

Gel s liposomski uklopljenim kloramfenikolom: karakterizacija i oslobađanje in vitro

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Liposomal gel with chloramphenicol: Characterisation and *in vitro* release

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The aim of our study was to develop a liposomal carrier system for the local treatment of bacterial vaginosis, capable to efficiently deliver entrapped drug during an extended period of time. Chloramphenicol was entrapped in liposomes composed of egg phosphatidylcholine/egg phosphatidylglycerol-sodium (9:1, molar ratio) and prepared by two different methods, the proliposome method and the polyol dilution method. Both liposome preparations were characterised and compared for particle size, polydispersity, entrapment efficiency and tested for *in vitro* stability in media that simulate human vaginal conditions (buffer pH 4.5 and vaginal fluid simulant). To achieve application viscosity of liposomes and to further improve their stability, liposomes prepared by the proliposome method were incorporated in the bioadhesive gel made of Carbopol 974P NF resin. *In vitro* release studies of liposomes incorporated in the gel have shown a prolonged release of entrapped chloramphenicol compared to control gel. Even after 24 hours of incubation in the vaginal fluid simulant, more than 40% of the originally entrapped drug was still retained in the gel. Storage stability studies have proven the ability of the Carbopol 974P NF gel to preserve the original size distribution of incorporated liposomes.

All the performed experiments confirm the applicability of liposomes as a novel drug carrier system for the local treatment of bacterial vaginosis.

Keywords: liposomes, chloramphenicol, bioadhesive gel, stability, vaginal application

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Novel vaginal delivery systems for local therapy as well as systemic administration of peptides and proteins have been extensively investigated over the last decade. Advantages of the vaginal route of drug administration include: avoidance of hepatic first pass metabolism, a reduction in the incidence and severity of gastrointestinal and/or hepatic side effects, easiness of application and complete privacy of the therapy for women.

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However, the currently available vaginal drug delivery systems (*e.g.*, foams, creams, gels and tablets) have some limitations, such as leakage, messiness and low residence time, which contribute to poor subject or patient compliance. Furthermore, intravaginal route of drug administration may lead to a systemic rather than a localised effect due to the remarkable absorption of the drug from the vaginal wall (1, 2). For that reason, attempts are being made to develop novel vaginal drug delivery systems that can provide prolonged and/or controlled release of drugs.

Liposomes are well established as drug carriers in topical treatment of diseases, especially in dermatology. They can enhance penetration of encapsulated hydrophilic drugs into the skin to enable a proper therapeutic effect (3). Because of their ability to provide sustained release of the incorporated drug, liposomes also have the potential to be applied vaginally (4, 5).

One of the most common infectious gynaecological diseases is bacterial vaginosis. It is a major cause of vaginitis, which is characterised by replacement of normal lactobacilli-dominated microflora with a mixed flora containing *Gardnerella vaginalis*, anaerobic bacteria and *Mycoplasma hominis* (6). Since bacterial vaginosis is a localised syndrome with no apparent inflammation of the vaginal epithelium, topical antimicrobial treatment provides an acceptable alternative to the systemic use (7). Local therapy of vaginitis generally includes administration of antibiotics and/or antifungal agents. The antibiotic chloramphenicol (CHL) is commercially available as a cream for local therapy, but only in dermatology and ophthalmology (8). Due to the broad spectrum of activity of the drug against Gram-positive as well as Gram-negative bacteria, it is reasonable to consider CHL application for the local vaginal treatment.

Previous studies have proven that application of liposomes containing CHL is possible in gynaecology (5). Continuing that research, here we report the development of a bioadhesive liposomal gel containing CHL for the local therapy of bacterial vaginosis. Liposomes composed of egg phosphatidylcholine (EPC) and egg phosphatidylglycerol-sodium (EPG) in the molar ratio 9:1 were prepared by the proliposome and the polyol dilution methods and incorporated in the bioadhesive gel. To be closer to the actual *in vivo* application in women, liposomal gels were tested in media simulating human vaginal conditions.

EXPERIMENTAL

Reagents

Egg phosphatidylcholine (EPC), egg phosphatidylglycerol-sodium (EPG) and Carbopol 974P NF were generous gifts from Lipoid GmbH (Germany) and BFGoodrich (Belgium), respectively. Chloramphenicol, bovine serum albumin and lactic acid were purchased from Sigma Chemicals (Germany).

Phosphate buffer, pH 7.4, was composed of 137 mmol L⁻¹ NaCl, 1.4 mmol L⁻¹ KH₂PO₄ and 16.7 mmol L⁻¹ Na₂HPO₄. Phosphate buffer, pH 4.5 was made of 100 mmol L⁻¹ KH₂PO₄ in demineralised water.

Vaginal fluid simulant (VFS) was prepared from 60 mmol L⁻¹ NaCl, 25 mmol L⁻¹ KOH, 3 mmol L⁻¹ Ca(OH)₂, 22.2 mmol L⁻¹ lactic acid, 16.7 mmol L⁻¹ acetic acid, 1.7 mmol L⁻¹ glycerol, 6.7 mmol L⁻¹ urea, 27.8 mmol L⁻¹ glucose. Bovine serum albumin was added in a concentration of 0.018 g L⁻¹. pH of the mixture was adjusted to 4.5 using 0.1 mol L⁻¹ HCl (2).

All chemicals used in the experiments were of analytical grade.

Preparation of liposomes

Liposomes containing CHL were prepared by two methods, the proliposome method (9) and the polyol dilution method (10). Concentrations of lipids as well as of the drug were the same for each preparation and were of 26 mmol L⁻¹ (total lipid) or 15 mmol L⁻¹ (CHL), respectively.

Proliposome method. – EPC (180 mg), EPG (20 mg), CHL (50 mg) were mixed with warm ethanol (160 mg) and the phosphate buffer pH 7.4 (400 mg) to achieve an initial proliposome mixture. The mixture was stirred for 2 minutes at 60 °C, cooled to room temperature and then converted to a liposome suspension by dropwise addition of 10 mL of the buffer, pH 7.4. During the last stage of this procedure, the suspension was stirred continuously (600 rpm) for 60 minutes.

Polyol dilution method. – EPC and EPG (molar ratio 9:1) and CHL were dissolved in propylene glycol (500 mg) at 60 °C, and 500 mg of phosphate buffer, pH 7.4 (previously warmed to the same temperature) was poured into the lipid-drug-polyol solution. The suspension was diluted by the addition of a buffer, pH 7.4 (10 mL) under continuous stirring (600 rpm) at 60 °C for 45 minutes and then cooled to room temperature.

Finally, both liposome suspensions were extruded three times through 0.4- μ m polycarbonate membrane filters (LiposoFast, Canada).

Lipid concentration was determined by quantification of inorganic phosphate using the method of Bartlett (11).

Entrapment efficiency determination

Untrapped (free) CHL was separated from liposomal CHL by gel chromatography on the Sepharose CL 4B (Pharmacia, Sweden) column using a phosphate buffer, pH 7.4, for elution. The concentration of the drug (both in liposomes and free) was determined in all collected fractions spectrophotometrically ($\lambda = 276$ nm) (5).

The recovery of CHL for all preparations was between 90.1–97.9% of the total amount involved in the procedure of liposomes preparation.

Size measurements

Morphology, size distribution and mean diameters of the liposomes were determined by image analysis microscopy (12). Morphology and size distribution of vesicles (based on the number of particles) were determined using an Olympus BH-2 microscope equipped with a computer-controlled image analysis system (Optomax V, Ai Cambridge Ltd., UK). A microscopic field was scanned with a video camera and was digitised or

broken into electronically defined individual picture elements. A representative sample of approximately 10000 liposomes was measured.

Also, the mean diameter, as well as the polydispersity index and zeta potential of the liposomes were determined by photon correlation spectroscopy, PCS (Zetamaster, Malvern Instruments, UK) 24 hours after their preparation (13).

Gel preparation

As a vehicle for incorporation of liposomes for vaginal delivery, a bioadhesive gel was made (13). Carbopol 974P NF (1 g) was dispersed in demineralised water (88 g) by stirring at 800 rpm for 60 minutes. Then, propylene glycol (10 g) was added and the mixture was neutralised by dropwise addition of 10% NaOH. Mixing was continued until a transparent gel appeared, while the amount of the base was adjusted to achieve a gel with pH 5.5.

Incorporation of liposomes into the gel

A previously described procedure (13) was applied. Liposomes containing CHL (separated from the untrapped drug) were mixed into the 1% (*m/m*) Carbopol gel with an electrical mixer (25 rpm, 2 min), the concentration of liposomes in the gel being 10% (*m/m*, liposome suspension/total). Control gels (10%, *m/m*) were made under the same conditions. Instead of liposomes, those samples contained free CHL.

In vitro stability studies

Liposome preparations (both suspensions and gels) were tested for *in vitro* stability in the phosphate buffer, pH 4.5. To be closer to human conditions, the same experiments were performed in the vaginal fluid simulant (VFS), pH 4.5.

Liposome suspensions. – The method reported by Pavelić *et al.* (5) was used. Liposomes (1 mL), separated from untrapped CHL, were dispersed in the buffer, pH 4.5 (5 mL), and incubated at 37 °C using a water bath. Samples were taken at certain time intervals (1, 2, 4, 6 and 24 hours), separated from the released drug and the amount of the drug was determined spectrophotometrically (as described earlier).

Liposomal gels. – The procedure modified by Peschka *et al.* (14) was applied. Samples of gel containing liposomal CHL (3 g) were put in glass vials and were separated from the receptor solution (phosphate buffer, pH 4.5 or VFS) by a layer of 2% agarose gel. The vials were incubated at 37 °C and the receptor solution was completely removed at certain time intervals (1, 2, 4, 6 and 24 hours). The amount of the released drug was determined spectrophotometrically before and after the addition of methanol.

Control experiments (Carbopol gels with free CHL) were done simultaneously and under the same conditions.

Storage stability study

Samples of gel with liposomes containing CHL were stored at 20 and 40 °C for 4 weeks. The physical stability of the incorporated liposomes was determined by image

analysis microscopy (as described above). In all experiments, approximately 5000 liposomes were examined.

RESULTS AND DISCUSSION

Several novel carrier systems were suggested to be appropriate for vaginal drug delivery, such as polycarbophilic gel (15, 16), bioadhesive tablets (6), microspheres (17) and liposomes (4, 5, 18). To achieve the desirable therapeutic effect of liposomes as drug carriers, they must be loaded with a sufficient amount of active compound. Therefore, liposomes with CHL were prepared by two methods and compared for entrapment efficiency, vesicle size and polydispersity (Table I). Regardless of the preparation procedure used, liposomes were of a mean diameter between 310–330 nm. There was no statistically significant difference (*t*-test, $p > 0.05$) between average diameters of liposomes estimated by different measuring technique, image analysis and PCS, or between proliposomes and PD liposomes. However, liposomes prepared by the polyol dilution method (PD liposomes) were of a more homogenous size distribution than liposomes prepared by the proliposome method (proliposomes). This was probably a consequence of the preparation procedure; in the better case, the proliposome mixture was converted to a stable liposome dispersion by dilution of the excess aqueous phase (9). Even after the extrusion was performed through 400-nm pore size polycarbonate membranes, a small amount of residual proliposome mixture remained in the final liposome dispersion, affecting the polydispersity index (0.198) in comparison to the polyol dilution liposomes (0.170). Since the same lipid composition was used for the preparation of liposomes, the zeta potential values were quite similar for both preparations (about -55 mV). Considering the entrapment of CHL, a marginally better entrapment of the drug was obtained in PD liposomes (30%) than in proliposomes (25%). Similar values obtained in the previous study, where liposomes were composed of EPC only (5), indicate that the surface charge of vesicles has no influence on the entrapment of CHL.

The next step in the investigation was to determine the *in vitro* stability of liposome dispersions with CHL in media that mimic human vaginal conditions. Since normal vaginal pH of healthy women is usually between pH 4.0 and 5.0 (19), experiments were

Table I. Characterisation of liposomes containing CHL

Parameter	Proliposomes	PD liposomes
Mean diameter (nm)	315.0 ± 3.5 ^a 325.3 ± 4.5 ^b	309.1 ± 3.6 ^a 317.5 ± 6.3 ^b
Polydispersity index	0.198 ± 0.029	0.170 ± 0.023
Zeta potential (mV)	-54.2 ± 0.8	-55.1 ± 0.6
Entrapment efficiency (%)	24.8 ± 3.1	30.2 ± 4.1

The values denote the mean ± SD ($n = 3$).

^a Determined by image analysis microscopy.

^b Determined by PCS.

performed in the buffer of pH 4.5. As can be seen in Fig. 1, liposomes were stable in the buffer of pH 7.4 (control). In the presence of a low pH, liposomes were less stable. After 1 hour of incubation at 37 °C, PD liposomes released almost 43% of the originally entrapped drug. During the following hours of testing, the release rate was much slower and after 24 hours about 30% of the drug was still present in PD liposomes. Similar behaviour was observed when proliposomes were incubated at the buffer pH 4.5; fast release of the drug in the first 60 minutes and a slower release during the following 23 hours. Comparison of the two preparation methods has shown proliposomes to be more stable; after 24 hours of incubation at pH 4.5 about 40% of originally entrapped CHL was retained in the liposomes (Fig. 1). Relatively fast release of the entrapped CHL from liposomes at pH 4.5 is probably a consequence of the pK_a value of the drug (20). Further investigations into the interaction of the CHL with membrane bilayer would be valuable to explain the lower association rate of the CHL than expected for lipophilic drugs.

In previous experiments performed with hydrophilic model substances of different molecular mass: [calcein, M_r 622.5 (13), FITC-dextran, M_r 4400, and FITC-dextran, M_r 21200 (21)], at the buffer pH 4.5, liposomes prepared by both methods could retain a higher amount of originally entrapped marker (*e.g.*, more than 60% calcein or even more than 80% FITC-dextran 21200 remained in PD liposomes after 24 hours). Comparison of those findings with the results obtained with CHL, demonstrate a slower release of water-soluble compounds. That could be explained by the hydrophilic nature of calcein at neutral pH in liposomes. Therefore, penetration of calcein across the membrane is very slow and liposomes could retain a higher amount of originally entrapped marker. In the case of FITC-dextran, even slower release in the buffer of pH 4.5 than in the study with calcein is probably a consequence of steric effects due to the higher molecular mass of encapsulated compounds.

To be closer to the application of liposomes in humans, an appropriate viscosity of liposomal preparations is required. This can be achieved by their incorporation in a vehicle suitable for vaginal self-administration. One of the limitations of conventional dosage forms in vaginal therapy is the relatively short residence time of the drug at the site

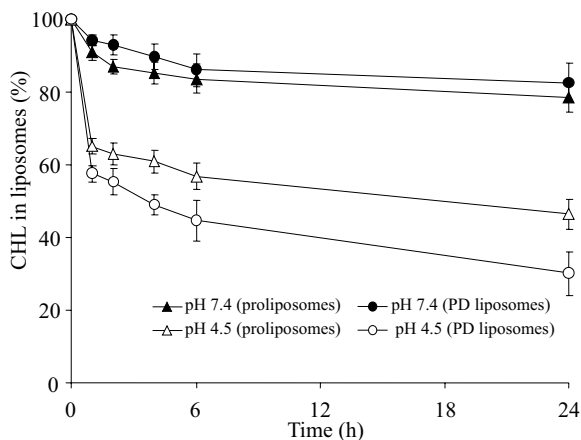


Fig. 1. *In vitro* stability of liposomes with CHL. The values denote the mean \pm SD ($n = 3$).

of application. Because a prolonged retention on the mucous wall is often required for the desired therapeutic effect, research efforts have been directed to using hydrophilic polymers with bioadhesive characteristics to improve drug delivery via the vagina (22). It has already been proven that liposomes are fairly compatible with polymers derived from crosslinked poly(acrylic acid) polymers (12, 13). Therefore it seemed logical to choose a gel made of Carbopol 974P NF resin as a vehicle for liposomes incorporation.

In this study, proliposomes with CHL were mixed into 1% Carbopol 974P NF gel and tested for *in vitro* release of the entrapped drug. As already described (13, 21), a method modified by Peschka *et al.* (14) was applied to follow the release of CHL from the liposomes incorporated in the gel. The porosity of the 2% agarose matrix allowed intact liposomes and released (free) drug to diffuse through the matrix into the receptor solution (buffer, pH 4.5 or VFS). The amount of CHL released from the gel was determined spectrophotometrically, before and after the addition of methanol to the supernatant over the agarose matrix.

Results presented in Tables II and III and Fig. 2a show a slower release of CHL from liposomes incorporated in Carbopol gel then from the control gel. After 24 hours of incubation at 37 °C in the buffer, pH 4.5, more than 47% of the originally entrapped CHL was still retained in the liposomes, in comparison to the control (13%). Slightly lower values were obtained in VFS (Fig. 2a). The model used to study the release of CHL from liposomes incorporated in the gel gave valuable information about intact liposomes released from the gel through the agarose matrix into the receptor media. CHL in intact liposomes was calculated as the difference between total CHL and free CHL, namely, after and before addition of methanol to the supernatant over the gel. As presented in Tables II and III, the amount of diffused intact liposomes was higher in the buffer of pH 4.5 (Table II) than in VFS (Table III), thus indicating a lower stability of liposomes in the presence of vaginal fluid components. Thus, after 24 hours of incubation in the buffer of pH 4.5, about 24% of CHL remained in the intact liposomes, while only 12% CHL remained in intact liposomes when experiments were performed in VFS. Statistically lower release of CHL from liposomes in the gel compared to the control gel was observed only after 24 h of incubation at 37 °C, both in the buffer of pH 4.5 and in VFS.

Table II. *In vitro* release of CHL entrapped in liposomes incorporated in the gel (buffer pH 4.5)

Time (h)	Free CHL (%) ^a	Total CHL (%) ^b	CHL in intact liposomes (%) ^c	Control (%)	Statistical inference ^d
1	9.27 ± 1.15	11.63 ± 1.15	2.36 ± 1.83	9.78 ± 2.68	NS
2	16.43 ± 3.27	23.75 ± 7.22	7.32 ± 4.92	17.83 ± 4.17	NS
4	24.36 ± 3.91	33.55 ± 7.92	9.18 ± 7.51	29.15 ± 7.82	NS
6	30.72 ± 4.70	45.55 ± 11.54	14.83 ± 12.83	36.93 ± 8.71	NS
24	52.76 ± 4.23	76.91 ± 9.01	24.15 ± 10.72	87.12 ± 6.20	S

The values denote the mean ± SD (*n* = 3).

^a Before disruption of liposomes by methanol.

^b After disruption of liposomes by methanol.

^c Difference between ^b and ^a.

^d Statistical analysis difference *vs.* control: NS – not statistically significant difference, S – statistically significant.

Table III. *In vitro* release of CHL entrapped in liposomes incorporated in the gel (VFS, pH 4.5)

Time (h)	Free CHL (%) ^a	Total CHL (%) ^b	CHL in intact liposomes (%) ^c	Control (%)	Statistical inference ^d
1	8.85 ± 1.89	14.45 ± 0.83	5.80 ± 3.45	9.78 ± 2.68	NS
2	16.88 ± 2.49	20.76 ± 0.40	5.09 ± 1.84	17.83 ± 4.17	NS
4	27.01 ± 1.43	29.39 ± 3.87	2.59 ± 1.92	29.15 ± 7.82	NS
6	35.66 ± 1.05	38.73 ± 0.63	2.64 ± 0.48	36.93 ± 8.71	NS
24	59.75 ± 3.35	71.18 ± 12.99	12.37 ± 8.87	87.12 ± 6.20	S

The values denote the mean ± SD (*n* = 3).

^a Before disruption of liposomes by methanol.

^b After disruption of liposomes by methanol.

^c Difference between ^b and ^a.

^d Statistical analysis difference *vs.* control: NS – not statistically significant difference, S – statistically significant.

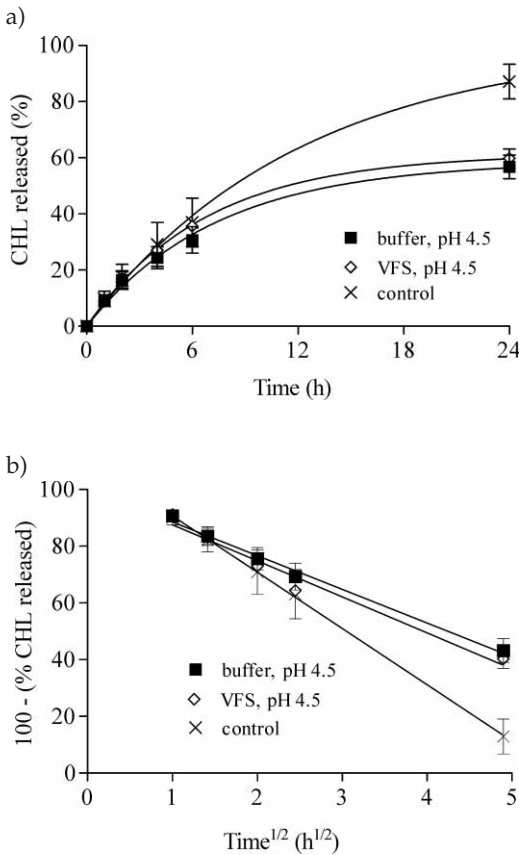


Fig. 2. Cumulative release of CHF from pro-liposomes incorporated in the gel in different media. a) The amounts of released drug were detected in receptor media before addition of methanol. b) The data are replotted according to the Higuchi equation (23). The values denote the mean ± SD (*n* = 3).

When the amount of CHL retained in the gel is plotted against the square root of time, a linear correlation according to the Higuchi equation (23) is obtained (Fig. 2b), showing matrix-controlled diffusion of the released drug.

In studies with hydrophilic compounds (13, 21), after 24 hours of incubation in the buffer of pH 4.5, only 16% of the originally encapsulated calcein, or 12% of FITC-dextran 4400, or 8% of FITC-dextran 21200 were released from the Carbopol gel. Such findings were expected because of the inverse correlation between the release rate and the molecular mass of the encapsulated marker. Release of CHL from liposomes incorporated in the gel was much faster probably due to the solubility of the lipophilic drug in acidic media. Since the gel pH was 5.5 and investigations were performed in media of low pH (pH 4.5), these results seem to be quite reasonable.

Regarding the physical properties of the vehicle for liposomes incorporation, it has been confirmed that the Carbopol 974P NH gel offers an adequate pH value and rheological properties (13). Due to the well-known loss of viscosity caused by sodium ions (24) from the buffer in which liposomes were made, the original gel was made in the concentration of 1% (*m/m*), and after addition of 10% liposomes, suitable viscosity was achieved (13). In order to provide a stable vehicle for vaginal application in which liposomes are distributed uniformly and their structure is preserved, a storage stability study was performed. Liposomal gels with CHL were kept for 4 weeks at 20 °C and 40 °C (stress testing). During those experiments, the size distribution and the mean diameter of the incorporated liposomes were determined. Results presented in Fig. 3 demonstrate the ability of the Carbopol 974P NF gel to preserve the original size distribution of liposomes with CHL. The mean diameter of proliposomes was 325 nm (immediately after incorporation in a vehicle). After 4 weeks of storage, the mean diameter changed from 325 to 361 nm (at 20 °C) and to 385 nm (at 40 °C) but the distribution remained almost unchanged. The data show that the percentage of smaller liposomes marginally decreased, while larger liposomes increased in number (Fig. 3).

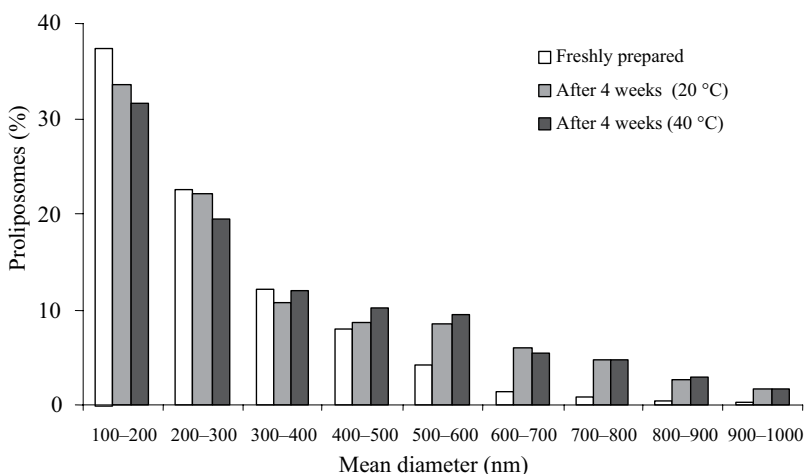


Fig. 3. Size distribution of proliposomes in the gel stored for 4 weeks at 20 and 40 °C.

CONCLUSIONS

Proliposome and polyol dilution methods yield similar entrapment efficiency for CHL. Since better stability of liposomes *in vitro* is observed with proliposomes, the proliposome method would be the proper choice of the preparation method. Incorporation of proliposomes in bioadhesive Carbopol gel improved their stability and enabled a sustained release of the drug, confirming the applicability of liposomes containing CHL as a novel delivery system for the local treatment of bacterial vaginosis.

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S A Ž E T A K

Gel s liposomski uklopljenim kloramfenikolom: karakterizacija i oslobađanje *in vitro*

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Cilj ovog rada bio je priprava i razvoj liposomskog terapijskog sustava s uklopljenim kloramfenikolom namijenjenog lokalnoj terapiji bakterijskog vaginitisa. Kloramfenikol je uklopljen u liposome fosfolipidnog sastava fosfatidilkolin/fosfatidilglicerol (molarni omjer 9:1), priređene dvjema metodama, proliposomskom i metodom razrjeđenja poliola. Obje preparacije su uspoređene i liposomi su karakterizirani s obzirom na veličinu čestica, polidisperznost, te uspješnost uklapanja. Provedena su ispitivanja stabilnosti liposoma *in vitro* u medijima koji svojim pH i sastavom oponašaju humanu vaginalnu sluznicu (pufer pH 4.5 i umjetni vaginalni medij). Kako bi se postigla prikladna viskoznost pripravka i dodatno povećala stabilnost liposoma namijenjenih vaginalnoj primjeni, liposomi su umiješani u bioadhezivni gel pripravljen iz karbopola 974P NF. *In vitro* ispitivanja s liposomima uklopljenima u gel pokazala su produljeno oslobađanje uklopljenog kloramfenikola. Pritom se čak nakon 24 sata inkubacije u umjetnom vaginalnom mediju više od 40% početno uklopljenog kloramfenikola zadržalo u gelu. Ispitivanja stabilnosti liposoma u gelu tijekom skladištenja potvrdila su povoljan učinak gela na očuvanje početne raspodjele uklopljenih liposoma s obzirom na veličinu. Ispitivanja provedena u ovom radu potvrđuju mogućnost primjene liposoma kao novog terapijskog sustava u lokalnoj terapiji bakterijskih vaginalnih infekcija.

Ključne riječi: liposomi, kloramfenikol, bioadhezivni gel, stabilnost, vaginalna primjena

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