Chitosan-coated liposomes for topical vaginal therapy: Assuring localized drug effect

May Wenche Jøraholmen[a], Željka Vanić[b], Ingunn Tho[a,c] and Nataša Škalko-Basnet[a,*]

[a] University of Tromsø, Drug Transport and Delivery Research Group, Department of Pharmacy, Faculty of Health Sciences, Universitetsveien 57, 9037 Tromsø, Norway

[b] University of Zagreb, Department of Pharmaceutical Technology, Faculty of Pharmacy and Biochemistry, A. Kovačića 1, 10 000 Zagreb, Croatia

[c] *present address*: University of Oslo, PharmaLuxLab, School of Pharmacy, Faculty of Mathematics and Natural Sciences, P.O.Box 1068 Blindern, 0316 Oslo, Norway

* Corresponding author: Tel.: +47-776-46640; Fax: +47-776-46151; E-Mail: natasa.skalko-basnet@uit.no (N. Škalko-Basnet)

Abstract

The choice of drug therapy in pregnant patients suffering from vaginal infections is limited by the safety profile of the drug. Assuring the efficient topical therapy to avoid systemic absorption is considered the best therapy option. Chitosan-coated liposomes have been developed and optimized to assure localized therapy of clotrimazole. Chitosan was selected as mucoadhesive polymer both to prolong system's retention at the vaginal site and act on biofilms responsible for high recurrence of infections. Sonicated liposomes were coated with chitosan in three different concentrations, namely 0.1, 0.3 and 0.6 % (w/v). Clotrimazole-containing (22 μg/mg lipid) chitosan-coated liposomes were in the size range of 100-200 nm. The *in vitro* release studies confirmed prolonged release of clotrimazole from both non-coated and chitosan-coated liposomes as compared to control. The *ex vivo* penetration experiments performed on the pregnant sheep vaginal tissue showed that coated liposomes assured increased clotrimazole tissue retention and reduced its penetration as compared to the control. Mucin studies revealed that the coating with lower chitosan concentration increased the system's mucoadhesive potential, as compared to coating with higher concentrations. These results provide a good platform for further *in vivo* animal studies on mucoadhesive liposomes destined to localized vaginal therapy.

Keywords: mucoadhesive liposomes; vaginal therapy; penetration, pregnancy, clotrimazole

1. Introduction

Although the occurrence of vaginal infections in pregnancy is common, the choice of drug therapy is rather limited (das Neves et al., 2008). In particular, topical antifungal therapy is preferred due to the systemic toxicity of antifungal drugs (Chang et al., 2002). In pregnant patients, the two main therapy goals can be summarized as i) assuring the high local drug concentration with concomitant avoidance of systemic absorption and ii) prevention of infection recurrence (Vanić and Škalko-Basnet, 2013). We propose that coating of liposomal surfaces with chitosan can assure both of the goals. When vagina is the site of drug administration, it is also important that both the drug and corresponding delivery system are safe and non-irritating to the delicate vaginal mucosa (Woodrow et al., 2009). The selection of mucoadhesive polymer will be therefore based on its biodegradability, biocompatibility and confirmed mucoadhesivness. Chitosan fulfils all the above mentioned criteria (Bernkop-Schnürch and Dünnhaupt, 2012; Bhattarai et al., 2010). Moreover, chitosan as mucoadhesive polymer is suited for repeated adhesion, as it does not become inactivated after the first contact with mucus; no reduction in its mucoadhesiveness has been reported (Valenta, 2005). In respect to recurrence, it is now clear that bacterial biofilms play an important role, as the negatively charged polysaccharide matrix coats the bacteria in the biofilm and restricts the penetration of antimicrobial in deeper parts of biofilm. Recently, chitosan was proposed to be able to disrupt bacterial biofilms in vaginal environment more efficiently than other polymers (polycarbophil). Even more importantly, its anti-biofilm effect was found to be pH-independent (Kandimalla et al., 2013).

The mucoadhesiveness of chitosan-based delivery systems has been studied in various routes of drug administration (das Neves et al., 2011a; Gradauer et al., 2012; Sugihara et al., 2012; Takeuchi et al., 2001; Takeuchi et al., 2005; Wang et al., 2011); however, its potential in vaginal drug delivery was comparatively less studied (Valenta, 2005; Bonferoni et al., 2008; Kast et al., 2002; Perioli et al., 2008; Perioli et al., 2009; Berginc et al., 2014). Based on its confirmed mucoadhesiveness, it is reasonable to expect that chitosan-based delivery systems will be superior in vaginal drug delivery, as some recent studies on chitosan nanoparticles indicate (Meng et al., 2011).

The success of non-invasive drug delivery via vaginal mucosa will be result of the interplay between the local vaginal environment, drug and physicochemical properties of drug carrier (Berginc et al., 2014). However, the interaction between drug delivery system and cervicovaginal mucus can affect the performance of drug nanocarrier, as the carrier must migrate through the vaginal or cervical fluid in order to deliver drug to the underlying mucosal surface (das Neves et al., 2012; Vanić and Škalko-Basnet, 2013). Vaginal mucosal tissue has relatively low turnover, which would be beneficial for prolonged residence time (Andrews et al., 2009). Vaginal absorption of drugs occurs in two main steps, namely the drug dissolution in vaginal lumen followed by the membrane penetration (Husain and Ahsan, 2005).

As a model drug we selected clotrimazole, often prescribed in vulovaginal candidosis. Its local therapy is recommended to pregnant and breast-feeding patients, as well as to patients not using reliable birth control methods, or planning to become pregnant (das Neves et al., 2008).

2. Materials and methods

Materials

Lipoid S 100 (PC, soybean lecithin, > 94 % phosphatidylcholine) was a generous gift from Lipoid GmbH, Ludwigshafen, Germany. Chitosan, low Mw (Brookfield viscosity 20.000 cps, degree of deacetylation (DD of 92 %), acetonitrile (CHROMASOLV® gradient grade), bovine serum albumin, clotrimazole, glycerol, methanol CROMASOLV®, mucin from porcine stomach (type III, bound sialic acid 0.5 % - 1.5 %, partially purified) and sodium chloride were the products of Sigma-Aldrich, Chemie GmbH, Steinheim, Germany. Acetic acid (glacial), anhydrous potassium phosphate and sodium hydrogen phosphate were purchased from Merck KGaA, Darmstadt, Germany. Calcium hydroxide, glucose, lactic acid, potassium hydroxide, propylene glycol, sodium hydroxide and urea were obtained from NMD, Oslo, Norway. Ammonium acetate was the product of BHD Prolab, Leuven, Belgium.

2.1. Preparation of liposomes with clotrimazole

Liposomes were prepared by the method described earlier (Berginc et al., 2014). In brief, clotrimazole (20 mg) and PC (200 mg) were dissolved in methanol in a round bottom flask. The solvent was evaporated using rotoevaporator system (Büchi rotavapor R-124 with vacuum controller B-721, Büchi Vac® V-500, Büchi Labortechnik, Flawil, Switzerland)

for at least 1 hour at 50 mm Hg and 40 °C. The remaining film was then re-suspended in 10 mL of distilled water. If necessary, ultrasonic bath was used to completely dislodge the film from the flask. Liposomal suspensions were stored in the refrigerator (4-8 °C) overnight prior to further use.

2.2. Vesicle size reduction

Liposomes (4 ml) were transferred to a 10 mL beaker and placed on ice bath. The needle probe tip of probe sonicator was placed in the centre of the beaker containing liposomal suspension. The sonicator (Ultrasonic processor 500 watt, Sigma-Aldrich, St. Louis, Missouri, USA) was set to 40 % amplitude and the liposomes were exposed to ultrasonic irradiation for 1, 2 or 2x2 minutes, respectively. The sonicated liposomes were stored in the refrigerator for at least 6 hours prior to further use.

2.3. Particle size analysis

The particle size distributions of liposomes were determined by photon correlation spectroscopy (Submicron particle sizer model 370, Nicomp, Santa Barbara, California, USA). In order to avoid interference from dust particles, the test tubes to be used for the determination were filled with distilled water and sonicated for 10 min in ultrasonic bath, then rinsed with filtered water (using 0.2 μm filter) prior to the experiments. Small aliquots of the samples were diluted with the filtered water to obtain particle intensity of

approximately 200-350 kHz (di Cagno et al., 2011). All formulations were prepared in a laminar airflow bench and analyses run in vesicle mode and the intensity-weightdistribution at 23 °C. Three parallels were determined (run time 10 min) for each sample measurement.

2.4. Zeta potential determination

Zeta potential measurements were performed on a Malvern Zetasizer Nano Z (Malvern, Oxford, UK). To ensure the validity of the measurements, the instrument was calibrated throughout the measurements using the Malvern Zeta Potential Transfer Standard (−50 ± 5 mV). The liposomal suspensions were diluted in 1:40 ratio in filtrated water before measurements to achieve the proper count rate. All measurements were performed at 23°C and the results were expressed as the average of at least three independent samples.

2.5. Entrapment efficiency determination

To separate free from liposomally entrapped drug, the sonicated liposomes were ultracentrifuged (Beckman model L8-70M preparative ultracentrifuge with SW 60 Ti rotor, Beckman Instruments, Palo Alto, California, USA) for 30 minutes, at 10 °C and 85 000 *g*. The pellet (containing unentrapped drug and liposomes larger than 200 nm) was separated from the supernatant (smaller liposomes containing clotrimazole), re-suspended in 500 µL of distilled water and finally diluted to 2 mL with methanol. Drug content in both supernatant and pellet was determined by the HPLC method. A reversed phase column

(XTerra® RP18 5µm, 3.9 x 150 mm column, Waters, Dublin, Ireland) installed in a Waters e2795 Separations Module coupled with a Waters 2489 UV/VIS detector was used in the measurements. The mobile phase consisted of acetonitrile and MilliQ water in a gradient starting at 30% acetonitrile (A), increasing to 90% A over 10 minutes, then to 100% A after 11 minutes. The HPLC measurements settings were as follows: flow rate 1 mL/min, column temperature of 25 °C, sample temperature 25 °C, injection volume 20 µL, run time 11 min and the detection wavelength 210 nm. The correlation coefficient was 0.9997 and the minimum detectable amount of clotrimazole 0.5 µg/mL. The entrapment was expressed as the amount of drug present in sonicated vesicles. The measurements were performed in triplicates.

2.6. Phospholipid content

An enzymatic assay was used to determine the amount of lipid present in liposomes in order to calculate the entrapment efficiency. For this purpose a commercial test kit (Phospholipids B; Wako Chemicals USA, Inc., Richmond, Virginia, USA) was applied in the method described earlier (Basnet et al., 2012). Measurements were performed in triplicates.

2.7. Coating of liposomes

The chitosan solutions (0.1, 0.3 and 0.6 %, w/v) used for liposome coating were prepared in 0.1 % and 0.5 % (v/v) glacial acetic acid, respectively. The chitosan solution was added

drop-wise to an equal volume of liposomes free from unentrapped clotrimazole, under controlled magnetic stirring at room temperature for 1 hour, followed by incubation in the refrigerator overnight. The rate of stirring was kept constant for all preparations (Karn et al., 2011).

2.8. In vitro release study

Drug release was followed by the method described earlier (Hurler et al., 2012). The Franz cell manual diffusion system (Perme Gear Ink, Diffusion cells and Systems, Hellertown, USA) was properly cleaned with methanol, demineralized water and distilled water, respectively. The heating circulator (Julabo Laboratechnik, F12-ED, Seelback, Germany) was set to 37 °C. The acceptor chamber (12 mL) was filled up with acetate buffer (pH 4.6; 77.1 g of CH_3COONH_4, 70 mL of glacial acetic acid and distilled water up to 1000 mL). Cellophane membrane (Max Bringmann KG, Wendelstein, Germany) was pre-soaked in the same buffer. Liposomal samples (both chitosan-coated and non-coated and sonicated for 2x2 min) or controls (clotrimazole in propylene glycol) were added in the donor chamber and the system was properly sealed. The drug content in all tested samples was determined (HPLC) prior to the study and the volumes of formulations in donor chambers normalized to assure the same drug amount. The samples (500 µl) were collected after 1, 2, 3, 4, 8 and 24 hours. The samples were replaced by an equal volume of buffer upon removal of sample from the acceptor chamber. The drug content was determined by the HPLC method. The experiments were performed in triplicates.

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183 *2.9. Preparation of vaginal tissue*

184 The sheep vaginal tissue (from pregnant animals) was obtained from the Laboratory
185 Animal Centre, University of Oulu, Finland. The vaginal tissue was carefully removed
186 from the underlying tissue and cleaned with the physiological solution (pH 7.4). Adequate
187 sized pieces were moisten by 0.9 % (w/w) NaCl and packed in clinging film, and frozen (-
188 20 °C). They were left to defrost at room temperature for at least 2 hours prior to
189 experiments. The thickness of the tissue was determined to be ranging from 900 to 1140
190 microns. It was earlier confirmed that no significant differences were observed in using
191 fresh or snap-frozen tissue samples (Sassi et al., 2004). We have earlier also compared the
192 barrier properties of the fresh vaginal tissue and frozen (-20 °C) and thawed tissue (both
193 animal skin and vaginal tissue) and have not observed any difference in the properties.

194

195 *2.10. Ex vivo penetration study*

196 The defrosted vaginal tissue was cut to fit the Franz diffusion cells surface (1.77 cm^2). The
197 acceptor chamber was filled up with phosphate buffer (pH 7.4, 8 g/L NaCl, 0.19 g/L
198 KH_2PO_4, and 2.38 g/L Na_2HPO_4) and the vaginal tissue fixed between donor and acceptor
199 chamber. Samples (600 µL) were added into the donor cells and the system was properly
200 sealed. The amount of the drug in each cell was the same, as confirmed by the HPLC
201 analysis. Samples of 500 µL were collected at 1, 2, 3, 4, 8 and 24 hours and replaced with

an equal amount of buffer. Drug content was determined by the HPLC method. The measurements were performed in triplicates.

2.11. In vitro mucin-binding test

The mucoadhesion was measured by determining the binding of liposomes to pig mucin. Liposomes (1 mL) were mixed with equal volume of pig mucin (PM) suspension (400 µg/mL) in 0.05 M PBS (pH 7.4) and incubated at room temperature for 2 hours, followed by ultracentrifugation for 1 hour, at 10 °C at 216 000 *g* (Optima LE-80; Beckman Instruments, Palo Alto, USA). Aliquots of 200 µL (4 from each sample) of the supernatants (free PM) 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). The PM binding efficiency was calculated according to Naderkhani et al. (2014).

2.12. Statistical evaluation

The student's *t*-test was used for comparison of two means. A significance level of $p<0.05$ was considered to be appropriate.

3. Results and Discussion

In order to achieve optimal therapeutic outcome, the delivery system is expected to provide a sufficient amount of the active ingredient (in our case clotrimazole) at the desired site of action (vaginal mucosal tissue) for a sufficiently long period of time to enable drug to perform its therapeutic action. Therefore, in the first step in optimization of chitosan-coated liposomes for topical vaginal delivery, we were focusing on the relationship between encapsulation yields *vs* particle size. Smaller particles are expected to provide larger surface area, however those particles carry less clotrimazole load, whereas larger particles will incorporate more of the drug, but will provide smaller contact surface for possible mucoadhesion.

3.1. Liposomal characteristics

Liposomes have been studied as drug delivery systems for almost 5 decades. A number of liposomes-based products are on the market and many more are in pipelines (Allen and Cullis, 2013). We have previously reported on the potential of liposomes to enhance the anti-inflammatory properties of associated curcumin destined for vaginal therapy (Basnet et al., 2012). Guided by those promising results, we selected liposomes as carrier for clotrimazole and used chitosan-coating to i) assure prolonged and controlled release of clotrimazole and ii) assure its retention at vaginal site, without significant penetration through the vaginal tissue.

242 Size and size distribution of liposomes are important characteristics of liposomes destined
243 for topical drug delivery. The effect of liposomal size on the efficacy of liposomal delivery
244 to skin is rather well studied (Cevc, 2004); however, relatively little is known about the
245 effect of vesicle size on the delivery of drugs intended for mucosal targeting at vaginal site.
246 Takeuchi and co-workers (2001) have found that the amount of liposomes penetrating into
247 the intestinal mucous layer increased when the size of the liposomes was reduced to
248 approximately 100 nm for both non- and chitosan-coated liposomes. In previous
249 experiments (Berginc et al., 2012) we observed that curcumin in smaller vesicles penetrated
250 less into the upper layers of vaginal tissue as compared to curcumin in mulitilammelar
251 vesicles. Both types of liposomes exhibited better tissue retention as compared to curcumin
252 in solution form. Similarly, polymer nanoparticles smaller than 200 nm were reported to
253 successfully deliver small-interfering RNA and provided sustained gene silencing
254 throughout the female reproductive tract for at least 14 days (Woodrow et al., 2009).
255 The particle size shown in Table 1 indicates that the liposomes sonicated for 2x2 minutes
256 were in the desired size range. Liposomal dispersions exhibited two distinguished peaks in
257 distributions, indicating bimodal distribution and rather high polydispersity (Table 1). With
258 the increase in sonication time, the polydispersity index values decreased, as expected
259 (Table 1).
260 We tried to minimize the exposure of liposomes to the sonication force, as it is known that
261 extensive sonication can lead to the release of originally incorporated drug and lipid
262 degradation (di Cagno et al., 2011).
263 Due to highly lipophilic nature of clotrimazole (log *P* of 3.5), clotrimazole was dissolved in
264 the organic solvent together with lipid during the preparation of liposomes and was

265 expected to incorporate itself within lipid bilayers of liposomes. We observed similar
266 entrapment efficiencies for liposomes sonicated for 1 and 2 minutes, and the loss of
267 originally entrapped clotrimazole was remarkable only after sonication for 2x2 minutes,
268 resulting in smaller liposomes (Table 1). In comparison to literature data, our entrapment
269 (up to 22 μg clotrimazole/mg lipid) was found to be lower; however none of the published
270 articles describes the use of the same liposome preparation method and phospholipid
271 composition. Although Ning et al. (2005) reported a very high entrapment of clotrimazole
272 (over 90 %) using the film method in preparation of liposomes, they used dialysis to
273 separate unentrapped from liposomal drug. The fact that clotrimazole has aqueous
274 solubility of only 5.5 μmol/L (Mw 344) (Bilensoy et al, 2006), and that no data is available
275 on whether or not the sink conditions were assured in the experimental set up of Ning and
276 coworkers (2005), it might be possible that part of the drug was accumulated in the
277 dialysis-tube in a form of precipitates, falsely contributing to high entrapment values.
278 Liposomes prepared by the proliposome and polyol dilution methods were reported to
279 incorporate more clotrimazole (Pavelić et al., 1999; Pavelić et al., 2005); however, the
280 methods of preparations differed from the method used in our experiments, and, more
281 importantly, the vesicle size was larger than in our case. Proliposome method is known to
282 yield multilamellar liposomes thus enabling high incorporation of lipophilic drug (Pavelić
283 et al., 1999). An additional difference between the previous and current experiments was
284 the liposomal composition (Pavelić et al., 2005). We choose to prepare liposomes from a
285 simple lipid mixture to be able to follow the effect of chitosan coating in a rather
286 straightforward manner, avoiding the interference of possible ionic interactions between
287 lipid and chitosan and consequently mucin.

The coating of liposomes is expected to result in an increase in their original size (Filipović-Grčić et al., 2001; Karn et al., 2011). Although the entrapment of clotrimazole was lower than we have desired, considering that the obtained vesicle size was in the optimal range for the purpose of development of mucoadhesive liposomal delivery system, we proceeded with coating of those vesicles. Prolonged retention time would be a rationale for lowering the dose needed to induce antifungal effect; therefore lower drug load should not limit the applicability of the system.

3.2. Coating of liposomes

It is well established that the polymer concentration significantly influences the strength of mucoadhesion. Moreover, the optimal polymer concentration depends on the physical state of the delivery system (Andrews et al., 2009) and, in the case of coated liposomes; the liquid nature of the system needs to be taken into consideration. Therefore, we have used three different concentrations in the coating of liposomes, all resulting in liquid formulations. The coating resulted in an increase in particle size (Table 2), in agreement with the results reported by Karn and colleagues (2011) and Gradauer and coworkers (2012). The coating of liposomes with chitosan also resulted in an increase of liposome zeta potential (Table 2) in agreement with Berginc et al. (2014). Moreover, the pH of liposomal suspensions changed upon coating; non-coated liposomal suspensions had a pH of 6.0 whereas 0.1, 0.3 and 0.6 % coated liposomal suspensions had a pH of 4.12, 4.02 and 4.07, respectively. This would represent additional advantage of chitosan-coated liposomes; it is well known that *C. albicans* adheres to vaginal tissue with higher affinity at pH 6 than at

311 pH 4 and that acidic formulations also restore the physiological acid pH of vagina (Chang
312 et al., 2002).
313 It was previously reported that the amount of polymer used for coating of nanoparticles and
314 found on vesicle surface was similar regardless of the molecular weight of chitosan used
315 (Llabot et al., 2011). However, nanoparticles coated with low molecular weight chitosan
316 where found to be more bioadhesive than those coated with high molecular weight
317 chitosan. This could be explained by the interpenetration mechanisms with the mucin
318 chains, as it is known that long polymer chains reduce the interpenetration, reducing the
319 bioadhesive strength (LLabot, 2011). This was the reason why we have selected low Mw
320 chitosan as a coating material.
321
322 Although there is no consensus on the optimal size range of nanocarriers expected to
323 penetrate mucus layer, it was suggested that the nanocarriers in the size range of 200-500
324 nm are superior to both much smaller and also larger nanosystems (das Nevas et al.,
325 2011b). Takeuchi et al. (2005) confirmed the superiority of nanosize chitosan-coated
326 liposomes in prolonging the retention time in the intestinal mucosa as compared to larger
327 vesicles. However, the vaginal mucosa has unique features which make direct translation of
328 the results rather difficult.
329
330 Regarding the optimal size, it is also important to consider that some of the particles tend to
331 agglomerate, and that agglomerates behave in a different manner than single particles (das
332 Nevas et al, 2011a). We have tested our liposomes for one month stability (at 4 °C) and
333 have not observed significant increase in the original size of chitosan-coated vesicles (data

not shown). It can be hypothesized that the chitosan-coating is working as a stabilizer by prohibiting agglomeration of the liposomes, even though the chitosan is not bound to the lipid surface either by covalent or ionic bonds. Although liposomes made of phosphatidylcholine have no surface charge, the electrostatically driven binding of chitosan to the lipid membrane is energetically favoured, even for neutral liposomes, leading to further stabilization of the vesicle suspension, as reported recently (Mertins et al., 2010; Mertins et al., 2011).

3.3. In vitro release of liposomally-associated clotrimazole

There are several means to achieve the prolonged release of drugs destined for vaginal administration. Most of the approaches rely on the use of polymer in a form of hydrogel, to assure both prolonged release and intimate contact between drug and vaginal mucosa, simultaneously using liposomes as solubilizers for poorly soluble drugs (Pavelić et al., 2005). Other approaches use the complexation of drug with cyclodextrine, followed by incorporation of a complex in a hydrogel, such as for example Pluronic thermosensitive gel, exhibiting prolonged release of clotrimazole when the drug was complexed with β-cyclodextrine prior to inclusion into the hydrogels (Bilensoy et al., 2006).

We have followed the release of liposomally-associated clotrimazole (Figure1) in comparison to clotrimazole in a free form (propylene glycol as vehicle). All liposomal formulations exhibited prolonged release as compared to free drug (control). All formulations, even the control, also failed to release all of the incorporated clotrimazole, which may be explained by the fact that clotrimazole, a very hydrophobic molecule, has very limited solubility in water. In the case of the control (propylene glycol), an osmotic

357 effect was observed drawing water from the acceptor medium into the sample in donor
358 chamber. As a consequence, clotrimazole started to precipitate (in donor chamber) when a
359 critical amount of drug dissolved in propylene glycol is mixed with water. Therefore, we
360 assume that it would be necessary to add some sort of the solubilizing agent into the
361 acceptor medium (acceptor chamber) to secure sink conditions. The limitation of Franz
362 diffusion system is the limited volume which can be used in the acceptor chamber, causing
363 the problems for the poorly soluble drugs. However, we could detect the differences
364 between control (free drug) and the drug released from liposomes. Although we have
365 expected the slowest release from chitosan-coated liposomes, interestingly, the slowest
366 release of the drug was perceived from non-coated (plain) liposomes ($p<0.05$). Non-coated
367 liposomes act as solubilizer for clotrimazole in the lipid membrane and drug only diffuses
368 from the lipid membrane as liposomes become leaky, if not coated. It seems that the
369 partitioning of clotrimazole between outer aqueous medium and liposomal bilayers is in
370 favour of liposomal bilayers, whereas in coated liposomes the release is supported by the
371 presence of chitosan coating. We have also observed the difference in the release from
372 liposomes coated with three different concentrations of chitosan; those coated with higher
373 concentration of chitosan exhibited more pronounced sustained release, however not on a
374 significant level. Chitosan is hydrophilic in nature and makes the surface of liposomes less
375 hydrophobic, as in chitosan-coated liposomes. The thicker coatings (0.3 and 0.6 %, w/v,
376 respectively) will cause the diffusion obstacle for the drug released from the surface,
377 resulting in the slower release. Berginc et al. (2014) reported that increasing the amount of
378 chitosan in the liposomal coating had no beneficial effect on the permeability of
379 liposomally-associated curcumin. This is very interesting, and could be relevant for

different types of mucoadhesive coatings on the surface of various nanoparticles and requires further studies. This finding is in agreement with report by Fang and colleagues (2001) who proposed that even the lowest chitosan mole fraction is able to reduce the cooperative unit of the DPPC bilayer and lead to certain degree of membrane bilayer perturbation. Reduction of pH increased the number of protonated amines on the chitosan backbone and caused further disruption on the membrane organization. Although we have used different lipid in our experiments, the interactions between chitosan and polar head of phospholipids are well established (Mertins et al., 2010, 2011). Whether the observation we made for chitosan-coated liposomes incorporating clotrimazole would also apply for liposomally-entrapped hydrophilic drugs and for liposomes of various phospholipid compositions remains to be determined.

3.4. Ex vivo penetration of liposomally-associated clotrimazole

The mucoadhesive properties of chitosan are mostly result of its cationic character. Mucoadhesion of chitosan-based delivery systems can therefore be achieved through ionic interactions between the cationic primary amino groups of chitosan and the anionic substructures of the mucus. In addition, the hydrophobic interactions might contribute to its mucoadhesive properties (Bernkop-Schnürch and Dünnhaupt 2012). Although thiolated chitosans have stronger mucoadhesive properties than non-modified chitosan, their compatibility with vaginal environment remains to be proven, and we have therefore opted for non-modified chitosan as a coating material. Chitosans of high degree of deacetylation and of a high molecular mass were reported to cause an increase in the epithelial permeability (Bernkop-Schnürch and Dünnhaupt, 2012), which needs to be taken into

403 consideration when choosing the type of chitosan for the coating of liposomes intended for
404 administration into pregnant patients, and was the reason that we selected low molecular
405 weight chitosan. We are currently evaluating the system in the *in vivo* conditions in
406 pregnant sheep to confirm the safety and non-irritability of the system, as it is well-known
407 that vaginal irritation can lead to increased susceptibility to foreign pathogens and
408 inflammation.
409 Mucus is a viscous coating on many epithelial surfaces and consists mainly of water (up to
410 95 % weight), inorganic salts, carbohydrates, lipids and glycoproteins, termed mucins.
411 Mucins are hydrosoluble and responsible for the gel-like properties of the mucus (Serra et
412 al., 2009). In order for mucoadhesion to take place, the wetting and swelling of polymer
413 should enable an intimate contact with the mucosal tissue, followed by interpenetration of
414 the polymer chains and entanglement between the polymer and mucin chains. Chitosan
415 exhibits strong bioadhesive properties through the electrostatic interactions with sialic
416 groups in mucins of the mucosal layer. The high positive charge density of chitosan
417 enhances its mucoadhesiveness (Meng et al., 2011). It is also important to consider the
418 concentration of liposomes applied to vaginal tissue, as this may affect the normal structure
419 of mucus and cause the collapsing of mucin fibres (das Nevas et al., 2011b). The
420 concentration of liposomes used in our experiments was low and not expected to cause
421 changes in mucosal structure and was considered to be safe. However, it remains to be
422 evaluated in the *in vivo* studies in suitable animal model.
423
424 Although the mucoadhesive behaviour of bulk material such as polymer-based hydrogels
425 made of chitosan for example is well characterized, rather little is known about the

behaviour of chitosan at the nanoscale (das Nevas et al., 2011b). We have previously confirmed the bioadhesion potential of chitosan-based hydrogels onto the skin (Hurler and Skalko-Basnet, 2012); however, the bioadhesiveness of chitosan-coated liposomes in nanosize range cannot be directly compared to hydrogels.

We have used the vaginal tissues of pregnant sheep to mimic closer the *in vivo* evaluation of our formulation in pregnant animals. The results presented in Figure 2 indicate that liposomes are able to retain associated clotrimazole on the vaginal tissue and in the tissue, thus preventing undesired penetration through the vaginal tissue. This is of great importance considering clotrimazole therapy in pregnant patients. The free drug, a form of propylene glycol solution, penetrated through vaginal tissue in remarkable manner (almost 40 % after 24 hours), whereas the liposomally-associated drug penetrated to significantly lower extent ($p<0.01$). We are aware that propylene glycol is a known skin penetration enhancer and acts as a carrier-solvent for poorly soluble substances such as clotrimazole. Moreover, propylene glycol may induce the osmotic effects which result in the changed barrier properties of the tissue. However, due to a very low solubility of clotrimazole, the choice of a solvent which we could use to prepare clotrimazole solution was very limited. Most of the other solvents are expected to directly damage the barrier properties of the vaginal tissue, resulting in the even higher penetration.

In respect to the drug retention on and within vaginal tissue, It appears that liposomes coated with 0.1 % chitosan solution exhibited lowest penetration of clotrimazole and highest amount of clotrimazole retained on top of the tissue (Figure 2), both in comparison to plain, non-coated liposomes and liposomes coated with different polymer concentrations.

449 One has to consider that the increased retention time on the target site would outweigh
450 more sustained release from non-coated liposomes (Figure 1), as reported for mucoadhesive
451 liposomes incorporating curcumin (Berginc et al., 2014).
452 The vulovaginal candidosis is characterized by the infection reaching the deeper epithelial
453 layers (das Nevas et al., 2008); thus prolonged retention time on the vaginal mucus would
454 be beneficial. To confirm that chitosan coating on liposomal surface is available for close
455 interaction with mucin, we tested non-coated and coated liposomes for mucin-binding
456 potential (Figure 3). The results clearly indicate ($p < 0.01$) that liposomes coated with 0.1 %
457 polymer concentration exhibit superior interaction with mucin in comparison to all other
458 formulations. This indicates that they exhibit potential to retain at the vaginal site. This type
459 of liposomes may improve the effectiveness of model drug, yet prevent the undesired
460 systemic absorption.
461

462 It is expected that lipophilic substances/drugs are absorbed from vagina as administration
463 site through the transcellular pathway (Sassi et al., 2004). The passive diffusion was found
464 to be the main mechanism of curcumin penetration into vaginal mucosa when liposomally-
465 associated curcumin was tested in the *ex vivo* conditions. Our current findings are in full
466 agreement. Depending on the liposomal size, the concentration of curcumin in different
467 layers of vaginal tissue was found to be significantly higher as compared to concentration
468 of curcumin applied in a form of solution. The permeability from 0.6 % chitosan-coated
469 liposomes was found to be similar or even lower than from 0.1 % coated liposomes when
470 tested in artificial and isolated bovine mucus. This can be explained by the fact that an

increase in the liposomal size, as well as changes in zeta potential, lead to the major part of the polymer being hindered in the deeper layers and unavailable for immediate adhesion. Only when the uppermost polymer sheets of higher polymer concentrations coatings are removed by erosion or by detachment from mucus, more chitosan becomes available for adhesion. This was proposed as an explanation for the superiority of liposomes coated with lower concentration of polymer (Berginc et al., 2014).In addition, liposomal curcumin administered vaginally exhibited negligible potential for systemic absorption, which would greatly support its administration in pregnant patients (Berginc et al., 2012) in agreement with our findings.

Currently, there is a vivid discussion in the field of vaginal drug delivery whether the mucoadhesiveness of delivery system is advantageous for improved drug therapy or rather disadvantageous (das Neves et al., 2011a). The physical properties of mucus are complex and often described as non-Newtonian behaviour with properties between those of a viscous liquid and an elastic solid. In women with bacterial vaginosis, the viscosity of vaginal fluid is reduced, leading to increased risk of infections and reduced barrier properties of vaginal mucus (Lai et al., 2009). Nevertheless, there is a consensus that the nanocarrier should first be retained at the vaginal site to increase the residence time and avoid vaginal leakage, and subsequently migrate through mucus towards the mucosal surface (das Neves et al., 2012). Antifungal agents used to treat vaginal candidosis need to penetrate deep into the epithelium to reach invasive *Candida* hyphae and exert a local antifungal action (das Neves et al., 2008). One approach to achieve this purpose has been

493 recently suggested by our groups by using deformable propylene glycol liposomes (Vanić
494 et al., 2014). Another approach is based on applying chitosan-based mucoadhesive
495 liposomes developed in this study.
496 Our findings clearly indicate that liposomes are a suitable drug delivery system in respect to
497 both prolonged release and limited tissue penetration. Liposomes are able to assure
498 sustained release of associated drug either with or without chitosan coating. However,
499 coated liposomes are expected to prolong the residence time in the vaginal cavity in the *in*
500 *vivo* conditions and are therefore, considered to be superior. The next step involves the
501 determination of bioadhesiveness of the system by the modified method originally
502 developed for skin (Hurler et al., 2012).
503 We are aware of a need to address current limitations of experimental set up, such as that
504 our experiments were performed on the vaginal tissue in the absence of cervicovaginal
505 fluid. The reason that we did not use cervicovaginal fluid surrogate, is that it was reported
506 that differences between the surrogate and native mucus may be noticeable. We also did not
507 vary the pH of the donor medium, although it is known that the pH is affecting the transport
508 across mucus (das Neves et al., 2012). The effect of semen and the changes in vaginal pH
509 related to age and disease conditions remain to be evaluated.

510

511 **4. Conclusions**

512 Chitosan-coated liposomes were shown to exhibit prolonged release of associated
513 clotrimazole. The penetration of liposomally-associated clotrimazole through the vaginal
514 tissue was reduced as compared to non-coated liposomes, an important fact regarding

system's potential in topical vaginal therapy, especially in pregnant patients. We are currently evaluating the system in the *in vivo* conditions in pregnant sheep to confirm the safety and non-irritability of the system as it is well-known that vaginal irritation can lead to increased susceptibility to foreign pathogens and inflammation.

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List of Tables and Figures

Tables:

Figures with legends

The concentration of clotrimazole in all formulations was the same. Control (*) contained clotrimazole in propylene glycol; non-coated liposomes (**) were diluted to obtain the same concentration of clotrimazole as in coated liposomes.

The concentration of clotrimazole was the same in all formulations. Control contained clotrimazole in propylene glycol; non-coated liposomes were diluted to obtain the same concentration of clotrimazole as in coated liposomes. Liposomal formulations assured significantly less ($p < 0.01$) drug penetration as compared to the control.

678

679 **Figure 3. Mucin-binding (PM) efficacy for non-coated and chitosan-coated liposomes**

680 **(n=3)**

681

682

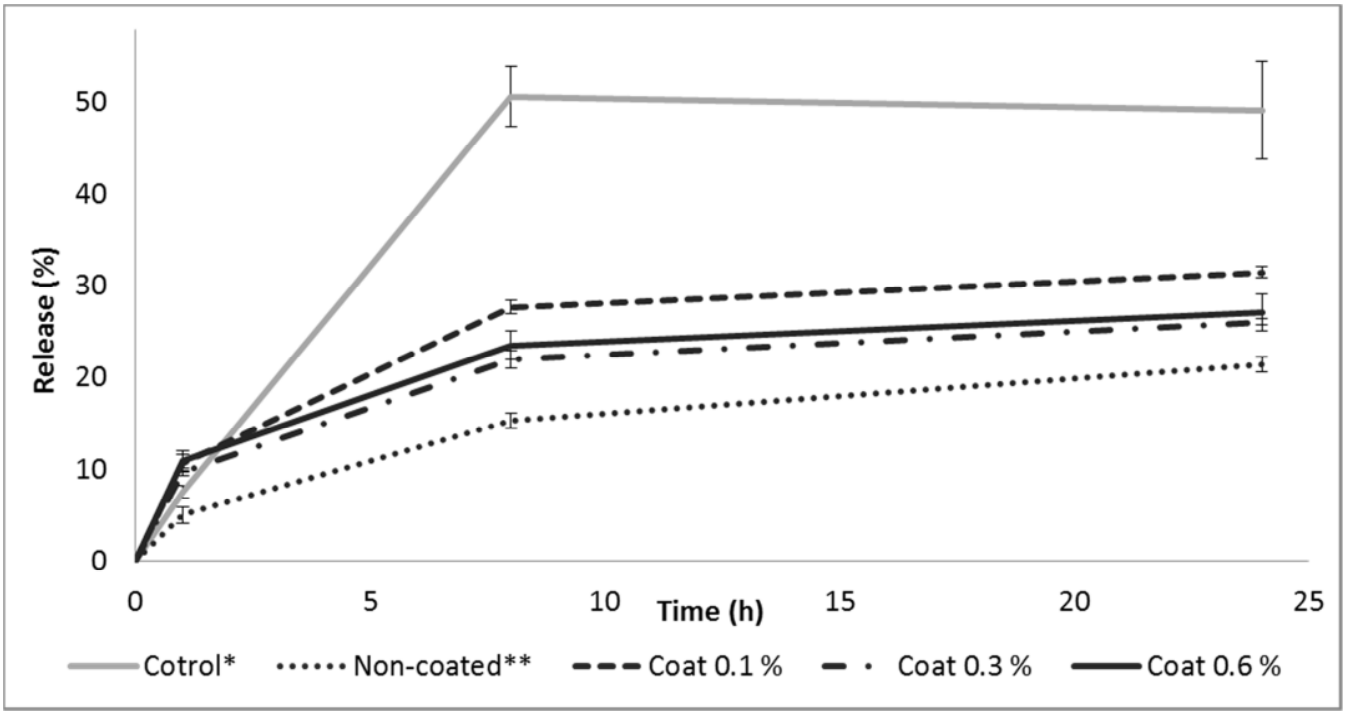
683

Table 1: Liposomal characteristics (n=3)

Time of sonication (min)	Vesicle size				PI	Entrapment (%)
	Peak 1 (nm)	Weight intensity (%)	Peak 2 (nm)	Weight intensity (%)		
1	317 ± 47	56.8	40 ± 5	41.0	0.58	23.2 ± 2.5
2	234 ± 31	53.9	36 ± 5	43.8	0.46	25.0 ± 0.5
2x2	111 ± 16	83.1	29 ± 4	16.9	0.46	16.5 ± 4.5

Table 2: The effect of chitosan-coating on liposomal size distribution and zeta potential (n=3)

Coating (%, w/v)	Vesicle size				PI	Zeta potential (mV)
	Peak 1 (nm)	Weight intensity (%)	Peak 2 (nm)	Weight intensity (%)		
-	107 ± 3	54 ± 3	27 ± 3	46 ± 3	0.34	- 1.6 ± 0.2
0.1	135 ± 21	53 ± 6	42 ± 9	45 ± 5	0.29	25.9 ± 4.0
0.3	141 ± 6	64 ± 6	48 ± 5	35 ± 6	0.27	35.6 ± 1.9
0.6	190 ± 8	58 ± 6	54 ± 2	42 ± 6	0.29	43.8 ± 3.3



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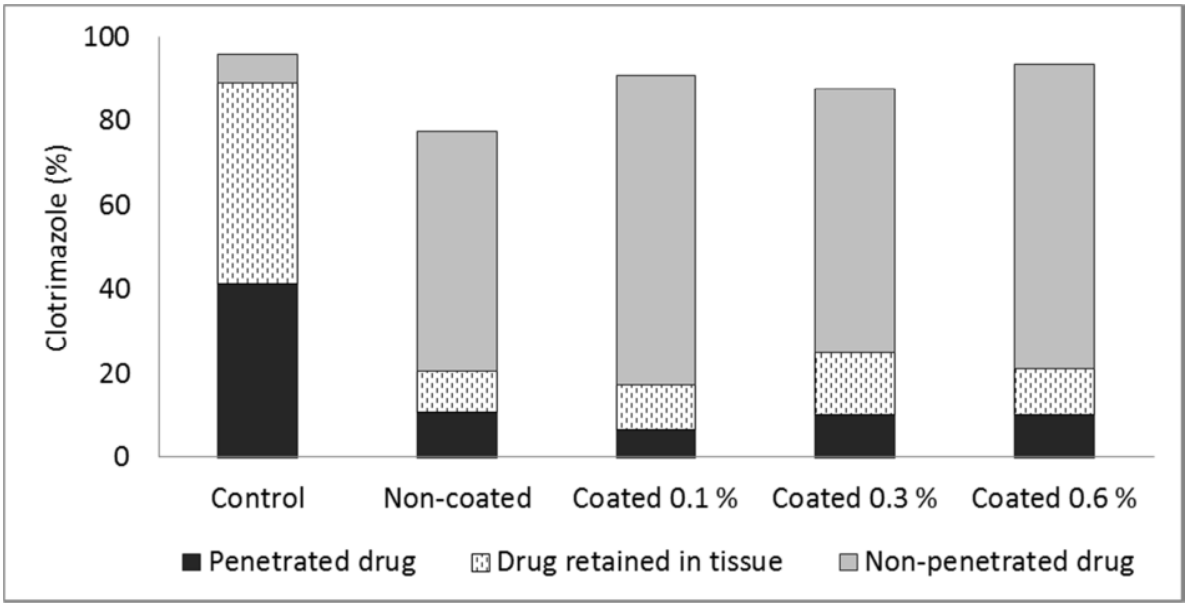

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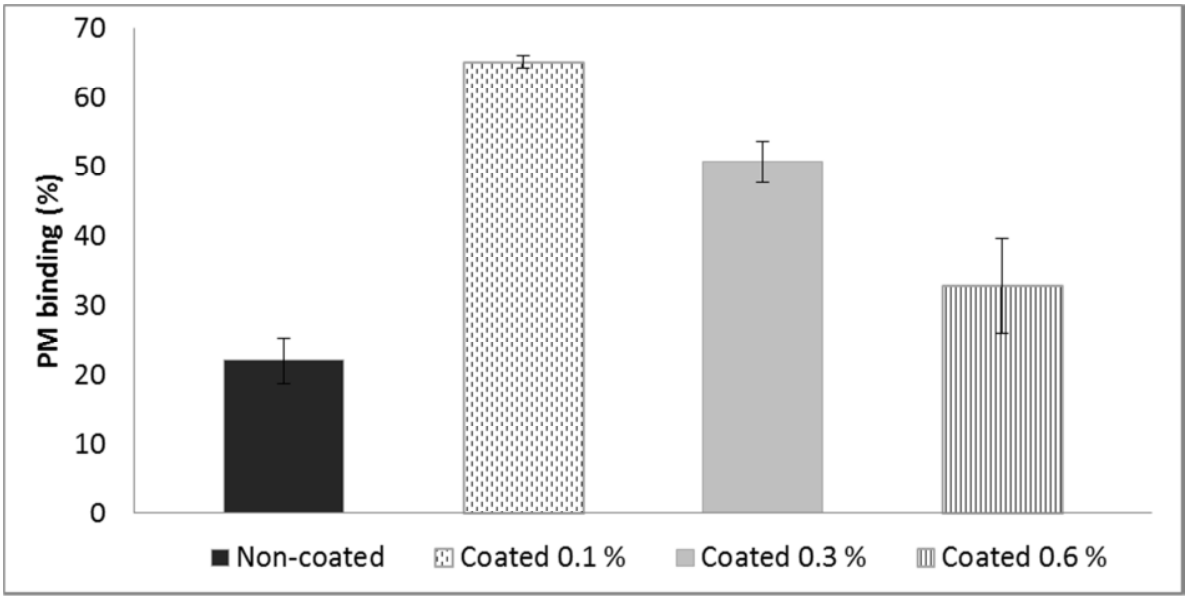
70
60
50
40
30
20
10
0
PM binding (%)
Non-coated
Coated 0.1 %
Coated 0.3 %
Coated 0.6 %

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