Development and optimization of a new processing approach for manufacturing topical liposomes-in-hydrogel drug formulations by dual asymmetric centrifugation

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Abstract

Objectives: The objective of the present study was to utilize dual asymmetric centrifugation (DAC) as a novel processing approach for the production of liposomes-in-hydrogel formulations.

Materials and Methods: Lipid films of phosphatidylcholine, with and without chloramphenicol (CAM), were hydrated and homogenized by DAC to produce liposomes in the form of vesicular phospholipid gels with a diameter in the size range of 200-300 nm suitable for drug delivery to the skin. Different homogenization processing parameters were investigated along with the effect of adding propylene glycol (PG) to the formulations prior to homogenization. The produced liposomes were incorporated into a hydrogel made of 2.5 % (v/v) soluble β-1,3/1,6-glucan (SBG) and mixed by DAC to achieve a homogenous liposomes-in-hydrogel-formulation suitable for topical application.

Results and Discussion: CAM-containing liposomes with a vesicle diameter of 282 ± 30 nm and polydispersity index (PI) of 0.13 ± 0.02 were successfully produced by DAC after 50 minutes centrifugation at 3500 rpm, and homogenously (< 4 % content variation) incorporated into the SBG hydrogel. Addition of PG decreased the necessary centrifugation time to 2 minutes and 55 seconds, producing liposomes of 230 ± 51 nm and PI of 0.25 ± 0.04. All formulations had an entrapment efficiency of approximately 50%.

Conclusions: We managed to develop a relatively fast and reproducible new method for the production of liposomes-in-hydrogel formulation by DAC.

Key words: Skin therapy, liposomes, hydrogels, dual asymmetric centrifugation, chloramphenicol, soluble beta-glucan

Introduction

Skin disorders are the fourth leading cause of nonfatal diseases at the global level (Hay et al., 2014). The potential of nanopharmaceuticals in treating local skin diseases, such as skin infections and wounds, has yet to be fully realized, however the extensive research efforts are expected to result in improved therapy outcome (DeLouise, 2012, Hurler and Škalko-Basnet, 2012). Nanosized delivery systems offer an opportunity for extensive innovation in nanomedicine, making them an attractive target in drug product development (Vanić et al., 2015). Nanosized drug delivery systems designed for improved skin therapy are expected to exhibit all, or at least some of the desired features, namely to be able to protect drug from degradation as well as improve penetration of drug into/through the skin (Couvreur and Vauthier, 2006). Among various nanosystems developed to improve skin therapy, our focus is on liposomes. Liposomal encapsulation of drugs for enhancement of drug deposition into the skin was first introduced by Mezei and his colleagues in the early 1980s (Mezei and Gulasekharam, 1980). Already in 1988, the first topical liposomal preparation, Pevaryl Lipogel®, produced by Cilag A.G. became available on the market. The fact that most topical liposomal preparations brought to the industrialization phase carry drugs that are targeted to the dermal region of the skin, shows that liposomal carriers are well-suited for this kind of application (Vanić et al., 2015).

However, liposomes are aqueous dispersions and their fluid nature could be a limitation for skin administration in respect to residence time and leakage from the skin surface. For these reasons, liposomes should be incorporated into suitable vehicles (bases) of appropriate rheological features. The ideal vehicle exhibits appropriate rheological and textural properties, is compatible with both nanosystem and incorporated drug, stable during the storage, and safe to use (Hurler et al., 2013).

Although liposomes have been widely studied as superior drug carriers destined for topical administration onto the skin, the manufacturing methods applied in their production remain to be difficult to scale up and limited in many aspects of drug development (Mozafari, 2005).

To overcome the limitations of liposomal manufacturing based on the classical methods, we have in the present study investigated dual asymmetric centrifugation (DAC) as a novel manufacturing method for preparing liposomes-in-hydrogel for topical application. DAC was first introduced by Massing and his group as a new method for producing liposomes for parenteral application (Massing et al., 2008), and the method was further exploited by others (Hirsch et al., 2009, Tian et al., 2010, Adrian et al., 2011, Helm and Fricker, 2015). The DAC-technology offers rapid mixing of viscous material, based on the rotation of the sample around two axes, the central axis and a second axis in the center of the sample container. The homogenizing effect is obtained when the material is semisolid and sticks to the wall of the container, bringing the combination of the two contra-rotating movements into effect. Since topical products usually are viscous and semisolid and should adhere to the skin and retain there for a sufficient amount of time to allow the product to have the intended medical effect (Elnaggar et al., 2014), the desirable viscous consistence of the DAC-samples is ideal for topical products. Typically, liposomes for topical application are dispersed in polymeric materials such as hydrogels to form “classical liposomal hydrogel” (Elnaggar et al., 2014). These liposomes-in-hydrogel formulations must not be mistaken for vesicular phospholipid gels (VPGs), semisolid gels formed from concentrated liposomal dispersions. Here, numerous densely packed phospholipid vesicles (liposomes) form a gel-like structure, due to steric interactions between neighboring vesicles. VPGs were traditionally produced by high-pressure homogenization (Brandl, 2010). However, DAC seems more promising when aiming for production of bigger liposomes, and also to us, the closed system and possibility to process smaller batches is attractive for early phase formulation optimization to keep the laboratory expenses down.

Our initial goal was to prove the suitability of DAC in preparing small-scale samples, therefore a custom-made sample holder fitting 30 ml injection vials was selected for the VPG production, whereas the

liposomes-in-hydrogel mixing process was carried out in a Max 40 cup (Synergy Devices, Buckinghamshire, UK) with a capacity of 40 ml. Propylene glycol (PG) has a viscous nature, and is widely used as excipient in topical formulations, suitable to serve as a solvent for phospholipids and poorly water soluble drugs, and able to increase the deformability of liposome vesicles (Elmoslemany et al., 2012). Those were the reasons for preparing both PG-liposomes and conventional liposomes in this study. Finally, the suitability of using DAC-based mixing of liposomes into final "liposomes-in-hydrogel preparations" was tested. Thus, DAC was applied in two critical steps in the production process, namely both for the size reduction of the vesicles and for mixing and incorporation of vesicles into a hydrogel. The hydrogel chosen for this study was 2.5 % (v/v) soluble β-1,3/1,6-glucan (2.5 % SBG) in aqueous solution. SBG is known to activate macrophages and promote wound healing, and it has already shown promising results in the treatment of diabetic ulcers in the lower extremities (Zykova et al., 2014). Chloramphenicol (CAM) was chosen as a model antimicrobial drug, as it is a well-known antimicrobial, so far used most extensively in the topical treatment of ear and eye infections, but with potential in treatment of skin infections (Heal et al., 2009).

Materials and methods

Materials

Lipoid S 100 (soybean lecithin, > 94 % phosphatidylcholine) was provided as a gift from Lipoid GmbH (Ludwigshafen, Germany). Water-soluble β-1,3/1,6-glucan gel (2.5 % SBG) was provided by Biotec BetaGlucans AS (Tromsø, Norway). Chloramphenicol, Fiske-Subbarow reducer, monobasic potassium phosphate, sodium chloride, 99.8 % anhydrous acetic acid, ≥ 37 % hydrochloric acid (HCl), chloroform ($CHCl_3$) and methanol CHROMASOLV® (MeOH) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Ammonium molybdate, disodium hydrogen phosphate dihydrate and 30 % hydrogen peroxide (H_2O_2) were purchased from Merck (Darmstadt, Germany). Concentrated sulphuric acid (H_2SO_4) was the product of May and Baker LTD (Dagenham, England). Polyamide membranes (pore size = 0.2 mm) used in drug release studies and cellulose acetate filters (0.2 µm) for filtration of buffer solution and mobile phase were purchased from Sartorius (Göttingen, Germany). Propylene glycol (PG), rubber stoppers and capsules for the injection vials were purchased from NMD - Norwegian Medical Depot AS (Oslo, Norway).

Preparation of liposomes and liposomes-in-hydrogel formulations

Formation of homogeneous lipid and lipid-drug films

Lipid films were prepared in brown injection vials (Ø 36 mm, height 62.8 mm, capacity 30 ml) by dissolving 200 mg Lipoid S 100 (PC) in 10 mL of chloroform:methanol (2:1, v/v). The organic solvents were evaporated at 24 °C under a stream of N_2 for 1.5 hour in order to create a dry lipid film. A custom-made mixing device was used to provide constant rotation of the vial during evaporation to achieve an evenly distributed film on the bottom of the vial. The mixing device (Figure 1) was built using an Arduino Uno R3 microcontroller (Hobbykomponenter AS, Egersund, Norway), an Arduino Motor Shield (version 2) and a NEMA-17 size - 200 steps/rev (50 rpm), 12V 350 mA stepper motor (Adafruit Industries, New York, USA). Epoxy clay was finally used to mold a custom holder were the injection vial could fit during rotation.

For the lipid-drug films that contained CAM, 20 mg of drug was dissolved with 200 mg of PC prior to evaporation and formation of the lipid films.

Hydration of lipid films

For hydration of the lipid films, 200 µl phosphate buffered saline (PBS) pH 7.4 (2.98 g/L disodium hydrogen phosphate dihydrate, 0.19 g/L monobasic potassium phosphate and 8 g/L sodium chloride) was added when making conventional liposomes (C-Lip), whereas propylene glycol-containing liposomes (PG-Lip) were prepared by adding 100 µl of PG together with the 200 µl of PBS pH 7.4. The hydration procedure was as follows: Injection vials containing the samples was mixed for 5 minutes on a MS2 Minishaker vortex mixer (IKA-Werke, Staufen im Breisgau, Germany), and allowed to rest for a minimum of 1 hour at room temperature. Finally, glass beads (GB) (Ø 2 mm) were added to the formulations and samples were stored at 4 °C overnight prior to size reduction.

Homogenization of liposomes by dual asymmetric centrifugation

Hydrated lipid films were processed into viscous liposome dispersions, or VPGs, by DAC in a SpeedMixer DAC 150 FVZ (Hauschild GmbH & Co KG, Hamm, Germany). A custom-made sample holder insert fitted for injection vials, described earlier by Massing and colleagues (Massing et al., 2008) was used during liposome processing, making it possible to insert the hydrated lipid films, directly into the SpeedMixer, facilitating closed handling. For formulations that demanded more than 5 minutes of centrifugation, the machine was restarted immediately after each 5-minute run until the desired total centrifugation time was achieved. Produced VPGs were diluted with PBS pH 7.4 to liposome dispersions (total volume of 2 mL) before characterization and dispersion in SBG gels to form the final liposomes-in-hydrogel formulation

Mixing of liposomes into hydrogel

To produce the liposomes-in-hydrogel formulations, 1 g of liposome dispersion and 9 g 2.5 % SBG was transferred to a Max 40 cup (Synergy Devices, Buckinghamshire, UK). Mixing was carried out at 3500 rpm for 5 minutes in the same SpeedMixer that was used for homogenization of liposomes.

Comparison of probe-sonication with dual asymmetric centrifugation

In order to compare the entrapment efficiency of our DAC-produced liposomes with a more well-known method, liposomes of the same composition as for the ones produced by DAC were prepared by probe-sonication. The lipid-drug films were prepared using a rotary evaporator system (Büchi Labortechnik AG, Flawil, Switzerland) with a vacuum pump (20 minutes at 150 mbar, followed by 1 hour at 50 mbar) and a water bath (45 ± 1 °C) to remove the organic solvents. Dried films were then hydrated into more dilute liposome dispersions (10 mg/ml PC) with PBS pH 7.4 and 2 mL samples were sonicated (40 % amplitude) for 2 x 2 minutes on ice bath using a GEX500 high intensity ultrasonic processor (Sonics & Materials Inc., Newtown, USA) with a 19 mm probe.

Liposome and liposomes-in-hydrogel characterization

Photon correlation spectroscopy

Mean vesicle diameter and polydispersity index (PI) of the different liposome formulations was determined by photon correlation spectroscopy (PCS) with a Submicron Particle Sizer Model 370 (Nicomp Particle Sizing Systems, Santa Barbara, USA) as described earlier (di Cagno et al., 2011, Jøraholmen et al., 2014) with minor modifications. All sample tubes were sonicated in PBS pH 7.4 for 10 minutes prior to use,

to eliminate any dust particles. The sample tube to be used during measurement were then filled with freshly filtered PBS pH 7.4 using syringe filters with a pore size of 0.2 µm (Acrodisc, PALL Life Sciences, Michigan, USA). The liposomal aliquots were diluted with PBS pH 7.4 until its intensity were within the range of 250 – 350 kHz. The samples were allowed to equilibrate in the Particle Sizer for 5 minutes before the analysis was started. Every analysis was performed in three 10-minutes cycles at 24 °C. Vesicle mode, automatic choice of channel width and volume weighting were used for data representation. Nicomp distribution was used for all measurements that had a Chi^2-value > 3 and did not fit the Gaussian distribution accordingly. In order to acquire a mean value for the measurements that fit a Nicomp distribution the size of the peaks were weighted according to their percentage and a weighted mean value was calculated.

Entrapment efficiency

Entrapment efficiency of the liposome formulations containing CAM were performed after the removal of free drug from the formulations by dialysis. The amount of drug present as free drug and liposome-associated drug was then quantified by high-performance liquid chromatography (HPLC) analysis.

Liposome formulations were diluted to a lipid concentration of 20 mg/ml prior to dialysis using a membrane with a M_w cut-off of 12–14000 Da (Medicell International Ltd., London, UK). PBS (pH 7.4) was used as dialysis medium. Duration of dialysis was 4 hours and was carried out at room temperature (23 °C) and under sink conditions.

Samples from both the dialysate and the liposome fraction were diluted with mobile phase prior to analysis. The mobile phase that was used, was a mixture of filtered water, MeOH and anhydrous acetic acid (55:44:0.1, v/v). Instrumentation setup consisted of a Waters e2795 Separations Module with a Symmetry C18 column guard and a XSELECT CSH C18 (2.5 µm 3.0x75 mm) column XP connected to a Waters 2489 UV/Visible detector (Waters, Dublin, Ireland). The flow rate was set to 0.4 mL/min, and both column and sample temperature were set to 30 ± 2 °C during separation.

Lipid loss during processing

Lipid content was determined using a modification of the Bartlett assay (Bartlett, 1959). To determine lipid loss on the glass beads used during processing of liposomes, the glass beads were immersed and rinsed in 1 mL 0.5 M HCl. After rinsing, the beads were removed and the solution diluted up to a total volume of 10 mL in a volumetric flask.

For determination of total amount of lipid used by liposomes in the formulations, 50 µL samples of liposome dispersion were withdrawn after dialysis. All samples were diluted up to a total volume of 10 mL prior to the lipid quantification in the same manner as samples for lipid loss.

A volume of 1 mL was withdrawn from all the diluted samples and mixed with 0.5 mL 10 N H_2SO_4, before heating them for 3 hours at 155 °C, and subsequent cooling to room temperature. When room temperature was reached, 2 drops of 30 % (v/v) H_2O_2 was added, and the mixtures was heated for an additional 1.5 hour at 155 °C. After all samples had reached room temperature again, they were mixed with 4.6 mL 0.22 % (v/v) ammonium molybdate and 0.2 mL Fiske-Subbarow reducer reagent, before the mixtures were heated for a final 7 minutes at 100 °C. The samples were analyzed calorimetrically at λ = 830 nm using a SpectraMax 190 Microplate Reader (Molecular Devices, California, USA).

Homogeneity of CAM in the formulations

Homogeneity of active ingredient, CAM, in the liposomes-in-hydrogel formulations was determined by sampling (ca. 200 mg) from five different places within the hydrogel formulations. The withdrawn samples were diluted up to a total volume of 10 mL with HPLC mobile phase, filtered through a 0.2 µm filter (Acrodisc, PALL Life Sciences, Michigan, USA) and then analyzed by HPLC as described earlier.

Drug release studies

In vitro drug release studies were performed in order to compare the drug release from both our liposomes-in-hydrogel formulations compared to free drug in PG, free drug in SBG and liposomally entrapped drug in PBS (pH 7.4). The Franz cells (PermeGear, Bethlehem, USA) that were used had a volume of 12.0 or 12.1 mL and a surface area of 1.77 cm^2. Polyamide membranes (Sartorius, Göttingen, Germany) were used as the model barrier, HPLC mobile phase were used as the acceptor medium and the temperature was set to 32 °C. A volume of 300 µL of each formulation were applied in the donor chambers. Samples (250 µL) were then withdrawn from the acceptor chamber at the following time points: 1, 2, 3, 4, 5, 6, 7 and 8 hours after application of the formulations in the donor chambers. The drug concentration in all samples were determined by HPLC as described earlier.

Stability testing

Measurements of mean vesicle size, PI and quantification of total CAM content were repeated after 8 weeks for liposome dispersions stored at 4 °C to check the stability of the formulation upon storage. Liposomes-in-hydrogel formulations stored at room temperature for 8 weeks were only retested for homogeneity of active ingredient.

Statistical analysis

Student's t-test was used to identify differences between formulations and sample sets. A significance level where $p < 0.05$ was used when data was analyzed.

Results and discussion

The entrapping ability, biodegradability and non-toxic nature of liposomes make them attractive as drug delivery carriers. Moreover, they are also able to increase the amount of drug deposited into the upper layers of the skin and have been demonstrated to lower systemic drug levels, as compared with conventional formulations containing the same drug (Elsayed et al., 2007). This makes them highly promising for improved drug delivery to the skin (Ferreira et al., 2004, Chen et al., 2013).

However, their successful application as drug delivery systems, relies not only on the proof of superiority as compared to more conventional formulations, but is also strongly dependent on the technical and economic feasibility of the liposomal formulation and its manufacturing process. Production procedures that easily can be brought from lab scale to an industrial scale, would speed up the development process, and finally bring new products to the market faster and within feasible economical frames.

To formulate liposomal preparations acceptable as pharmaceuticals, some criteria are frequently mentioned to be important (Wagner et al., 2002, Huang et al., 2014): (1) A unimodal narrow-size distribution, (2) a satisfactory chemical and physical stability, (3) a reproducible production process, (4) a high and constant entrapment efficiency, (5) a retained, depot-type drug-release from the vehicle is often anticipated, and (6) a production procedure that facilitates sterile and pyrogen-free products. The

relatively small number of liposomal products approved for human use so far, relative to the enormous research and development works on liposomes, might be explained by failure to meet any of these criteria, but high cost of the production process, especially on a larger scale, might as well keep products from entering the market (Mozafari, 2005).

In the present work, we studied the potential use of DAC as a method for producing liposomes-in-hydrogel formulations suitable for topical wound treatment.

Optimization of the methodology

In an effort to keep the production process as simple as possible, we wanted to develop a method that utilized the full potential of the SpeedMixer and facilitated its use throughout the whole production of our liposomes-in-hydrogel formulations.

When we began establishing the DAC-procedure we aimed to investigate the possibility of integrating the polyol dilution method as the first step of liposome production, inspired by the work of Kikuchi and colleagues (Kikuchi et al., 1994) and others (Pavelić et al., 1999, Pavelić et al., 2005), but in combination with DAC. The polyol dilution method makes it possible to avoid the use of harmful organic solvents in the production process, replacing them with PG to dissolve both CAM and PC. By dissolving the two compounds in PG at 60 °C prior to hydration and processing in the SpeedMixer, we were able to successfully produce liposomes. However, due to a disappointing entrapment efficiency of only 5 % CAM at best, we decided to try the conventional film hydration method. Since we wanted to avoid loss of material, unnecessary transfer and the need to produce our formulations in excess, we modified the film hydration method so, that the lipid film could be formed directly in an injection vial, and a custom-made setup for drying of lipid films was built (Figure 1). After evaporating solvents from the samples for 1.5 hour under a continuous stream of N_2, the amount of residual solvent left in the vials was found to be < 0.8 % and judged as satisfactory for our purpose.

It is generally accepted that for optimal dermal drug delivery into the skin, liposomes applied topically should be controlled for their physiochemical properties, such as vesicle size (Verma et al., 2003), drug entrapment efficiency, and lipid bilayer composition and elasticity/fluidity (Kirjavainen et al., 1999). Du Plessis et al. (du Plessis et al., 1994) suggested that the intermediate vesicle size of 300 nm gave the best deposition of drug in the deeper skin layers, and the highest drug concentration in the reservoir. During our investigation and method optimization, we therefore aimed for a liposome size distribution between 200-300 nm. Furthermore, we wanted to keep the amount of glass beads (GB) used during the processing as low as possible based on the findings by Massing and colleagues concerning the generation of glass particles from the beads (Massing et al., 2008).

Table 1 shows the vesicle size and polydispersity index (PI) of empty conventional liposomes after 40 minutes centrifugation time at 2400 and 3500 rpm with 25 % (w/w) GB, respectively. Mean diameter of liposomes produced at the two different speed levels were both within the desired size range of 200-300 nm. The size difference is significant ($p < 0.05$) and points towards the importance of centrifugation speed on vesicle size, but there is no significant change in the PI. The lack of change in polydispersity of the samples when the speed is increased from 2400 to 3500 rpm might be due to the relatively small interval of 1100 rpm that was tested. When such a small interval is used it is difficult to observe anything other than the most significant factors, which in this case was vesicle size.

Entrapment of chloramphenicol

When CAM was incorporated into the conventional liposomes, mean vesicle size increased from 214 ± 12.5 nm to 426 ± 181.3 nm, whereas the PI changed from 0.20 ± 0.04 to 0.23 ± 0.10. Based on the increase of vesicle size, PI, and standard deviations, we decided to increase the content of GB from 25 to 50 % (w/w) and the centrifugation time from 40 to 50 minutes aiming to get a smaller and narrower size distribution of drug-containing vesicles. We found that although there was no significant change in vesicle size or PI when increasing the amounts of GB, the reproducibility improved (Figure 2). After 50 minutes centrifugation under the new settings, a mean vesicle diameter of 282 ± 30 nm with a PI equal to 0.13 ± 0.02, was obtained. The significant ($p < 0.05$) increase in vesicle size for CAM-liposomes compared to empty liposomes, and the increase in amount of energy needed to produce liposomes within the desired size range (200-300 nm) indicate that CAM is mainly located in the lipid bilayer of liposomes. However, based on CAM's solubility; 2.5 mg/ml in water (25 °C) and 150.8 mg/ml in propylene glycol, it is expected that CAM is in equilibrium with a dissolved portion of drug in the aqueous phase of the liposome dispersion.

Effect of propylene glycol on liposomal characteristics

We obtained an entrapment efficiency close to 50 % for both conventional liposomes and PG-liposomes (Table 2), and PG did not seem to affect the entrapment of CAM in the lipid bilayer. It was however critical to hydrate lipids and drug from a lipid film, as only 5% entrapment was obtained when liposomes were prepared with the conventional polyol method. Results from the comparison with the probe-sonication showed that even though bigger liposomes with a higher PI were obtained (836.1 nm and PI = 0.512), entrapment was only 30.13 ± 0.15 %, making DAC a superior method. DAC also seem to be superior to extrusion when it comes to entrapment of CAM as Engesland and colleagues achieved an entrapment efficiency of only 32.5 ± 2.8 % (drug:lipid ratio of 32.5 ± 2.8 µg/mg) after size reduction of liposomes (667 nm) by nitrogen-driven extrusion while using exactly the same lipid and drug composition as applied in this study (Engesland et al., 2015). In the studies by Pavelić and colleagues (Pavelić et al., 2004), the starting CAM:lipid ratio was 2.5 times higher than in this present study; 4:4 (w/w) relative to 1:10 (w/w), respectively. From this a final drug entrapment of 24.8 ± 3.1% (proliposome method) and 30.2 ± 4.1% (polyol method) was obtained. It seems that our results give the most successful method regarding entrapping the highest fraction of the added drug into the liposomes. This is very promising as it makes it feasible to skip the time consuming process of separating free drug from the liposomes before mixing them into the hydrogel/vehicle. To avoid the separation step is also critical when a relative high content of liposomes and drug in the hydrogel is aimed for, as both dialysis and size exclusion chromatography will demand the VPGs to be diluted for removal of the unentrapped drug. However, when looking at the drug entrapment values and calculating the drug to lipid ratio, Pavelić and co-workers obtained a ratio of 76.1 ± 8.3 µg/mg (polyol dilution) and 62.7± 8.6 µg/mg (proliposomes) (Pavelić et al., 1999). For liposomes composed of PC:PG (molar ratio 9:1) they obtained a similar values of 75.5 ± 10.3 µg/mg (polyol dilution) and 62.0 ± 7.8 µg/mg (proliposome) (Pavelić et al., 2004). The entrapment was slightly higher than what we achieved with DAC; 52.2 ± 1.0 µg/mg (C-Lip-CAM) and 50.8 ± 4.2 µg/mg (PG-Lip-CAM) respectively, which indicate that it might still be possible to increase the drug:lipid ratio for our DAC-method. However, the preliminary results indicate that increasing the initial drug:lipid ratio, results in a higher content of free drug, which is not ideal if we want to avoid the separation of free drug from the formulation before incorporation into the hydrogel vehicle.

After the addition of 24.8 % (w/w) PG to the formulation prior to the size reduction and homogenization, we observed a significant decrease in the time needed to obtain liposomes with a diameter of 200-300 nm. Centrifugation time decreased from 50 minutes to 2 minutes and 55 seconds with 50 % (w/w) GB

(Table 2). Entrapment efficiency of CAM did not change compared to the formulations without PG; however the PI increased significantly (Table 2). The observed increase in PI is both a result of the reduced centrifugation time and addition of PG. Reduction of centrifugation time seems to give a decreased homogenization and more polydisperse samples (Figure 2 and Table 2). The presence of PG seems to lower the amount of energy needed to reduce the size of the liposomes; this observation is consistent with PG's solubilizing effect on lipids and the PG molecules ability to intercalate with the lipid bilayer and thus allow a tighter packing of the lipids and smaller vesicle diameter (Castangia et al., 2013, Manca et al., 2014, Palac et al., 2014).

Lipid loss and degradation during processing

Neither CAM, amount of GB nor PG affected significantly the amount of lipids lost during processing (Figure 3). The result, however, may indicate a trend towards less lipid loss with a decrease in the amount of GB and addition of PG. This is also reasonable, and might be explained by a decrease in the overall surface area available for the lipids to stick to when the number of GBs is reduced, and an increase in lipid solubility when PG is added. However, the lipid loss was judged acceptable for all settings used.

The temperature of samples were recorded with an infrared thermometer, both before and after centrifugation, to determine the possibility for lipid degradation. However, the observed elevation in sample temperature was only in the range of 7-10 °C from a starting temperature of 25 °C for both types of formulations. As Massing and colleagues previously showed (Massing et al., 2008), the degree of lipid degradation within the same temperature range is negligible. Our sample temperature did not exceed 50 ± 1 °C, and we therefore found the possibility of significant lipid degradation unlikely.

Homogeneity of liposomes-in-hydrogel formulations

In comparison to other methods used for the mixing of liposomal dispersion into hydrogels, such as using a standard electrical mixer (Škalko et al., 1998) or mixing it in by hand (Hurler et al., 2012), the SpeedMixer has the advantage of assuring fast, homogenous mixing of the formulations without the introduction of air bubbles/pockets. Actually, we observed that mixing with the SpeedMixer also removed air bubbles from viscous material during processing. Mixing of formulations using the SpeedMixer also allows production of more concentrated samples, for instance by mixing VPGs directly into the hydrogel vehicle. Hence, it makes it possible to increase the overall lipid and drug content, which is advantageous when aiming for the skin treatment of both chronic wounds and burn wounds with antimicrobials, where a higher drug concentration than what have been possible with liposomes-in-hydrogel formulations previously, may be required. The test for homogeneity of active ingredient in the final liposomes-in-hydrogel formulations produced with the SpeedMixer showed high homogeneity of the samples with an average content of 8.36 ± 0.12 mg CAM per 10 g of gel formulation, and a variation < 4 % (Table 3).

Drug release studies

Preliminary results from the *in vitro* drug release studies comparing the release from hydrogel formulations for both conventional liposomes (Figure 4) and PG-liposomes (Figure 5) with liposomes dispersed in PBS (pH 7.4), free drug dissolved in PG and free drug in hydrogel, indicate that our liposomes-in-hydrogel formulations has a slightly delayed and sustained release compared to free drug dissolved in PG.

Storage stability

The liposomes dispersed in SBG gel are difficult to analyze with regard to mean vesicle diameter and PI due to polymer network and presence of polymer aggregates. Thus, the liposome dispersions stored at 4 °C were used as stability indicators, and change in size and PI were determined after 8 weeks storage and compared with the liposome size of the same samples right after preparation.

Our results showed that the mean vesicle diameter changed for two of the three samples (Table 4). However, the change was minor (11 and 3 nm, respectively), and the PI immediately after preparation and after 8 weeks of storage shows that the values have decreased ($p < 0.05$) for all of the three samples, indicating that system is further stabilized after preparation and remained stable upon storage.

Total CAM content in liposome dispersions containing PG stored at 4 °C decreased 5 ± 3 % after eight weeks of storage, while we observed a significant difference with an average decrease of 11 ± 1 % for the liposomes-in-hydrogel formulations stored at 23 °C (Figure 6). The difference in stability of the drug in these two preparations might be explained both by the difference in storage temperature and the dispersion media. In addition, the final concentration of the drug also differed. The choice of storage conditions for liposomes-in-hydrogel formulations was however done in accordance with the manufacturer recommendations, which was that storage at 4 °C was not possible for hydrogels made of SBG due to changes in the integrity of the polymer network at lower temperatures (personal communication). Thus, liposomes-in-hydrogel formulation made with SBG had to be stored at 23 °C to ensure optimal conditions for the polymer network. In both instances, the formulations were also protected from light. A study performed by Boer and Pijnenburg with CAM solutions (pH 7.2) under similar condition in the dark (4 °C and 21 °C) (Boer and Pijnenburg, 1983), showed the same kind of temperature-dependent difference in degradation of CAM as observed for our samples. The stability limitations of CAM is well-known, and is explained by CAM hydrolysis into a glycol-derivative and $CHCl_2COOH$ in the presence of water (and light) (Lv et al., 2005). However, the rate of degradation observed by Boer and Pijnenburg was lower at both temperatures compared to our results, suggesting an increased rate of degradation in our liposome-in-hydrogel formulations. The positive effects of incorporating liposomes in other types of hydrogels, such as obtaining a sustained drug release effect, preventing leakage of entrapped drug and improved bioadhessiveness of the formulation to the skin, has already been proven (Škalko et al., 1998, Hurler et al., 2012). Thus, the main focus for further improvement of this specific formulation should be on the ways to minimize drug degradation and assure a satisfactory shelf-life. Overall, our findings might suggest that instant mixing of CAM-containing liposomes into hydrogel right before application might be the simplest way to solve the stability issue, but further studies and investigation on alternative strategies are still needed.

Conclusions

We successfully developed a new, straightforward and reproducible method for the production of liposomes-in-hydrogel formulations by the use of DAC. The method enabled the production of homogenous liposomes-in-hydrogel formulations with a higher lipid and drug content than what have previously been possible with the conventional film hydration and hand mixing methodology.

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Declaration of interest

The authors report no conflict of interest.

References

ADRIAN, J. E., WOLF, A., STEINBACH, A., RÖSSLER, J. & SÜSS, R. 2011. Targeted delivery to neuroblastoma of novel siRNA-anti-GD2-liposomes prepared by dual asymmetric centrifugation and sterol-based post-insertion method. *Pharmaceutical Research,* 28**,** 2261-2272.

BARTLETT, G. R. 1959. Phosphorus assay in column chromatography. *Journal of Biological Chemistry,* 234**,** 466-468.

BOER, Y. & PIJNENBURG, A. 1983. HPLC determination of chloramphenicol degradation in eye drops. *Pharmaceutisch Weekblad Scientific Edition,* 5**,** 95-101.

BRANDL, M. 2010. Vesicular phospholipid gels. *In:* WEISSIG, V. (ed.) *Liposomes - Methods and Protocols, Volume 1: Pharmaceutical Nanocarriers.* 1 ed.: Humana Press.

CASTANGIA, I., MANCA, M. L., MATRICARDI, P., SINICO, C., LAMPIS, S., FERNÀNDEZ-BUSQUETS, X., FADDA, A. M. & MANCONI, M. 2013. Effect of diclofenac and glycol intercalation on structural assembly of phospholipid lamellar vesicles. *International Journal of Pharmaceutics,* 456**,** 1-9.

CHEN, G., LI, D., JIN, Y., ZHANG, W., TENG, L., BUNT, C. & WEN, J. 2013. Deformable liposomes by reverse-phase evaporation method for an enhanced skin delivery of (+)-catechin. *Drug Development and Industrial Pharmacy,* 40**,** 260-265.

COUVREUR, P. & VAUTHIER, C. 2006. Nanotechnology: Intelligent design to treat complex disease. *Pharmaceutical Research,* 23**,** 1417-1450.

DELOUISE, L. A. 2012. Applications of nanotechnology in dermatology. *Journal of Investigative Dermatology,* 132**,** 964-975.

DI CAGNO, M., STYSKALA, J., HLAVAC, J., BRANDL, M., BAUER-BRANDL, A. & ŠKALKO-BASNET, N. 2011. Liposomal solubilization of new 3-hydroxy-quinolinone derivatives with promising anticancer activity: a screening method to identify maximum incorporation capacity. *Journal of Liposome Research,* 21**,** 272-8.

DU PLESSIS, J., RAMACHANDRAN, C., WEINER, N. & MÜLLER, D. G. 1994. The influence of particle size of liposomes on the deposition of drug into skin. *International Journal of Pharmaceutics,* 103**,** 277-282.

ELMOSLEMANY, R., ABDALLAH, O., EL-KHORDAGUI, L. & KHALAFALLAH, N. 2012. Propylene glycol liposomes as a topical delivery system for miconazole nitrate: comparison with conventional liposomes. *AAPS PharmSciTech,* 13**,** 723-731.

ELNAGGAR, Y. S. R., EL-REFAIE, W. M., EL-MASSIK, M. A. & ABDALLAH, O. Y. 2014. Lecithin-based nanostructured gels for skin delivery: An update on state of art and recent applications. *Journal of Controlled Release,* 180**,** 10-24.

ELSAYED, M. M. A., ABDALLAH, O. Y., NAGGAR, V. F. & KHALAFALLAH, N. M. 2007. Lipid vesicles for skin delivery of drugs: Reviewing three decades of research. *International Journal of Pharmaceutics,* 332**,** 1-16.

ENGESLAND, A., SKALKO-BASNET, N. & FLATEN, G. E. 2015. Phospholipid Vesicle-Based Permeation Assay and EpiSkin® in Assessment of Drug Therapies Destined for Skin Administration. *Journal of Pharmaceutical Sciences,* 104**,** 1119-1127.

FERREIRA, L. S., RAMALDES, G. A., NUNAN, E. A. & FERREIRA, L. A. M. 2004. In Vitro Skin Permeation and Retention of Paromomycin from Liposomes for Topical Treatment of the Cutaneous Leishmaniasis. *Drug Development and Industrial Pharmacy,* 30**,** 289-296.

HAY, R. J., JOHNS, N. E., WILLIAMS, H. C., BOLLIGER, I. W., DELLAVALLE, R. P., MARGOLIS, D. J., MARKS, R., NALDI, L., WEINSTOCK, M. A., WULF, S. K., MICHAUD, C., J.L. MURRAY, C. & NAGHAVI, M. 2014. The global burden of skin disease in 2010: an analysis of the prevalence and impact of skin conditions. *Journal of Investigative Dermatology,* 134**,** 1527-1534.

HEAL, C. F., BUETTNER, P. G., CRUICKSHANK, R., GRAHAM, D., BROWNING, S., PENDERGAST, J., DROBETZ, H., GLUER, R. & LISEC, C. 2009. Does single application of topical chloramphenicol to high risk sutured wounds reduce incidence of wound infection after minor surgery? Prospective randomised placebo controlled double blind trial. *BMJ,* 338**,** a2812.

HELM, F. & FRICKER, G. 2015. Liposomal conjugates for drug delivery to the central nervous system. *Pharmaceutics,* 7**,** 27-42.

HIRSCH, M., ZIROLI, V., HELM, M. & MASSING, U. 2009. Preparation of small amounts of sterile siRNA-liposomes with high entrapping efficiency by dual asymmetric centrifugation (DAC). *Journal of Controlled Release,* 135**,** 80-88.

HUANG, Z., LI, X., ZHANG, T., SONG, Y., SHE, Z., LI, J. & DENG, Y. 2014. Progress involving new techniques for liposome preparation. *Asian Journal of Pharmaceutical Sciences,* 9**,** 176-182.

HURLER, J., BERG, O. A., SKAR, M., CONRADI, A. H., JOHNSEN, P. J. & ŠKALKO-BASNET, N. 2012. Improved burns therapy: Liposomes-in-hydrogel delivery system for mupirocin. *Journal of Pharmaceutical Sciences,* 101**,** 3906-3915.

HURLER, J. & ŠKALKO-BASNET, N. 2012. *Advancements in burn therapy: Promise of nanomedicine, in Burns: Prevention, Causes and Treatment,* New York, Nova Science Publishers.

HURLER, J., ŽAKELJ, S., MRAVLJAK, J., PAJK, S., KRISTL, A., SCHUBERT, R. & ŠKALKO-BASNET, N. 2013. The effect of lipid composition and liposome size on the

release properties of liposomes-in-hydrogel. *International Journal of Pharmaceutics,* 456**,** 49-57.

JØRAHOLMEN, M. W., VANIĆ, Ž., THO, I. & ŠKALKO-BASNET, N. 2014. Chitosan-coated liposomes for topical vaginal therapy: Assuring localized drug effect. *International Journal of Pharmaceutics,* 472**,** 94-101.

KIKUCHI, H., YAMAUCHI, H. & HIROTA, S. 1994. A polyol dilution method for mass production of liposomes. *Journal of Liposome Research,* 4**,** 71-91.

KIRJAVAINEN, M., URTTI, A., VALJAKKA-KOSKELA, R., KIESVAARA, J. & MÖNKKÖNEN, J. 1999. Liposome–skin interactions and their effects on the skin permeation of drugs. *European Journal of Pharmaceutical Sciences,* 7**,** 279-286.

LV, F.-F., ZHENG, L.-Q. & TUNG, C.-H. 2005. Phase behavior of the microemulsions and the stability of the chloramphenicol in the microemulsion-based ocular drug delivery system. *International Journal of Pharmaceutics,* 301**,** 237-246.

MANCA, M. L., CASTANGIA, I., MATRICARDI, P., LAMPIS, S., FERNÀNDEZ-BUSQUETS, X., FADDA, A. M. & MANCONI, M. 2014. Molecular arrangements and interconnected bilayer formation induced by alcohol or polyalcohol in phospholipid vesicles. *Colloids and Surfaces B: Biointerfaces,* 117**,** 360-367.

MASSING, U., CICKO, S. & ZIROLI, V. 2008. Dual asymmetric centrifugation (DAC)—A new technique for liposome preparation. *Journal of Controlled Release,* 125**,** 16-24.

MEZEI, M. & GULASEKHARAM, V. 1980. Liposomes - a selective drug delivery system for the topical route of administration I. Lotion dosage form. *Life Sciences,* 26**,** 1473-1477.

MOZAFARI, M. R. 2005. Liposomes: an overview of manufacturing techniques. *Cellular & Molecular Biology Letters,* 10**,** 711-9.

PALAC, Z., HURLER, J., ŠKALKO-BASNET, N., FILIPOVIĆ-GRČIĆ, J. & VANIĆ, Ž. 2014. Elastic liposomes-in-vehicle formulations destined for skin therapy: the synergy between type of liposomes and vehicle. *Drug Development and Industrial Pharmacy,* 41**,** 1247-1253.

PAVELIĆ, Ž., ŠKALKO-BASNET, N., FILIPOVIĆ-GRČIĆ, J., MARTINAC, A. & JALŠENJAK, I. 2005. Development and in vitro evaluation of a liposomal vaginal delivery system for acyclovir. *Journal of Controlled Release,* 106**,** 34-43.

PAVELIĆ, Ž., ŠKALKO-BASNET, N. & JALŠENJAK, I. 1999. Liposomes containing drugs for treatment of vaginal infections. *European Journal of Pharmaceutical Sciences,* 8**,** 345-351.

PAVELIĆ, Ž., ŠKALKO-BASNET, N. & JALŠENJAK, I. 2004. Liposomal gel with chloramphenicol: characterisation and in vitro release. *Acta Pharm,* 54**,** 319-30.

ŠKALKO, N., ČAJKOVAC, M. & JALŠENJAK, I. 1998. Liposomes with Metronidazole for Topical Use: The Choice of Preparation Method and Vehicle. *Journal of Liposome Research,* 8**,** 283-293.

TIAN, W., SCHULZE, S., BRANDL, M. & WINTER, G. 2010. Vesicular phospholipid gel-based depot formulations for pharmaceutical proteins: Development and in vitro evaluation. *Journal of Controlled Release,* 142**,** 319-325.

VANIĆ, Ž., HOLSÆTER, A. M. & ŠKALKO-BASNET, N. 2015. (Phospho)lipid-based nanosystems for skin administration. *Current Pharmaceutical Design,* 21**,** 4174-4192.

VERMA, D. D., VERMA, S., BLUME, G. & FAHR, A. 2003. Particle size of liposomes influences dermal delivery of substances into skin. *International Journal of Pharmaceutics,* 258**,** 141-151.

WAGNER, A., VORAUER-UHL, K. & KATINGER, H. 2002. Liposomes produced in a pilot scale: production, purification and efficiency aspects. *European Journal of Pharmaceutics and Biopharmaceutics,* 54**,** 213-219.

ZYKOVA, S. N., BALANDINA, K. A., VOROKHOBINA, N. V., KUZNETSOVA, A. V., ENGSTAD, R. & ZYKOVA, T. A. 2014. Macrophage stimulating agent soluble yeast β-1,3/1,6-glucan as a topical treatment of diabetic foot and leg ulcers: A randomized, double blind, placebo-controlled phase II study. *Journal of Diabetes Investigation,* 5**,** 392-399.

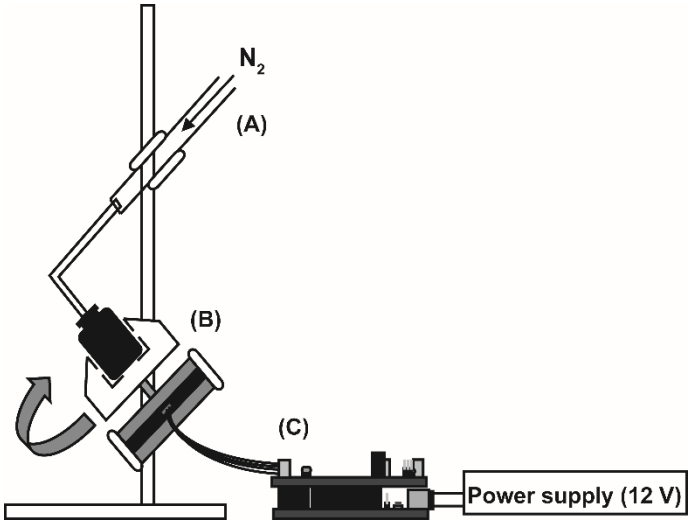

Figure 1: Illustration of the custom-made mixing device: (A) tube for N2 with a glass tip that directs the gas towards the vial; (B) injection vial with holder mounted on stepper motor; (C) Motor shield that drives the motor, stacked on top of the microcontroller, which in turn is connected to a 12 V power supply.

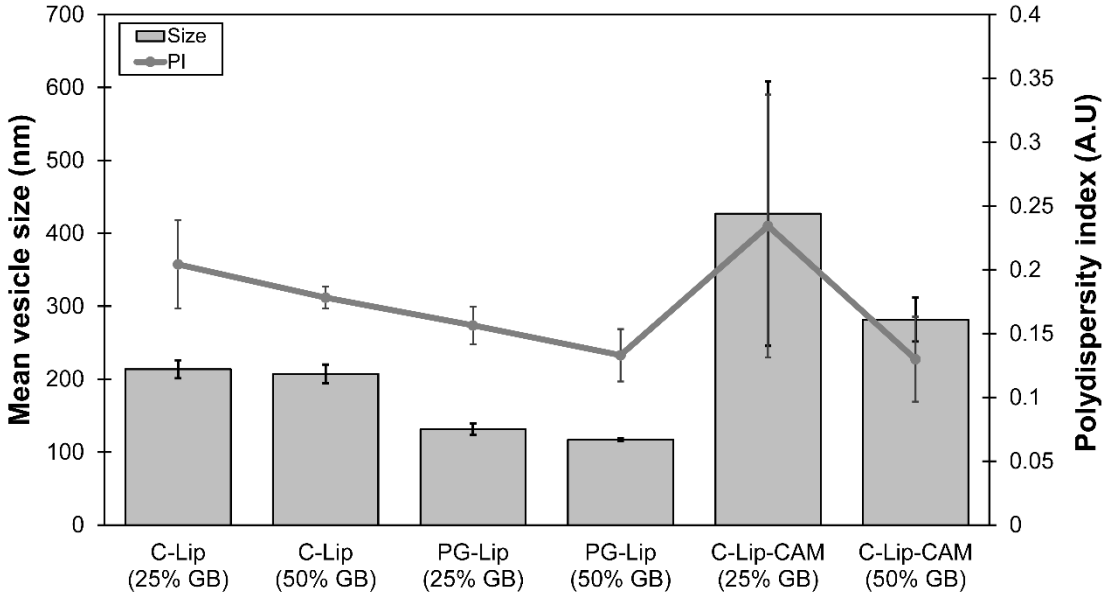

Figure 2: Effect of glass beads and chloramphenicol (CAM) on mean vesicle size (diameter) and polydispersity index (PI) (n = 3). Results obtained after 40 minutes centrifugation time at 3500 rpm.

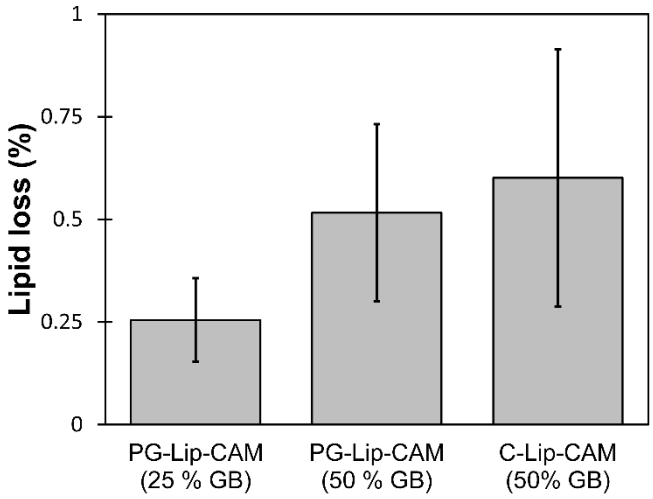

Figure 3: Effect of glass beads on the lipid loss (n ≥ 3).

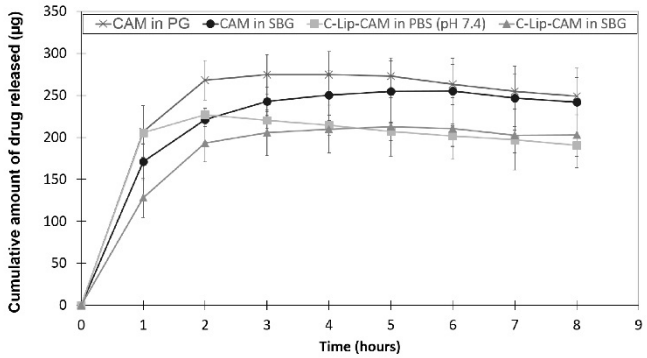

Figure 4: Drug release from conventional liposomes-in-hydrogel formulation compared to the release of conventional liposomes in PBS (pH 7.4), free drug dissolved in PG and free drug in SBG (n = 3).

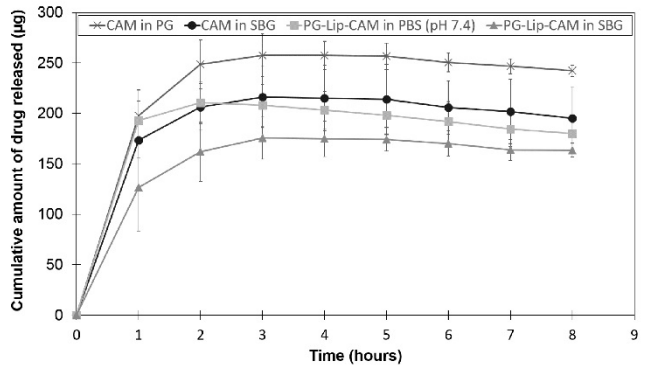

Figure 5: Drug release from PG-liposomes-in-hydrogel formulation compared to the release of PG-liposomes in PBS (pH 7.4), free drug dissolved in PG and free drug in SBG (n = 3).

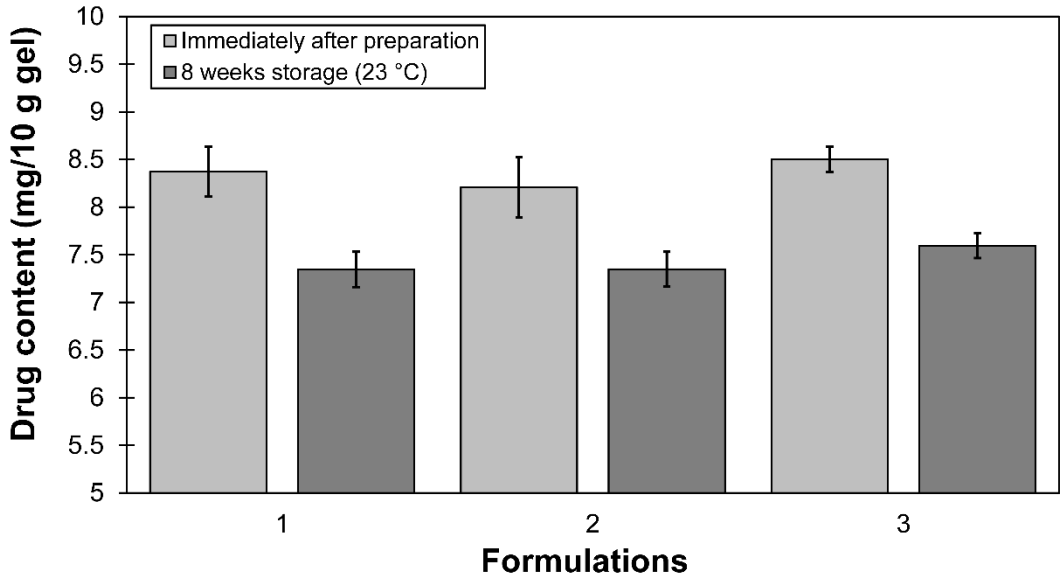

Figure 6: Changes in drug content in liposomes-in-hydrogel formulations upon storage (n = 3).

Table 1. Effect of centrifugation speed on liposomal characteristics (n = 3).

Centrifugation speed (rpm)	**Vesicle diameter (nm ± SD)**	**PI ± SD**
2400	264 ± 8.3	0.23 ± 0.026
3500	214 ± 12.5	0.20 ± 0.035

Table 2. Characteristics of liposomes (n ≥ 3).

Formulation	**Centrifugation time**	**Mean diameter (nm ± SD)**	**PI ± SD**	**Entrapment efficiency (% ± SD)**
C-Lip-CAM (50 % GB)	50 min	282 ± 30	0.13 ± 0.02	51.2 ± 2.3
PG-Lip-CAM (25 % GB)	2 min 55 sec	278 ± 66	0.31 ± 0.03	47.7 ± 3.6
PG-Lip-CAM (50 % GB)	2 min 55 sec	230 ± 51	0.25 ± 0.04	49.7 ± 2.8

Key: C = Conventional, PG = propylene glycol, Lip = Liposomes, CAM = Chloramphenicol, GB = Glass beads.

Table 3. Drug homogeneity in the final liposomes-in-hydrogel formulations expressed as the average drug content (n ≥ 3).

Formulation	**Drug content (mg/10 g gel ± RSD)**
1	8.37 ± 3.15 %
2	8.21 ± 3.90 %
3	8.50 ± 1.53 %

Table 4. Changes in liposomal size upon storage (n = 3).

	Immediately after preparation		***8 weeks (4 °C)***	
Formulation	**Mean diameter (nm ± SD)**	**PI ± SD**	**Mean diameter (nm ± SD)**	**PI ± SD**
1	201.2 ± 3.5	0.240 ± 0.01	201.2 ± 2.9	0.197 ± 0.02*
2	288.8 ± 59.0	0.288 ± 0.03	242 ± 23.5*	0.239 ± 0.02*
3	199.8 ± 0.9	0.206 ± 0.01	203.9 ± 2.3*	0.190 ± 0.01*

*) Significant difference ($p < 0.05$) observed upon storage.