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

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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.

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

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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 the 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 (Hussain and Ahsan, 2005).

As a model drug we selected clotrimazole, often prescribed in vulovaginal candidiasis. 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

2.1. Materials

Lipoid S 100 (PC, soybean lecithin, >94% phosphatidylcholine) was a generous gift from Lipoid GmbH, Ludwigshafen, Germany. Chitosan, low molecular weight (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.

2.2. 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 h 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.

2.3. 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 W, Sigma–Aldrich, St. Louis, MO, USA) was set to 40% amplitude and the liposomes were exposed to ultrasonic irradiation for 1, 2 or 2 × 2 min, respectively. The sonicated liposomes were stored in the refrigerator for at least 6 h prior to further use.

2.4. Particle size analysis

The particle size distributions of liposomes were determined by photon correlation spectroscopy (Submicron particle sizer model 370, Nicomp, Santa Barbara, CA, 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–weight distribution at 23 °C. Three parallels were determined (run time 10 min) for each sample measurement.

2.5. 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.

2.6. 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, CA, USA) for 30 min, 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 5 μm, 3.9 × 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 min, then to 100% A after 11 min. 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.

2.7. 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, VA, USA) was applied in the method described earlier (Basnet et al., 2012). Measurements were performed in triplicates.

2.8. 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,