Successful co-encapsulation of benzoyl peroxide and chloramphenicol in liposomes by a novel manufacturing method - dual asymmetric centrifugation (DAC)

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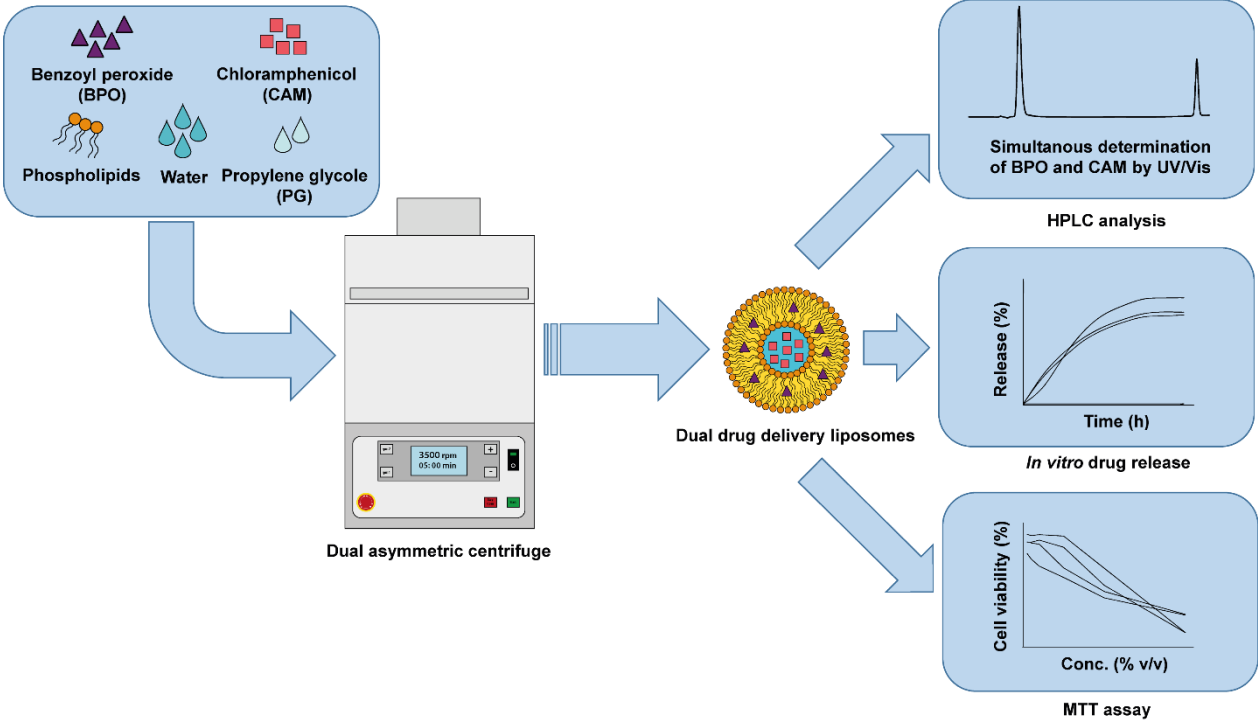

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Graphical abstract

Abstract

Encapsulation of more than one active pharmaceutical ingredient into nanocarriers such as liposomes is an attractive approach to achieve a synergic drug effect and less complicated dosing schedules in multi-drug treatment regimes. Liposomal drug delivery in acne treatment may improve drug efficiency by targeted delivery to pilosebaceous units, reduce adverse effects and improve patient compliance. We therefore aimed to co-encapsulate benzoyl peroxide (BPO) and chloramphenicol (CAM) into liposomes using the novel liposome processing method – dual asymmetric centrifugation (DAC). Liposomes were formed from soybean lecithin, propylene glycol and distilled water (2:1:2 w/v/v ratio), forming a viscous liposome dispersion. Liposomes containing both drugs (BPO-CAM-Lip), single-drug (BPO-Lip and CAM-Lip), and empty liposomes were prepared. Drug entrapment of BPO and CAM was determined by a newly developed HPLC method for simultaneous detection and quantification of both drugs. Encapsulation of around 50% for BPO and 60% for CAM respectively was obtained in both single-drug encapsulated formulations (BPO-Lip and CAM-Lip) and co-encapsulated formulations (BPO-CAM-Lip). Liposome sizes were comparable for all liposome formulations, ranging from 130 to 150 nm mean diameter, with a polydispersity index < 0.2 for all formulations. CAM exhibited a sustained release from all liposomal formulations, whereas BPO appeared retained within the liposomes. BPO retention could be attributed to its poor solubility. However, HaCaT cell toxicity was found dependent on BPO released from the liposomes. In the higher concentration range (4% v/v), liposomal formulations were less cytotoxic than the corresponding drug solutions used as reference. We have demonstrated that DAC is a fast, easy, suitable method for encapsulation of more than one drug within the same liposomes.

Key words: dual asymmetric centrifugation, co-encapsulation, liposomes, chloramphenicol, benzoyl peroxide, acne

1. Introduction

Acne vulgaris (or simply acne), is a chronical inflammatory skin disease, affecting approximately 90% of the global population during adolescence. However, the skin condition might also continue into adulthood, having a severe impact on self-esteem and general quality of life (Williams et al., 2012). Current guidelines for management of acne recommend combination therapy with topical retinoid and benzoyl peroxide (BPO) as first-line treatment in mild to moderate acne (Walsh et al., 2016; Zaenglein et al., 2016).

The use of multiple drugs in combination to achieve a synergistic effect in treatment of diseases is a well-known strategy for improved therapeutic outcome, and has been adapted as standard first-line treatment in several diseases (Durante-Mangoni et al., 2014; Fischbach, 2011; Landewé et al., 2002). The use of combination therapy is also likely to increase in the future as the emergence of antibiotic resistant pathogens is associated with therapeutic failure, forcing us to develop new drugs and treatment strategies in order to assure future availability of treatment for these pathogens (Durante-Mangoni et al., 2014; Walsh et al., 2016). The drawback of conventional combination therapy is that it is more complex compared to monotherapy and often involves the use of several dosage forms that increase the possibility of patient non-adherence during the treatment. Co-encapsulation and co-delivery of drugs using nanocarriers such as liposomes has the potential to simplify the therapy, since drugs incorporated into the same carrier will be delivered to the target simultaneously.

Today, chloramphenicol (CAM) is mainly used for treatment of eye and ear infections, as its oral and intravenous use are limited by its bone marrow toxicity. However, topical administration of CAM for treatment of local invasive skin infections offers rapid delivery, and since nanocarriers might circumvent the limitations of the drug associated with systemic toxicity, CAM is already recognized as a good candidate for encapsulation into nanocarriers for topical treatment of skin infections (Ingebrigtsen et al., 2016; Kalita et al., 2015). Moreover, it has been demonstrated that old antibiotics such as CAM remain active against prevalent resistant bacterial isolates owing to their rather low-level use in the past (Fayyaz et al., 2013).

Although BPO is preferred first-line therapy for the treatment of mild to moderate acne, it is known that BPO might cause local adverse reactions such as cutaneous irritation or dryness, erythema and

scaling (Foti et al., 2015). In addition, BPO is also prone to degradation and is unstable in solution (Chellquist and Gorman, 1992).

Liposomes were first suggested as a topical drug delivery system by Mezei and Gulasekharam already in 1980 (Mezei and Gulasekharam, 1980), and have been studied quite intensively as skin nanocarriers since then; however co-encapsulation of multiple drugs into liposomes is a relatively new approach. Recently, several studies have investigated the potential of this approach, and reported liposomal encapsulation of drug combinations such as topotecan/vincristine (Zucker et al., 2012), irinotecan/doxorubicin for anticancer therapy (Shaikh et al., 2013), and BPO/tretionin combination for the treatment of acne (Gupta et al., 2010).

Dual asymmetric centrifugation (DAC), is a laboratory mixing system, which differs from conventional centrifugation by adding a secondary rotation around its own axis (Massing et al., 2008). The suitability and versatility of this new technique for liposome production has already been demonstrated in a number of studies (Adrian et al., 2011; Ingebrigtsen et al., 2016; Massing et al., 2008; Meier et al., 2015; Parmentier et al., 2014; Tian et al., 2010). Liposomes can be produced in a gentle way, and aseptic manufacturing is facilitated, as the sample holder is a closed container (Hirsch et al., 2009). Since the technique provides a closed system and eliminates the need of organic solvents, it has several advantages compared to other available production methods (Massing et al., 2008; Wagner and Vorauer-Uhl, 2011). Concentrated vesicular phospholipid gels (VPGs) are formed as an intermediate product in this process, making it especially suitable for obtaining high liposome content in secondary vehicles such as creams, lotions or hydrogels (Ingebrigtsen et al., 2016).

In this study, we aimed to investigate the suitability of DAC for the production of co-encapsulated BPO and CAM in liposomes. In addition, a new HPLC method for simultaneous detection of BPO and CAM was developed to facilitate fast and easy drug quantification during characterization of the investigated formulations. The system's toxicity was compared to single-drug liposomes and corresponding drug solutions.

2. Materials

Acetone, acetonitrile CHROMASOLV®, 99.8% anhydrous acetic acid, ammonium molybdate, Luperox® A75FP (75% benzoyl peroxide; remainder water), chloramphenicol, chloroform, Dulbecco's Modified Eagle's Medium with 1% L-glutamine, ethanol 96%, Fiske-Subbarow reducer agent, isopropanol, monobasic potassium phosphate, RPMI-1640 medium, sodium chloride and Triton X-100 were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Disodium hydrogen phosphate dihydrate, and 30% water-free hydrogen peroxide were purchased from Merck (Darmstadt, Germany). Concentrated sulfuric was the product of May and Baker LTD (Dagenham, England). Propylene glycol was purchased from NMD – Norwegian Medical Depot (Oslo, Norway). Lipoid S 100 (soybean lecithin, > 94% phosphatidylcholine) was a kindly provided gift from Lipoid GmbH (Ludwigshafen, Germany).

3. Methods

3.1. Liposome preparation

Liposomes were made by dissolving 200 mg Lipoid S 100 (PC) in 10 ml of acetone:chloroform (1:2 v/v) together with 3 mg BPO (equal to 4 mg Luperox® A75FP) and/or 20 mg of CAM.

For all formulations, the organic solvents were removed at room temperature under a stream of nitrogen using the setup described elsewhere (Ingebrigtsen et al., 2016). The lipid film was hydrated with 100 µl propylene glycol (PG) and 200 µl distilled water. Finally, glass beads (Ø = 2 mm) equal to 50% (w/w) of the total sample weight were added before storage overnight at 4 °C.

Liposome size was reduced by DAC using a Speedmixer (DAC 150.1 FVZ-K Speedmixer, Synergy Devices Ltd., High Wycombe, UK). All samples were processed for 40 minutes at maximum speed, corresponding to 3500 rpm. The VPGs were diluted with distilled water to produce liposomal dispersions with a total volume of 2 ml, suitable for further characterization.

3.2. Liposome characterization

3.2.1. Entrapment efficiency

Free unentrapped BPO crystals were separated from entrapped liposomal BPO by filtration through a 0.22 µm filter (Acrodisk, Pall Corporation, New York, USA). Total amount of BPO in the filtrate and in the original sample was quantified by HPLC analysis. Drug entrapment of BPO was calculated as described in Equation 1.

Equation 1:

$$\frac{\text{Amount of BPO}_{\text{filtrate}}}{\text{Amount of BPO}_{\text{original sample}}} \times 100 = \text{ Entrapment efficiency (\%)}$$

The entrapment of CAM was determined after separation of unentrapped CAM from the formulations by dialysis. Liposomal samples were dialyzed against distilled water using a cellulose membrane with a M_w cut-off of 12–14000 Da (Medicell International Ltd., London, UK). After 4 hours dialysis, CAM concentration was quantified by HPLC. Drug entrapment efficiency of CAM was calculated using Equation 2.

Equation 2:

$$\frac{\text{Amount of CAM}_{\text{dialyzed sample}}}{\text{Amount of CAM}_{\text{original sample}}} \times 100 = \text{ Entrapment efficiency (\%)}$$

3.2.2. Size measurements

Size measurements were performed on a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK), using the following setting; sample temperature 25 °C, equilibration time 180 seconds, and number of runs was set to automatic. All liposome dispersions were diluted 1:100 with filtered distilled water prior to measurement.

3.2.3. Zeta-potential measurements

The zeta-potential of our liposome dispersions was also determined using the Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK), with the same equilibration time and measurement temperature as for the size measurements (3.2.2). All measurements were performed in triplicate, using the automatic setting for drive voltage and number of runs per measurement. Liposome samples were diluted 1:10 with filtered distilled water prior to measurement.

3.2.4. *In vitro* drug release

Franz diffusion cells (PermeGear, Bethlehem, USA) with a surface area of 0.64 cm^2 and receptor volume of 5.0 ml were used for the drug release studies. The cells were mounted in a stirrer (V6A-02, PermeGear, Bethlehem, USA) and connected to a heating circulator (F12-ED, Julabo Laboratechnik, Seelback, Germany). All experiments were performed in triplicates at 25 °C using a cellophane membrane (Max Bringmann KG, Wendelstein, Germany). Phosphate buffered saline (PBS) (2.98 g/l disodium hydrogen phosphate dihydrate, 0.19 g/l monobasic potassium phosphate, and 8 g/l sodium chloride) with pH 7.4 was the chosen acceptor phase for these experiments. Free drug was removed from the liposome dispersion (as described in section 3.2.1) prior to testing. The sample volume (both liposome formulations and the control drug solution) in the donor compartment was 300 µl. Samples of 500 µl were collected from the acceptor chamber every hour for 8 hours. All samples were subsequently analyzed by HPLC. After each sampling, the acceptor cells were refilled with 500 µl fresh acceptor phase. To avoid evaporation during the experiments, a triple layer of parafilm was used to cover both the donor compartment and the sampling port to the acceptor chamber.

3.3. HPLC analysis

A HPLC method was developed to facilitate simultaneous detection and analysis of BPO and CAM. The instrumentation used comprised a Waters e2795 Separations Module, a Symmetry C18 column guard and a XSELECT CSH C18 (2.5 mm; 3.0 75 mm) column XP connected to a Waters 2489 UV/Visible detector (Waters, Dublin, Ireland). Detection wavelength was set to $\lambda = 278$ nm. Analyses were run using a flow rate of 0.6 ml/min and gradient flow conditions (Figure 1) at 25 °C, with an injection volume of 20 µl, run time of 10 minutes and a delay of 2 minutes between each injection.

For the *in vitro* drug release studies (section 3.2.4), the analyses were performed in dual wavelength mode, with detection at both 238 nm and 278 nm. Since BPO has a higher absorbance at 238 nm, this wavelength allowed a lower BPO concentration to be quantified.

3.4. Quantification of lipids

The amount of lipids in the liposome formulations were quantified by a phosphorus assay (Bartlett, 1959) with modifications previously described by our group (Ingebrigtsen et al., 2016). In short,

liposomes (50 µl) were diluted up to a total volume of 10 ml with distilled water. After dilution, 1 ml of each sample was mixed with 0.5 ml 10 N sulfuric acid and heated at 155 °C for 3 hours. Two drops of 30% water-free hydrogen peroxide were then added, before the samples were heated again at 155 °C for an additional 1.5 hour. After heating, samples were mixed with 4.6 ml 0.22% ammonium molybdate solution and 0.2 ml Fiske-Subbarow reducer agent before additional 7 minutes heating at 100 °C. Samples were finally analyzed calorimetrically at $\lambda = 830$ nm using a SpectraMax 190 Microplate Reader (Molecular Devices, California, USA).

3.5. Cell viability assay

Cell viability was tested according to a modified method originally described by Mosmann (Mosmann, 1983). HaCaT cells were seeded on 96-well plates with a density of 2×10^5 cells/ml. Cells were cultured overnight in a humidified atmosphere of 5% CO_2 at 37 °C. Liposomal samples were applied in the assay after removal of unentrapped drug (as described in section 3.2.1). The investigated formulations, both liposome dispersions and ethanol drug solutions, were added after dilution in growth medium resulting in final sample concentrations between 0.1 and 4.0 % (v/v), corresponding to between 5 to 200 µg/ml CAM and 0.75-30 µg/ml BPO. After the treatment, cells were incubated for 4 hours before the MTT solution was added (5 mg/ml in PBS). After two additional hours of incubation, the crystallized formazan was dissolved in acidified isopropanol (0.04 N). The absorbance of the reduced MTT (formazan) was determined at $\lambda = 590$ nm on a SpectraMax 190 Microplate Reader (Molecular Devices, California, USA).

Cell survival/viability was determined relative to the positive and negative controls; 2% (v/v) triton solution and growth medium, respectively.

3.6. Statistical analysis

All statistical analyses were performed using SigmaPlot for Windows version 13 (Systat Software, Erkrath, Germany) and a confidence interval of 95%.

4. Results and discussion

Liposomes have already been proposed as a carrier to improve the effect and reduce adverse effects of different drugs used in the topical treatment of acne (Castro and Ferreira, 2008; Vyas et al.,

2014), such as clindamycin (Škalko et al., 1992), retinoid derivative tretinoin (Patel et al., 2000), adapalene (Kumar and Banga, 2016) and BPO (Patel et al., 2001). Liposomes are regarded to be biocompatible carriers that have the ability to accommodate both hydrophilic and lipophilic compounds while also protecting the compounds from the external environment. Co-encapsulation of BPO and CAM into liposomal nanocarriers might therefore provide therapeutic advantages, such as controlled release, improved drug penetration, and improved efficiency through increased stability in the carrier (Vanić, 2015).

To the best of our knowledge, no studies have so far investigated the potential of using DAC as a processing method for the entrapment of more than one drug into the same liposomes. If proven feasible, this will make DAC an even more versatile manufacturing method for liposomes. The possibility of performing both the size reduction of liposomes, and mixing them into a final vehicle (such as liniments, hydrogels or creams) in high concentrations using only one machine, makes this method especially relevant for topical skin formulation intended for treatment of damaged or diseased skin. Moreover, the method offers a potential to perform the whole manufacturing process in aseptic conditions.

However, the production of liposomes containing more than one drug represents not only a processing challenge, but also an analytical one. Thus, we first had to develop a HPLC method for simultaneous quantification of CAM and BPO, needed in further characterization and evaluation of the prepared liposomal formulations.

4.1. Simultaneous detection of BPO and CAM

HPLC methods for determination and quantification of CAM (Boer and Pijnenburg, 1983; Burke et al., 1980; Ingebrigtsen et al., 2016; Sample et al., 1979) and BPO (Burton et al., 1979; Saiz et al., 2001) as a single drug have already been described in the literature. However, no reports on co-determination were available and we aimed to develop a time-efficient HPLC method capable of simultaneous detection of the two drugs.

The established in-house HPLC method for determination and quantification of CAM (Ingebrigtsen et al., 2016) was the starting point for the development of this novel HPLC method. First change made was regarding the mobile phase, where the organic solvent was changed from methanol to acetonitrile, as BPO has poor stability in methanol and decomposes readily even at 25

°C (Hongo et al., 2006). Next, the mobile phase composition and flow rate were adjusted, until BPO and CAM were adequately separated and proper retention times were obtained. The isocratic elution with 55% (v/v) acetonitrile and 45% (v/v) 0.1% acetic acid in MilliQ-water, was changed to a gradient elution, increasing the acetonitrile content linearly over 1 min to reach 95% (v/v) after 4 minutes run time (Figure 1). This, together with an increase in a flow rate from 0.4 to 0.6 ml/min allowed us to elute and detect both CAM and BPO with retention times of 1.5 and 6.8 minutes, respectively (Figure 2). As the optimal absorption wavelength for BPO and CAM is $\lambda = 238$ nm and $\lambda = 278$ nm, respectively, these wavelengths were initially chosen for our dual wavelength UV-detector. However, since both drugs gave sufficient absorption using single wavelength mode at $\lambda = 278$ nm, with drug concentrations ranging from 2.5 to 80.0 µg/ml, single wavelength mode was applied for most analyses. In the given concentration range of the drugs, the calibration curves had r^2-value of 0.9998 for CAM and 0.9999 for BPO, respectively. Limit of detection and limit of quantification for CAM were determined to be 0.6 and 1.9 µg/ml, and 0.2 µg/ml and 0.7 µg/ml for BPO, respectively. This HPLC method thus satisfied our analytical requirements for further characterization of liposomal drug formulations and a suitable run time of 10 minutes was applied in the method.
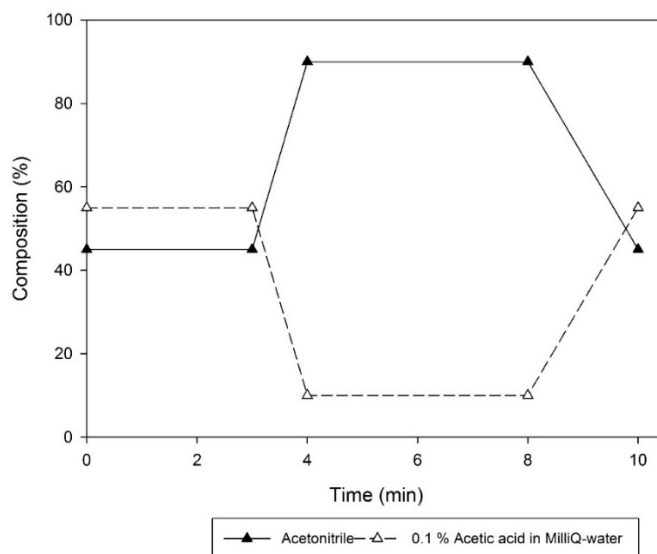

Figure 1: Mobile phase composition used for simultaneous detection of BPO and CAM by HPLC.
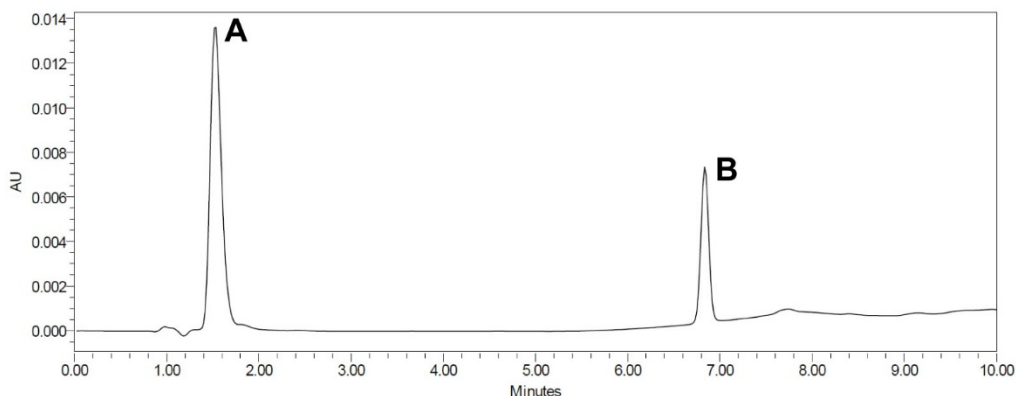
Figure 2: Representative HPLC chromatogram indicating simultaneous detection of both CAM (A, RT = 1.5 min) and BPO (B, RT = 6.8 min) at a concentration of 2.5 µg/ml, detected at 278 nm.

4.2. Liposome characteristics

Co-encapsulation of two or more active ingredients into the same liposomal bilayer may change the characteristics of the liposomes in different ways, depending on the lipophilicity, size and charge/polarity of the drugs entrapped/incorporated. If the two drugs interact with each other, the release profile of the individual drug from the liposome carrier might change, ultimately influencing the biological effects of the drug. We therefore aimed to compare single-drug liposomes with the dual-drug containing liposomes. The DAC method has already been proven to circumvent some limitation of conventional liposome preparation methods, enabling processing of concentrated liposome dispersions with defined size distribution, high drug encapsulation ratios and a low polydispersity index (PI) (Ingebrigtsen et al., 2016; Massing et al., 2008). For optimization of the different formulations, we therefore aimed to make as few changes as possible to the already established DAC liposome processing method (Ingebrigtsen et al., 2016).

4.2.1. Drug entrapment and size distribution

As seen in Table 1, only minor and insignificant differences in the size and size distribution were observed between empty, single- and dual-drug containing liposomes made by DAC. Results indicated low PI (< 0.2), and the mean liposome size varied from 131.6 ± 9.1 nm (empty liposomes) to 146.2 ± 7.6 nm (BPO-lip), respectively. These results shows that the DAC method give

reproducible results regarding the mean size and size distribution, independently of which drug is loaded into the carrier, at least for these four formulations. It is also encouraging that the PI values obtained are so low. Our previous work (Ingebrigtsen et al., 2016), showed that CAM-containing liposomes (CAM-Lip) prepared with DAC, but without PG added, exhibited a PI of 0.13 ± 0.03. This also demonstrate the robustness and reproducibility of the DAC method. Both BPO and CAM had no impact on the liposome size in the concentrations applied. Moreover, no significant difference in ζ-potential was observed between the different formulations. Considering the entrapment efficiency, the entrapment of BPO and CAM in the combined liposomes (BPO-CAM-Lip) proved to be very similar to the entrapment of the two drugs in respective single-drug liposomes (BPO-Lip and CAM-Lip), indicating that BPO and CAM are not affected by the presence of the other drug (Table 1). The observed lack of interaction between BPO and CAM might be explained by the higher lipophilicity of BPO (log $K_{ow} = 3.46$) compared to CAM (log $K_{ow} = 1.14$). The higher lipophilicity of BPO results in a stronger association with the lipid bilayer as compared to CAM; thus it can be postulated that BPO is accommodated within the lipid bilayers whereas CAM is mostly entrapped within the aqueous part of the vesicles.

Interestingly, the entrapment efficiency for CAM in both the combination and single-drug formulation was higher than the entrapment value of 49.7 ± 2.8 % obtained in our previous study (Ingebrigtsen et al., 2016). This increase in drug entrapment might be explained by the increased centrifugation time from 2 minutes and 55 seconds to a total of 40 minutes, allowing for a more thorough mixing of PC and CAM. The improved entrapment values obtained with increased mixing time; drug:lipid ratios of 60.6 ± 3.3 and 60.7 ± 2.0 µg/mg, compares well with previously reported liposomal CAM-entrapment values reported with the proliposome method; drug:lipid ratio of 62.7 ± 8.6 µg/mg (Pavelić et al., 1999). Compared to other preparation methods, the CAM-encapsulation values obtained by DAC, however clearly exceeds the reported encapsulation values, i.e. CAM-encapsulation values of 32.5 ± 2.8 µg/mg was obtained for filter extruded liposomes (Engesland et al., 2015), although the authors prepared larger liposomes of around 670 nm in diameter. Also, previous studies in our group found that the encapsulation efficiency of approximately 30% (drug:lipid ratio = 31.12 ± 0.15 µg/mg) was achievable for CAM-liposomes prepared by probe sonication (Ingebrigtsen et al., 2016). Thus, the increased encapsulation achieved by DAC might be explained by the reduced water content in-between the liposome vesicles during processing and the high lipid content of VPGs. The limited availability of water to

solubilize in for CAM, forces a higher amount of drug to associate either with the lipid bilayers of the liposomes or the aqueous space within the liposomes.

An entrapment efficiency of 48 ± 8.1% (BPO-CAM-Lip) to 51 ± 7.7% (BPO-Lip) was obtained for BPO, corresponding to a drug:lipid ratio of 7.2 to 7.7 µg/mg. This is comparable to the entrapment efficiency presented in percentage previously reported by Patel et al. (Patel et al., 2001), where six liposome formulations were made with different composition of the lipid bilayer, mixing PC and cholesterol in different molar ratios. However, since entrapment values were only given as percentage, the liposomes had different composition, and larger multilamellar vesicles were applied in this study, a direct comparison is not possible. The presence of cholesterol is known to influence packing and fluidity of the lipid bilayer (Ladbrooke et al., 1968). Interestingly, Patel et al. (Patel et al., 2001) report that the entrapment of BPO was improved by cholesterol presence until a threshold concentration was reached, where further increase in cholesterol content decreased the encapsulation efficiency of the liposomes. It would be therefore interesting to investigate if cholesterol could improve the encapsulation of BPO also in DAC processed liposomes in future studies.

Taking into account that most of the active pharmaceutical ingredients applied in transdermal and dermal drug delivery exhibit a low water solubility (Haque et al., 2015), the relatively high encapsulation efficiency obtained with CAM and BPO is promising, and is suggesting that an increased number of active ingredients could be successfully entrapped into liposomes using DAC.

Table 1: Liposomal characteristics (n ≥ 3).

Formulation	Mean vesicle size (nm ± SD)	PI (AU ± SD)	Z-potential (mV ± SD)	Entrapment efficiency (% ± SD)
Empty liposomes	131.6 ± 9.1	0.14 ± 0.02	6.5 ± 2.7	-
BPO-Lip	146.2 ± 7.6	0.11 ± 0.02	9.6 ± 1.7	51.0 ± 7.7
CAM-Lip	143.4 ± 6.4	0.19 ± 0.08	8.0 ± 2.4	60.6 ± 3.3
BPO-CAM-Lip	137.6 ± 5.5	0.08 ± 0.02	5.3 ± 3.0	48.1 ± 8.1 (BPO) 60.7 ± 2.0 (CAM)

4.2.2. Loss of lipids

As already mentioned, one of the main advantages of the DAC method is that all processing is performed in the same container, and that no transfer of material is required during the manufacturing. Consequently, the loss of material is expected to be avoided, contrary to other processing methods such as high-pressure homogenization, probe sonication and filter extrusion. To demonstrate this advantage, a phosphorus assay was performed to determine the lipid recovery from DAC. Since processing, subsequent dilution and mixing of the VPGs to liposomal dispersion take place in the same container, the only loss of lipids we were able to quantify was due to a volume loss during sample transfer and characterization. As seen in Figure 3, the lipid recovery was around 100% for all formulations except BPO-Lip, which had a recovery of 96%. The effect of volume loss on lipid recovery is further illustrated by evaluating the recovery after the separation of unentrapped drug from entrapped drug. BPO-Lip, which were filtered through a 0.22 µm filter to remove unentrapped drug, exhibited no loss of lipids, while CAM-Lip and BPO-CAM-Lip that were dialyzed or dialyzed and filtered, respectively, lost around 13% of original lipid content.
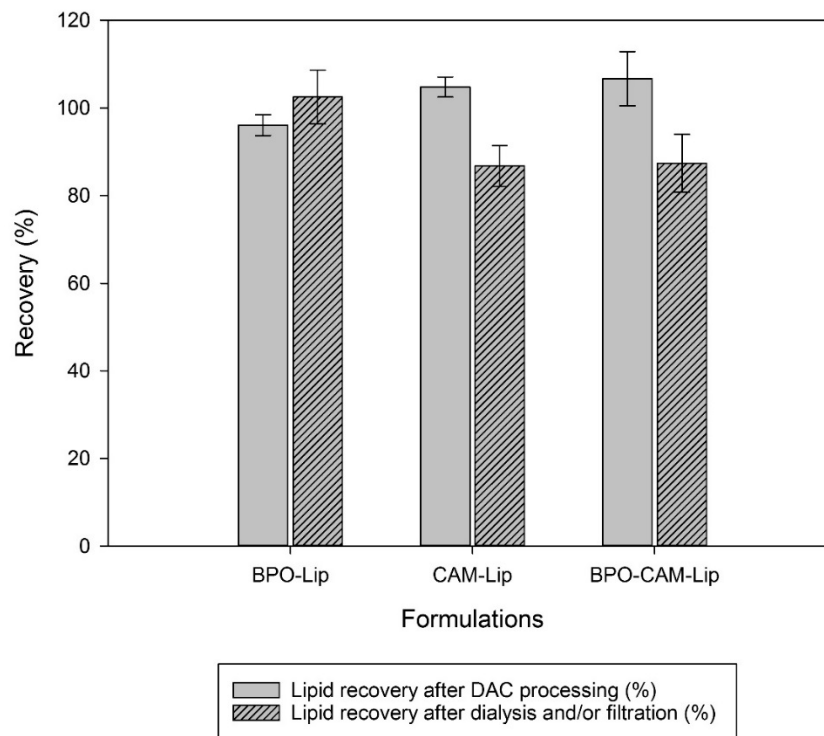

Figure 3: Lipid recovery (n = 3, except for BPO-Lip where n = 2).

4.3. *In vitro* **drug release**

Co-encapsulation of drugs into the same nanocarrier might change the drug release profile of the individual drugs if an interaction between the two drugs takes place in the liposome bilayer, or the packing or integrity of the liposome membrane is changed. To investigate if this was the case for our combination formulation, BPO-CAM-Lip, we performed an *in vitro* drug release study, using a standard Franz diffusion cell system, to compare the release profile from all the three liposome formulations (BPO-Lip, CAM-Lip and BPO-CAM-Lip, respectively). Since preliminary studies with the two drugs in solution alone and in combination showed that the release profile did not change for neither BPO nor CAM when combined, we decided to use a solution of BPO-CAM in PG as the common control during the release experiments of the liposomal formulations.

The liposomal dispersions are expected to provide a sustained release of the drugs as compared to drugs in solution. Our results (Figure 4) showed that CAM demonstrated a sustained release, and the release was not influenced in any way by the presence of BPO in the combined formulations, as the CAM release from BPO-CAM-Lip was the same as the release from CAM-Lip. However, BPO was retained in the control solution (BPO-CAM-Sol), the BPO-Lip formulation and the BPO-CAM-Lip formulation (Figure 4). The observed absence of release of BPO during the release experiment might be explained by limited water solubility of BPO. Although there are successful reports that demonstrate *in vitro* release from BPO- and BPO-tretinoin-containing niosomes (Goyal et al., 2015; Gupta et al., 2014), the presence and amount of organic solvent in the acceptor phase used in these studies would most likely interfere with the integrity of the liposomal carrier. Moreover, it would also limit our ability to observe the potential sustained release from the liposome formulation. To improve BPO solubility in the acceptor chamber, while avoiding disruption of the liposome membrane, we therefore performed some preliminary release studies using a dispersion of empty liposomes (size < 400 nm) and PEG 400:water (9:1 v/v) mixture as the acceptor phases, respectively. Despite these changes, we still failed to detect any release of BPO from any of the formulations, and thus decided to perform the release study with PBS as the acceptor phase. In order to fully reveal how the liposome formulation influence the partition of the drug into the skin, an artificial *in vitro skin model* or an *in vivo* model could have been used (Flaten et al., 2015). However, since liposomes for topical application to the skin also require a suitable vehicle for the final product development, such as hydrogel or a cream/lotion, our current main

objective was to observe possible interactions of the drugs in the liposomal carrier and determine whether the carrier influenced the drug release as compared to non-encapsulated drugs. Moreover, we indirectly proved that BPO is released from the nanocarrier in the cell viability assay (Table 2 and Figure 5), as BPO in all formulations gave the most prominent effect of the cell viability.
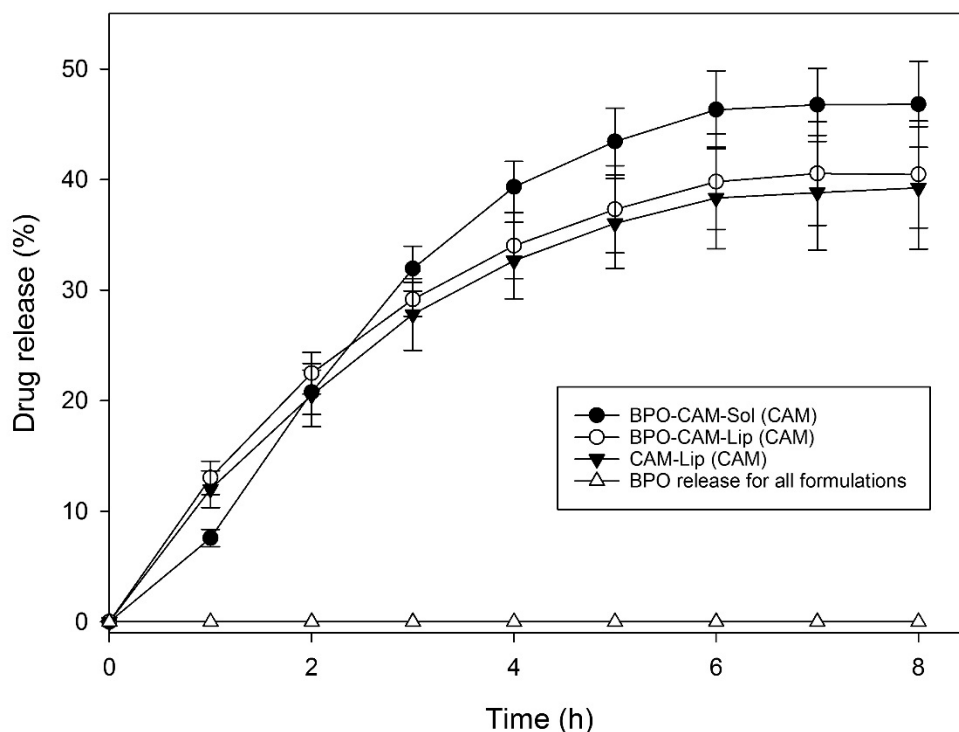

Figure 4: *In vitro* release of BPO and CAM from liposomes (n = 3).

4.4. Influence of liposomal BPO and CAM on cell viability

For skin infections such as acne, the topical route of administration is preferred due to the delivery of a higher concentration of drug into the desired infected area relative to what is achieved when the same drug is administered systemically. In addition, oral antibiotics are associated with possible severe adverse effects and high cost, as well as development of antimicrobial resistance. Local treatment of acne is less likely to cause systemic adverse effects and toxicity, but are usually associated with local irritation of the skin (Vyas et al., 2014). Liposomal drug delivery in topical treatment might be advantageous, as drug penetration and retention might be increased, a sustained release provided, lowering the required drug concentration needed to obtain effect from treatment

(Castro and Ferreira, 2008). In addition, poorly soluble drugs might be solubilized by the lipid bilayer of the liposomes (Yang et al., 2009), and liposomal targeting of pilosebaceus structures is achieved (Lieb et al., 1992). To evaluate the relative skin irritation as an effect of liposomal delivery, we applied the MTT assay on HaCaT cells. Cell viability was measured at drug concentrations corresponding to a liposome sample concentration between 0.1-4.0% (v/v), equivalent to 5-200 μg/ml CAM and 0.75-30 μg/ml BPO, respectively. To determine the potential cytotoxic effect of empty vesicles, we also investigated the effect of ethanol and empty liposomes in the same concentration range as applied in the drug formulations. The results showed that empty liposomes were more toxic (87.7 ± 1.3% cell viability) than ethanol (96.4 ± 1.8% cell viability). A decrease in proliferation of keratinocytes when incubated with liposomes *in vitro* has previously been reported by Bonnekoh et al. (Bonnekoh et al., 1991) and Pitto et al. (Pitto et al., 1999), and could be linked to increased cellular lipid fluidity of keratinocytes during the incubation (Bonnekoh et al., 1991). Pitto et al. also observed that the cells associated more efficiently with liposomes that contained soya lecithin, the lipid applied in this study (Pitto et al., 1999). An innate toxicity of the carrier itself or a closer association between the carrier and the cell might also explain the higher cytotoxicity exhibited by CAM in liposomal formulation (CAM-Lip) than in solution (CAM-Sol). Liposome formulations containing BPO generally showed a lower toxicity as compared to the BPO solution, indicating that liposomes are able to protect HaCaT cells from the toxic effects of BPO, most probably caused by a delayed release of the drug from the carrier, thereby reducing BPO's interaction with the cells. The presence of CAM in liposomes did not seem to increase the cytotoxicity of BPO, since both BPO-Lip and BPO-Sol exhibited the same toxicity as CAM-BPO-Lip and CAM-BPO-Sol (Table 2).

Table 2. Cell toxicity post dilution 1:25 in growth medium (n = 3).

Samples name	Formulation (diluted 1:25 (corr. to 4% v/v)	Cell viability post treatment (% ± SD)
RPMI	Growth media (negative control)	99.7 ± 1.5
Triton 2%	Positive control	9.1 ± 0.6
Ethanol	Vehicles	96.4 ± 1.8
Empty liposomes		87.7 ± 1.3
CAM-BPO-Lip	Liposome formulation	28.3 ± 1.3
CAM-Lip		78.4 ± 1.3
BPO-Lip		28.9 ± 0.6
CAM-BPO-Sol	Drug dissolved in 96% ethanol	12.7 ± 0.2
CAM-Sol		95.5 ± 2.3
BPO-Sol		12.8 ± 0.8

Since only BPO-containing liposomes exhibited cytotoxicity at higher concentration, only the BPO-containing formulation were included in concentration-dependency experiments (Figure 5). Interestingly, the results indicate that the cytotoxicity of the liposomes attributed more to the total toxicity of the sample at lower sample concentrations, further supporting the reported findings of Bonnekoh et al. (Bonnekoh et al., 1991) and Pitto et al. (Pitto et al., 1999) regarding the influence of liposomes on cell proliferation. At lower concentrations (< 3-4%, v/v), dual-drug liposomes exhibited relatively higher toxicity than the liposomes containing only BPO (Figure 5).
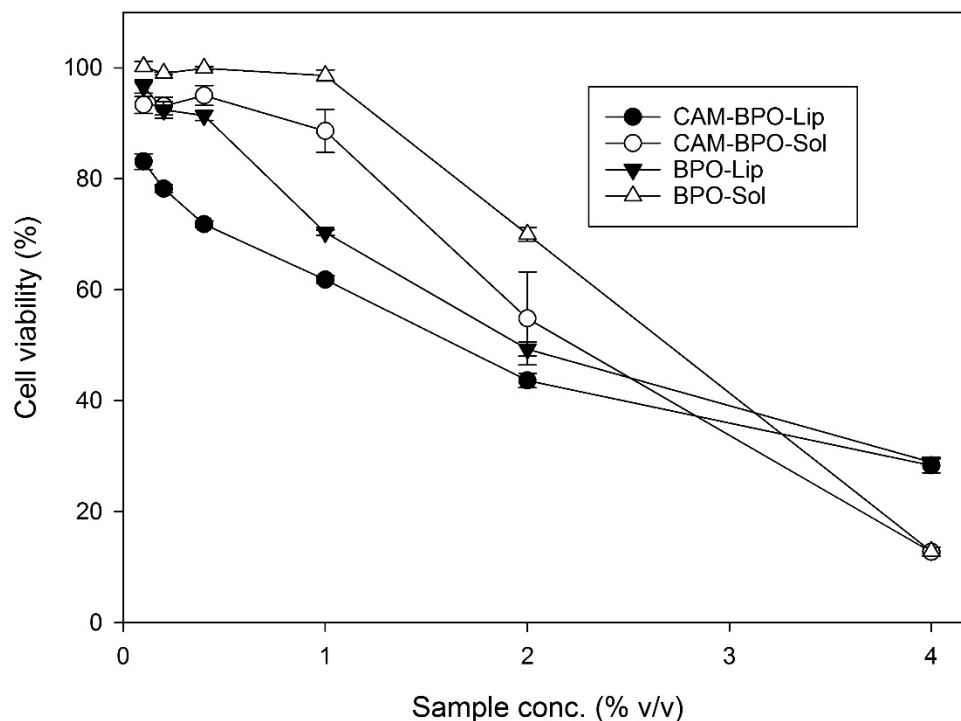
Figure 5. Effect of formulation concentration on the HaCaT cells viability (n = 3).

The general BPO toxicity observed in this study is not surprising, since BPO is known to exhibit a keratolytic effect in addition to its antimicrobial properties (Waller et al., 2006). BPO-containing drug products are also known to cause considerable skin dryness and irritation, sometimes leading to the discontinuation of the treatment (Tripathi et al., 2013). Marketed products such as ointments or washes containing BPO are today available in a higher concentrations range, from 2.5 to 10% (w/v). However, Mills et al. (Mills et al., 1986) compared the efficacy of 2.5, 5 and 10% BPO in gels after an 8 week, twice-daily treatment regime, and concluded that all three formulations had similar efficacy in relation to reduction of number of papules and pustules. Moreover, the use of 10% BPO gel formulation was associated with higher frequency and severity of adverse effects; peeling, erythema and burning, compared to the gel formulation containing only 2.5% BPO. Okamoto et al. (Okamoto et al., 2016) also reported the minimum inhibitory concentration (MIC) values for BPO in the range of 128 to 256 µg/ml while investigating *in vitro* activity of BPO against *P. acnes*, and Gupta et al. determined BPO's MIC-value for *S. epidermidis* to be 28 µg/ml (Gupta

et al., 2014). Based on these evidences, our liposome formulation containing approximately 750 µg/ml BPO should have a sufficiently high concentration to demonstrate a pronounced effect against both *P. acne* and *S. epidermidis*. It would therefore be interesting to see if it is possible to decrease BPO's toxicity further by optimization of the liposomal carrier system and to include the BPO-containing liposomes in a suitable gel vehicle. Finally, the lack of cytotoxicity observed for CAM in our cell toxicity study makes it an even more promising antibiotic for further application in the topical liposomal formulations.

5. Conclusion

We have successfully demonstrated that DAC is a suitable method for co-encapsulation of BPO and CAM into a liposomal carrier. Co-encapsulation could be performed without any detectable interaction or influence of the two drugs on liposomal characteristics or release profiles. In addition, we were also able to develop a novel HPLC method for the simultaneous detection of BPO and CAM by HPLC-UV. Toxicity of both drugs were independent of their presence as single- or dual-drug formulations.

6. References

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