynoxin®, Canestene®) or a solid system (Preventex®) and are not specifically intended to be adhesive. They have been chosen to have an idea of their mucoadhesive capacity, as no reference product and no reference values of mucoadhesion are available.

It is obvious that all the sponges are significantly more mucoadhesive than the pharmaceutical products, even at the smallest concentration of HEC (0.83%). Moreover, as demonstrated before, the concentration of HEC influences the mucoadhesion [19]; sponges with 1.67% HEC are more adhesive (~ 1.1 N) than sponges with 0.83% (~ 0.7 N) and this can be explained by the interpenetration mechanism involved in the mucoadhesive interactions [42]. Indeed, the

intimate contact between the two surfaces, sponge and mucin disc, induces interpenetration of glycoproteins chains of mucin with polymeric chains of HEC. Assuming that the surface of the mucin disc in each experiment is similar, the higher the HEC concentration is, the stronger the mucoadhesive bonds are. Finally, the presence of trehalose and lipoplexes has no significant influence ($p > 0.05$) on the mucoadhesion force. Lipoplexes in the sponges do not influence their mucoadhesion capacity.

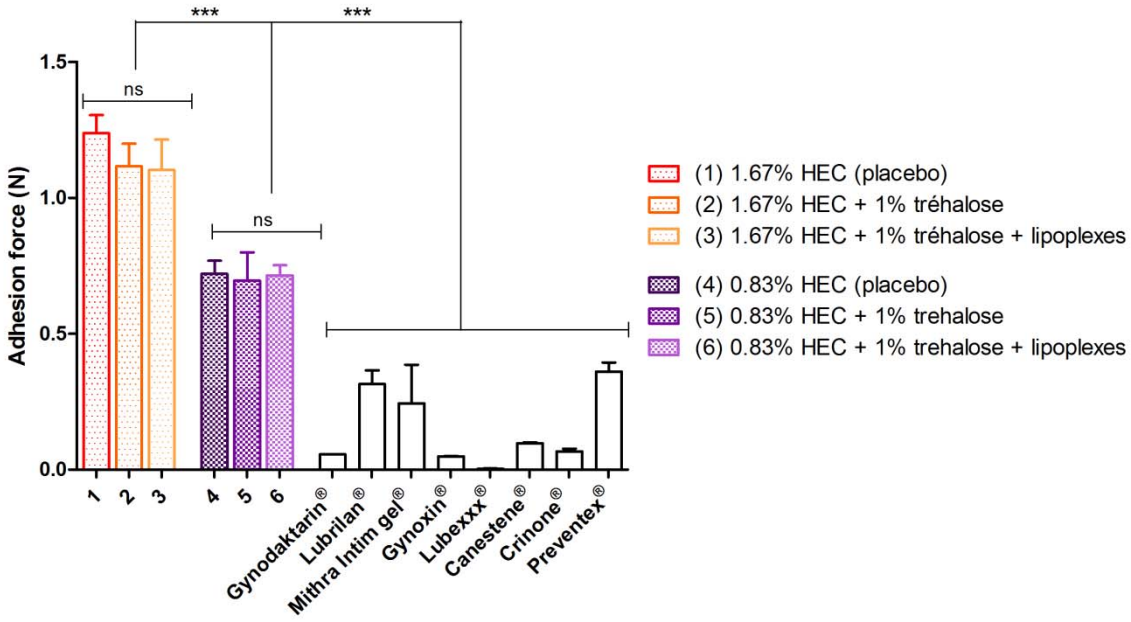


Figure 8: Adhesion force (N) of placebo sponges **(1)** - **(4)**, of sponges with 1% trehalose **(2)** - **(5)** and of sponges with 1% trehalose and lipoplexes **(3)** - **(6)** compared to pharmaceutical commercial products. Results are analyzed by a one-way ANOVA, followed by a Dunnett's test (n=6).

3.3.2. Hardness and deformability

Topical application of sponges requires an insight to their behaviour after compression stresses. These systems should possess suitable mechanical resistance to facilitate the application inside vagina and also enough resistance to deformation to ensure durability against shear stress encountered. The hardness is directly correlated with the polymer

concentration (figure 9.A); sponges with 1.67% are harder than sponges with 0.83% HEC (~ 1 N vs ~ 0.7 N). Moreover, the presence of trehalose and lipoplexes has no significant influence ($p > 0.05$) on this characteristic. Since there are no reference values for optimal hardness, the ability of sponges to be easily removed out of their containers and their malleability were also analysed. Indeed, all sponges met these conditions; they are hard enough to be extracted without being broken, they are malleable and retain their shape. Regarding the deformability (figure 9.B), the slopes of the curves provide information about the deformability of sponges. An increase in the slope corresponds to an increase deformability of the sponge. Specifically, the sponges with 1.67% show around 10% of deformability while those containing 0.83% are deformed at maximum 20%. Again, the presence of trehalose and lipoplexes does not change the deformability.

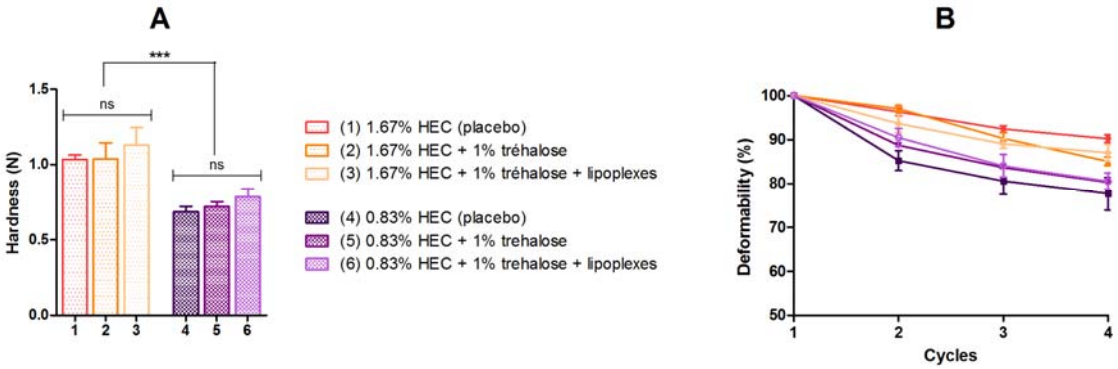
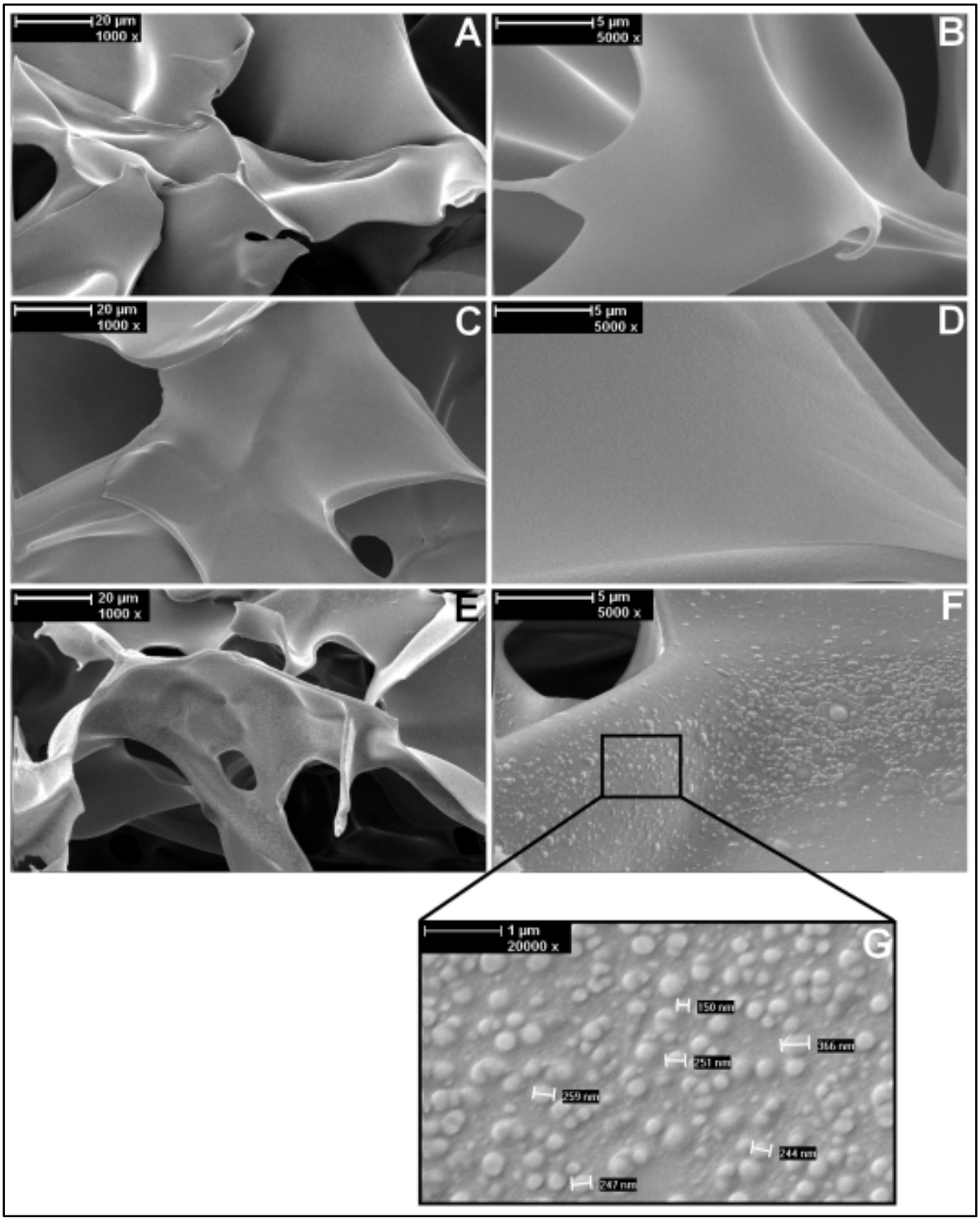


Figure 9: (A) Hardness (N) and **(B)** deformability (%) of placebo sponges **(1)** - **(4)**, of sponges with 1% trehalose **(2)** - **(5)** and of sponges with 1% trehalose and lipoplexes **(3)** - **(6).** One-way ANOVA, followed by the Dunnett's test is used (n=12).

3.3.3. Morphology

The morphology and integrity of lipoplexes in sponges were evaluated by SEM analysis. As demonstrated in figure 10, placebo sponges (A and B) and sponges with 1% trehalose (C and D) have smooth surfaces, without any irregularities and/or pores. On the contrary, sponges with lipoplexes (E, F and G) show a rough surface, with small individual spherical asperities.

Their size is around 250 nm, in agreement with the sizes obtained using DLS and fSPT in the previous sections (3.1.2. and 3.2.1.). In order to demonstrate that these spherical asperities correspond to intact lipoplexes, an elemental analyse was performed. This technique was used to confirm the presence of the phosphorous atom (P) and thus the presence of the siRNA in the observed vesicles. Figure 11 shows that the P atom was detected in the sponge containing 1.67% HEC, 1% trehalose and lipoplexes (same results were observed for sponges with 0.83% HEC while no P detection was observed with placebo sponges, data not shown). This confirms that observed vesicles are lipoplexes. After being incorporated into the hydrogel and freeze-dried, the lipoplexes retain their morphology, are intact and still have a size between 200 to 300 nm.



462

463 **Figure 10:** SEM images of sponges with 1.67% HEC. **(A)** - **(B)** are placebo sponges, **(C)** - **(D)**

464 are sponge with 1% trehalose and **(E)** - **(F)** - **(G)** are sponges containing 1% trehalose and

465 lipoplexes.

466

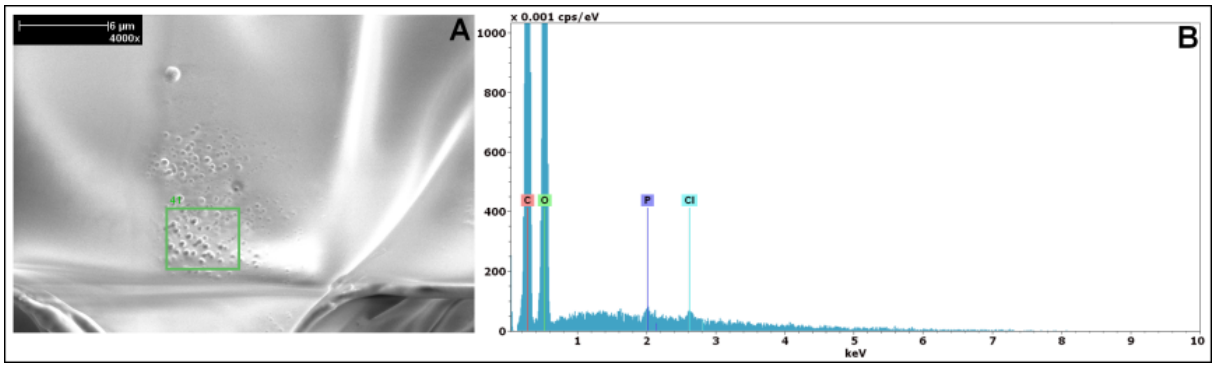


Figure 11: (A) SEM image and **(B)** phosphorous (P) elemental analysis (SEM-EDX) on sponge containing 1.67% HEC, 1% trehalose and lipoplexes.

3.4. Diffusion and stability of lipoplexes in rehydrated sponges

When applied in the vagina, sponges have to rehydrate with mucus to form a hydrogel. Lipoplexes will have to progressively diffuse through the gel and then through the vaginal mucus to reach vaginal epithelium. Therefore it is necessary to determine the diffusion ability of lipoplexes and their stability into the rehydrated sponges. Moreover, the influence of HEC concentration was also studied. fSPT and FCS techniques, as described previously, were used for these analyses. Sponges were rehydrated with SVF at 37°C, mimicking vaginal conditions. As shown in figure 12.A, lipoplexes have different diffusion profiles in the two types of gels (0.83% vs 1.67 % HEC). In gels with 0.83% HEC, there are two populations of particles: a large majority with a high peak value (D ~ 0.8 $\mu m^2/s$) and some others particles with a lower peak value of the diffusion distribution (D ~ 0.2 $\mu m^2/s$). Regarding the measured sizes of these lipoplexes, there are also two populations; a large majority with a diameter at around 200 nm and also a little fraction of particles with a peak diameter around 90 nm (data not shown). Indeed, particles with a diameter around 200 - 300 nm can better diffuse in mucus than smaller with a diameter below 100 nm [16]. Lipoplexes are also able to diffuse in gels with 1.67% HEC but slower than in the 0.83% HEC gels (D ~ 0.4 $\mu m^2/s$). The difference in viscosity of the two hydrogels before and after freeze drying can explain this (100 mPa.s for the 0.83% HEC hydrogel and 1300 mPa.s. for the 1.67% HEC hydrogel). Moreover, sponges were rehydrated

with SVF which also increases the viscosity of the final hydrogel. This can consequently further reduce the mobility of lipoplexes.

The stability of lipoplexes (siRNA release) in the hydrogel (figure 12.B) was next assessed. Within both types of rehydrated sponges, maximum 8% of siRNA are released after 4 hours at 37°C. The concentration of HEC does not influence the entrapment efficiency of lipoplexes. They diffuse through rehydrated sponges without releasing their content.

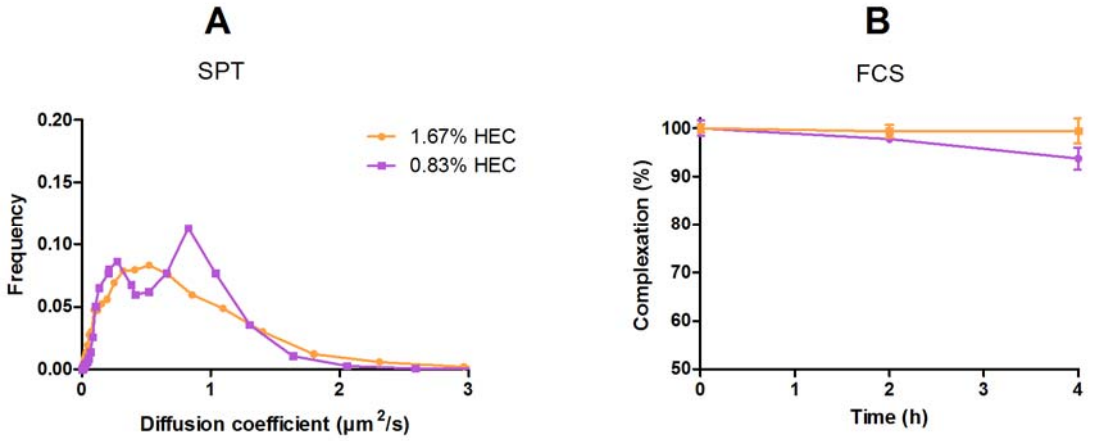


Figure 12: Diffusion distributions of lipoplexes **(A)** and percentage of complexed siRNA as a function of time **(B)** in 0.83% and 1.67% HEC sponges; respectively determined by fSPT and FCS analysis (n=3).

3.5. Release of lipoplexes outside rehydrated sponges

Finally, the release rate of lipoplexes from rehydrated sponges was monitored over time and compared for both types of sponges (0.83% vs 1.67% HEC). By employing fSPT, videos were recorded in order to demonstrate the presence of lipoplexes in the acceptor compartment filled with SVF and consequently their diffusion outside the rehydrated system (see on figure 1). Figure 13 represents screenshots of these videos (A=1.67% and B=0.83% HEC). This qualitative technique is used to show the number of lipoplexes diffusing in the mucus and if it increases with time.

It is first observed that no lipoplexes were in the acceptor compartment at time 0, independently of the HEC concentration. A lag time is necessary for the rehydration of sponges and to allow the diffusion of lipoplexes. Then, fluorescent spots appear progressively in the acceptor compartment with a delay difference of 2 hours between the two sponges. Lipoplexes appear in the acceptor compartment after two hours for 0.83% HEC sponges (B) while they appeared after four hours for 1.67% HEC sponges (A). These spots confirm that lipoplexes are able to diffuse through sponges rehydrated with artificial vaginal fluids (section 3.4.). The appearance delay of lipoplexes in the receiving compartment depends on the HEC concentration; the higher the concentration of HEC, the longer the rehydration duration of the sponges. Moreover, increasing the concentration of HEC results in gels with higher viscosity values. These two phenomena delay the diffusion of the lipoplexes in the gel and in the receiving compartment. Despite the different viscosities, lipoplexes were able in both cases to diffuse from the rehydrated sponges even after 6 hours at 37°C. The increased amount of fluorescent spots suggests an increase of lipoplexes release. This last point demonstrates that the sponges can be considered as a matrix system allowing a sustained delivery of lipoplexes.

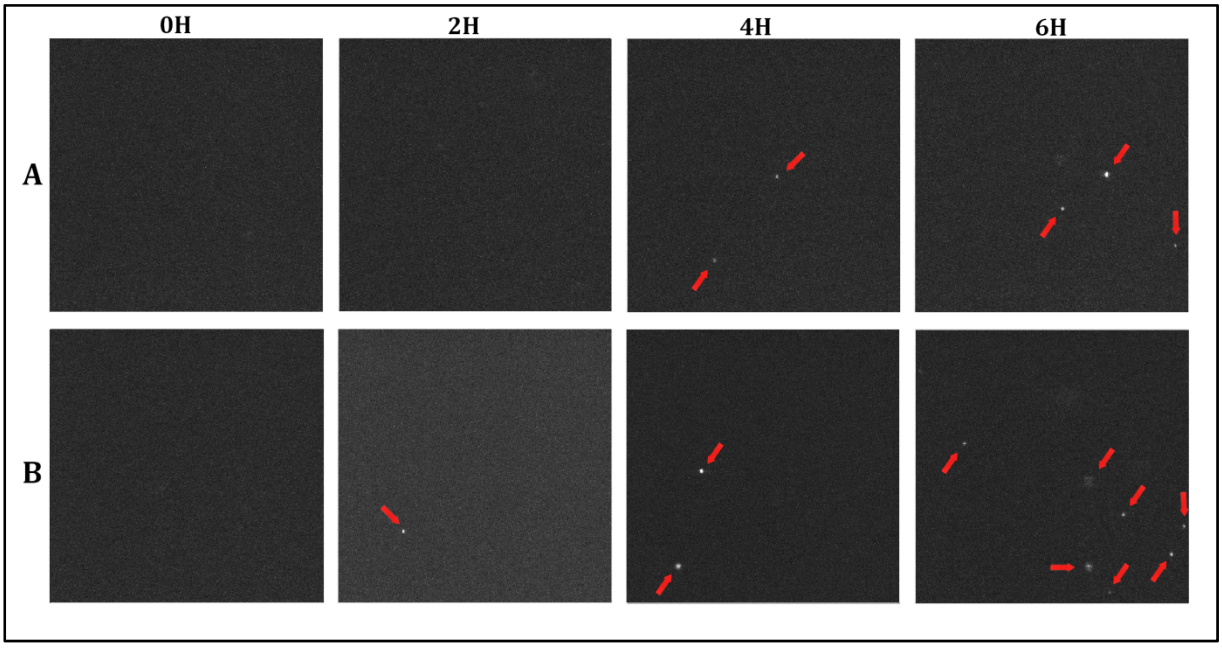


Figure 13: Screenshots of fSPT videos of SVF in the acceptor compartment after rehydration of sponges with 1.67% HEC **(A)** or with 0.83% HEC **(B)** and lipoplexes.

4. Conclusions

This study shows the feasibility of entrapping pegylated lipoplexes into a solid matrix system for a prolonged delivery of siRNA in vaginal mucus. The sponge system is obtained by freeze-drying and is intended to be administered directly inside vagina in order to treat pathologies using the gene silencing mechanism. For this, the sponges have to be *in situ* rehydrated to form a hydrogel and allow a sustained release of lipoplexes. Hydroxyethyl-cellulose (HEC) was chosen to prepare the sponges for its mucoadhesive properties.

Vaginal administration of lipoplexes is a challenge since the mucus presents a significant barrier to effective delivery. To overcome this, 30% of three types of PEG derivatives were grafted on the lipoplexes; DSPE-PEG_{2000}, DSPE-PEG_{750} and ceramide-PEG_{2000}. Their physicochemical characteristics, colloidal stability and their ability to diffuse inside simulated vaginal fluids (SVF) were tested. Altogether, lipoplexes with DSPE-PEG_{2000} are the best choice for the intended application. They have a size close to 200 nm, which is adequate for mucopenetration. They are stable after freeze-drying and have high complexation efficiency (> 95%). They have the highest diffusion coefficient and do not aggregate in SVF. Moreover, they do not release their content even after 4 hours at 37°C. Consequently, lipoplexes with DSPE-PEG_{2000} were chosen for incorporation inside HEC hydrogels/sponges.

In order to be administered into the vagina and to ensure a prolonged delivery of the lipoplexes, the sponges must meet acceptable mechanical characteristics such as ease of manipulation, low hardness and good bioadhesion. Furthermore, lipoplexes have to be intact inside the sponges. As our analysis shows, the sponges containing lipoplexes meet these criteria. Sponges are hard enough to be malleable and flexible; what is important for an easy application. The maximum percentage of deformation is around 20%, which could be enough to resist to shear stress inside vagina. The strength necessary to separate the hydrated mucin disc from the surface of the sponge is almost 0.7 N which is higher than for commercialized vaginal products and could be sufficient to obtain an appropriate retention during the therapeutic period. In addition, lipoplexes, incorporated in sponges, retain their morphology and their original size.

To mimic vaginal conditions, sponges were rehydrated with SVF. The diffusion of lipoplexes inside and outside the rehydrated sponges was measured. Depending on the HEC concentration, lipoplexes present two different diffusion profiles. The diffusion is slower in the sponges containing 1.67% of HEC than in those containing 0.83% of HEC. This observation is the same for the diffusion outside the system. The release rate is lower in the 1.67% HEC sponges. This can be explained by the fact that sponges with higher quantities of HEC need more time to be rehydrated and that they have a higher viscosity after *in situ* rehydration. These two phenomena delay the diffusion of the lipoplexes inside the gel and in the receiving compartment. Considering that it takes 4 hours for lipoplexes to diffuse outside rehydrated sponge with 1.67%, the sponges containing 0.83% HEC should be more suited for an optimal vaginal treatment.

In conclusion, a new mucoadhesive solid system adapted for a prolonged vaginal delivery of lipoplexes has been developed. It is easy to handle, able to protect pegylated lipoplexes and to be rehydrated with vaginal fluids. In future studies, this promising freeze-dried mucoadhesive sustained released system will be validated with active siRNA.

Acknowledgements

Authors thank the Belgium National Fund for Scientific Research (FNRS, http://www.frs-fnrs.be) for financial support.

References

1. Bigucci, F., et al., *Vaginal inserts based on chitosan and carboxymethylcellulose complexes for local delivery of chlorhexidine: preparation, characterization and antimicrobial activity.* Int J Pharm, 2015. **478**(2): p. 456-63.
2. Whaley, K.J., et al., *Novel approaches to vaginal delivery and safety of microbicides: biopharmaceuticals, nanoparticles, and vaccines.* Antiviral Res, 2010. **88 Suppl 1**: p. S55-66.
3. Ugaonkar, S.R., et al., *A novel intravaginal ring to prevent HIV-1, HSV-2, HPV, and unintended pregnancy.* J Control Release, 2015. **213**: p. 57-68.
4. Palmeira-de-Oliveira, R., A. Palmeira-de-Oliveira, and J. Martinez-de-Oliveira, *New strategies for local treatment of vaginal infections.* Adv Drug Deliv Rev, 2015. **92**: p. 105-22.
5. Yang, S., et al., *Advancements in the field of intravaginal siRNA delivery.* J Control Release, 2013. **167**(1): p. 29-39.
6. Rodriguez-Gascon, A., et al., *Vaginal gene therapy.* Adv Drug Deliv Rev, 2015. **92**: p. 71-83.
7. Chang, J.T., et al., *Highly potent and specific siRNAs against E6 or E7 genes of HPV16- or HPV18-infected cervical cancers.* Cancer Gene Ther, 2010. **17**(12): p. 827-36.
8. Berginc, K., et al., *Mucoadhesive liposomes as new formulation for vaginal delivery of curcumin.* Eur J Pharm Biopharm, 2014. **87**(1): p. 40-6.
9. Joraholmen, M.W., et al., *Chitosan-coated liposomes for topical vaginal therapy: assuring localized drug effect.* Int J Pharm, 2014. **472**(1-2): p. 94-101.
10. Ensign, L.M., R. Cone, and J. Hanes, *Nanoparticle-based drug delivery to the vagina: a review.* J Control Release, 2014. **190**: p. 500-14.
11. Wu, S.Y., et al., *Vaginal delivery of siRNA using a novel PEGylated lipoplex-entrapped alginate scaffold system.* J Control Release, 2011. **155**(3): p. 418-26.
12. Yang, M., et al., *Nanoparticle penetration of human cervicovaginal mucus: the effect of polyvinyl alcohol.* J Control Release, 2014. **192**: p. 202-8.
13. Rabanel, J.M., P. Hildgen, and X. Banquy, *Assessment of PEG on polymeric particles surface, a key step in drug carrier translation.* J Control Release, 2014. **185**: p. 71-87.
14. Suk, J.S., et al., *PEGylation as a strategy for improving nanoparticle-based drug and gene delivery.* Adv Drug Deliv Rev, 2016. **99**(Pt A): p. 28-51.
15. Xu, Q., et al., *Scalable method to produce biodegradable nanoparticles that rapidly penetrate human mucus.* J Control Release, 2013. **170**(2): p. 279-86.
16. Lai, S.K., et al., *Rapid transport of large polymeric nanoparticles in fresh undiluted human mucus.* Proc Natl Acad Sci U S A, 2007. **104**(5): p. 1482-7.
17. das Neves, J., M. Amiji, and B. Sarmento, *Mucoadhesive nanosystems for vaginal microbicide development: friend or foe?* Wiley Interdiscip Rev Nanomed Nanobiotechnol, 2011. **3**(4): p. 389-99.
18. das Neves, J. and M.F. Bahia, *Gels as vaginal drug delivery systems.* Int J Pharm, 2006. **318**(1-2): p. 1-14.
19. Furst, T., et al., *Mucoadhesive cellulosic derivative sponges as drug delivery system for vaginal application.* Eur J Pharm Biopharm, 2015. **95**(Pt A): p. 128-35.
20. Piette M., C.I.a.E.B., *Composition and method for treating HPV*, U.o. Liege, Editor. 2014: Belgium.
21. Mufamadi, M.S., et al., *A review on composite liposomal technologies for specialized drug delivery.* J Drug Deliv, 2011. **2011**: p. 939851.
22. Gupta, P.N., et al., *Development of liposome gel based formulations for intravaginal delivery of the recombinant HIV-1 envelope protein CN54gp140.* Eur J Pharm Sci, 2012. **46**(5): p. 315-22.
23. Lechanteur, A., et al., *Development of anti-E6 pegylated lipoplexes for mucosal application in the context of cervical preneoplastic lesions.* Int J Pharm, 2015. **483**(1-2): p. 268-77.
24. Margareth R. C. Marques, *simulated biological fluids with possible application in dissolution testing*

Dissolution Technologies, 2011.
25. das Neves, J., et al., *Interactions of microbicide nanoparticles with a simulated vaginal fluid.* Mol Pharm, 2012. **9**(11): p. 3347-56.
26. Braeckmans, K., et al., *Sizing nanomatter in biological fluids by fluorescence single particle tracking.* Nano Lett, 2010. **10**(11): p. 4435-42.
27. Chen, C., et al., *An overview of liposome lyophilization and its future potential.* J Control Release, 2010. **142**(3): p. 299-311.
28. Guan, P., et al., *Solidification of liposomes by freeze-drying: the importance of incorporating gelatin as interior support on enhanced physical stability.* Int J Pharm, 2015. **478**(2): p. 655-64.
29. Yadava, P., et al., *Effect of lyophilization and freeze-thawing on the stability of siRNA-liposome complexes.* AAPS PharmSciTech, 2008. **9**(2): p. 335-41.
30. Wieber, A., T. Selzer, and J. Kreuter, *Physico-chemical characterisation of cationic DOTAP liposomes as drug delivery system for a hydrophilic decapeptide before and after freeze-drying.* Eur J Pharm Biopharm, 2012. **80**(2): p. 358-67.
31. Fonte, P., S. Reis, and B. Sarmento, *Facts and evidences on the lyophilization of polymeric nanoparticles for drug delivery.* J Control Release, 2016. **225**: p. 75-86.
32. Groo, A.C. and F. Lagarce, *Mucus models to evaluate nanomedicines for diffusion.* Drug Discov Today, 2014. **19**(8): p. 1097-108.
33. Vanic, Z. and N. Skalko-Basnet, *Nanopharmaceuticals for improved topical vaginal therapy: can they deliver?* Eur J Pharm Sci, 2013. **50**(1): p. 29-41.
34. Elisa Zagato, K.F., Thomas Martens, Kristiaan Neyts, Jo Demeester, Stefaan De Smedt, Katrien Remaut, Kevin Braeckmans, *single particle tracking for studying nanomaterials dynamics: applications and fundamentals in drug delivery* Nanomedicine 2014: p. 913-927.
35. Martens, T.F., et al., *Measuring the intravitreal mobility of nanomedicines with single-particle tracking microscopy.* Nanomedicine (Lond), 2013. **8**(12): p. 1955-68.
36. Buyens, K., et al., *A fast and sensitive method for measuring the integrity of siRNA-carrier complexes in full human serum.* J Control Release, 2008. **126**(1): p. 67-76.
37. Dakwar, G.R., et al., *Colloidal stability of nano-sized particles in the peritoneal fluid: towards optimizing drug delivery systems for intraperitoneal therapy.* Acta Biomater, 2014. **10**(7): p. 2965-75.
38. Braeckmans, K., et al., *Advanced fluorescence microscopy methods illuminate the transfection pathway of nucleic acid nanoparticles.* J Control Release, 2010. **148**(1): p. 69-74.
39. Remaut, K., et al., *Can we better understand the intracellular behavior of DNA nanoparticles by fluorescence correlation spectroscopy?* J Control Release, 2007. **121**(1-2): p. 49-63.
40. Mansuri, S., et al., *Mucoadhesion: A promising approach in drug delivery system.* Reactive and Functional Polymers, 2016. **100**: p. 151-172.
41. Sosnik, A., J. das Neves, and B. Sarmento, *Mucoadhesive polymers in the design of nano-drug delivery systems for administration by non-parenteral routes: A review.* Progress in Polymer Science, 2014. **39**(12): p. 2030-2075.
42. Smart, J.D., *The basics and underlying mechanisms of mucoadhesion.* Adv Drug Deliv Rev, 2005. **57**(11): p. 1556-68.