Drug Development and Industrial Pharmacy

ISSN: 0363-9045 (Print) 1520-5762 (Online) Journal homepage: http://www.tandfonline.com/loi/iddi20

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To cite this article: Nina Dragicevic, Danina Krajisnik, Jela Milic, Alfred Fahr & Howard Maibach (2018): Development of hydrophilic Gels containing Coenzyme Q_{10}-loaded Liposomes: Characterization, Stability and Rheology measurements, Drug Development and Industrial Pharmacy, DOI: 10.1080/03639045.2018.1515220

To link to this article: https://doi.org/10.1080/03639045.2018.1515220

Accepted author version posted online: 22 Aug 2018.

Development of hydrophilic Gels containing Coenzyme Q_{10}-loaded Liposomes: Characterization, Stability and Rheology measurements

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Abstract

Objective: The aim of this study was to develop, characterize and evaluate stability of a gel containing coenzyme Q_{10} (Q_{10})-loaded liposomes, and enhance the stability of Q_{10} in the nanocarrier-containing gel compared to the conventional gel.

Methods: Q_{10}-loaded liposome dispersions prepared from unsaturated or saturated lecithin, were characterized for particle size, polydispersity index (PDI), zeta-potential, pH value, oxidation index, Q_{10}-content and morphology, and incorporated into carbomer gel. Liposome gels and liposome-free gel were analyzed for flow properties, pH values, Q_{10}-content, and liposomes size and PDI (liposome gels), 48h after preparation and in predetermined time intervals during 6 months storage at different temperatures in order to predict their long term stability.

Results: Liposomes were of small particle size, homogeneous, negatively charged, and their incorporation into gel did not significantly change ($p>0.05$) their particle size and PDI. All gels revealed non-Newtonian, shear-thinning plastic flow behavior during storage with no marked changes in rheological parameters. Storage of gels did not significantly influence the pH value ($p>0.05$), while it significantly decreased Q_{10}-content ($p<0.05$). Q_{10} was significantly more ($p<0.05$) stable in liposome gel containing unsaturated lecithin liposomes (G1) than in gel containing saturated lecithin liposomes (G2) and liposome-free gel (G3).

Conclusion. Q_{10}-loaded liposome gel G1 was the optimal formulation, since during storage at different temperatures, it did not show significant increase in liposome size and PDI, it provided significantly higher stability for Q_{10} than other gels and its pH value was suitable for skin application. Due to limited Q_{10}-stability it should be stored at 4°C.

Keywords: **liposome, liposome gel, coenzyme Q_{10}, carbomer gel, stability, rheology**

Introduction

The application of coenzyme Q_{10} (Q_{10}) in pharmaceutical industry has grown significantly in the past decade i.e. it has been successfully applied in medicine, cosmetics and nutraceuticals [1]. Coenzyme Q_{10} is a highly effective liposoluble non-enzymatic antioxidant which represents the first line of antioxidant defense [2]. Q_{10} has been known since 1950, but became commercially available since its isolation from tobacco plants. Biotechnological or synthetic production is nowadays possible [2].

Q_{10} has radical scavenger and bioenergetical properties. It is already known for its efficacy in the area of neurodermatitis, psoriasis, periodontitis, external substitution under stress, adiposity, immune support and has many other benefits [3]. Q_{10} is highly effective in protecting keratinocytes from DNA damage induced by UVA radiation and also in preventing photoageing *in vivo* with a reduction in wrinkle depth [4]. Because of its beneficial effects, i.e. its antioxidant activities against environmental aggressions and photoageing, Q_{10} has recently made its way into many pharmaceutical and cosmetic products. However, Q_{10} can be easily oxidized, especially under aerobic conditions and light exposure. In order to enhance the photostability of Q_{10}, it may be incorporated into different nanocarriers. Incorporation of Q_{10} into nanostructured lipid carriers (NLC) significantly improved the photo-stability of Q_{10} [5]. In addition, Q_{10} is liposoluble and hence its incorporation into cosmetic formulations is complicated. Therefore, Q_{10} has been incorporated into nanocarriers, like liposomes, nanoemulsion, nanoparticles which are further added into cosmetic formulations. Generally, Q_{10} can be incorporated into various novel drug delivery carriers, which include liposomes, polymeric nanoparticles, polymeric micelles, solid lipid nanoparticles, nanostructured lipid carriers, self-emulsifying drug delivery systems, nanoemulsions, solid and aqueous dispersions [1]. Moreover, the incorporation of Q_{10} into nanocarriers would enhance its

penetration into the skin, as it has been shown for Q_{10} encapsulated in solid lipid nanoparticles (SLN) being further incorporated in a carbomer gel [6]. The skin delivery of Q_{10} was doubled with gels containing Q_{10}-loaded SLN in comparison with gels containing free Q_{10}. As for cosmetic purpose most cosmetic actives should be delivered into the skin in order to exhibit their effect, the use of nanocarriers loaded with cosmetic actives has proven to be adequate as they deliver the actives into the skin. Nanostructured lipid carriers (NLC) with a size of about 230 nm have shown to be beneficial for the dermal delivery of Q_{10}, and they increased Q_{10} skin penetration when compared to an equally sized nanoemulsion [7]. These results were in accordance with the results reported by Chen et al. [8] who showed an epidermal uptake of Q_{10} from Q_{10}-NLC being 10.11 times higher compared to that achieved by the Q_{10}-emulsion. Furthermore, dependent on the drug delivery system, also transdermal delivery of Q_{10} was reported. Namely, a tocopheryl phosphate mixture which self-assembles to form vesicular structures in hydroethanolic solutions (mean size from 101-162 nm) increased the permeation of carnosine, vitamin D3, caffeine and coenzyme Q_{10} into or through the skin [9]. As to the penetration enhancing ability, liposomes have been widely used to enhance dermal and transdermal drug delivery [10-17]. In brief, liposomes, are small, spherical vesicles consisting of amphiphilic lipids, enclosing an aqueous core, which are still highly appreciated due to some advantages over other encapsulation technologies [18]. A variety of drugs can be entrapped within liposomes. Topical delivery of liposomally encapsulated actives may offer advantages over conventional formulations since liposomes have the potential to: (a) reduce serious side-effects and incompatibilities that may arise from undesirably high systemic absorption of drugs, (b) act as a local depot for sustained release of dermally active components, (c) serve as penetration enhancers and (d) serve as a rate-limiting membrane barrier for the modulation of systemic absorption of drugs [19]. Several independent studies have shown a higher stability against UV radiation of vitamins encapsulated in liposomes

compared to conventional formulations [20-22]. An improved stability was also found in the case of retinol and ascorbyl palmitate encapsulated in liposomes stored at room temperature compared to traditional formulations [23-24].

The purpose of this study was to develop, characterize and investigate the stability of a semisolid formulation (gel) containing Q_{10}-loaded liposomes. This Q_{10}-loaded liposome gel is aimed to be applied onto the skin as a cosmetic antiaging product containing Q_{10}. In this study, as it presents our first study on semisolids containing Q_{10}-loaded liposomes, we used a hydrophilic carbomer gel as a semisolid vehicle for the incorporation of liposomes. Among different semisolid vehicles, the carbomer gel was chosen as liposomes exhibit highest stability in hydrophilic gels, i.e. in creams their structure could be disturbed due to the presence of emulsifiers. Thus, in this study, firstly two kinds of Q_{10}-loaded liposomes (with saturated or unsaturated phospholipids) were prepared and afterwards (24 h after preparation) characterized for particle size, polydispersity index (PDI), zeta potential, pH value, oxidation index and Q_{10}-content. Moreover, these physical and chemical parameters (except the zeta potential) were determined in liposome dispersions also during storage for 6 months at 4 ºC, room temperature (RT, 20 ± 2 ºC) and 40 ºC. After their preparation, Q_{10}-loaded liposome dispersions were incorporated into the gel in order to obtain Q_{10}-loaded liposome gels. The obtained two kinds of Q_{10}-loaded liposome gels and the liposome-free gel containing Q_{10} were analyzed for flow properties, 48 h after their preparation and in predetermined time intervals during 6 months storage at RT and 40 ºC. In addition, pH value and Q_{10}-content, were determined in the three aforementioned gels, 48 h after their preparation and in predetermined time intervals during 6 months storage at 4 ºC, RT and 40 ºC. Further, the two Q_{10}-loaded liposome gels were analyzed for liposome size and liposome homogeneity during 6 months storage at 4 ºC, RT and 40 ºC. The Q_{10}-loaded liposome gels, as well as the Q_{10}-

loaded liposome dispersions, which were used to prepare the liposomal gels, were also analyzed for the shape and lamellarity of liposomes.

Materials and methods

Materials

The following substances were used: non-hydrogenated soybean lecithin (Phospholipon® 80, Lipoid GmbH, Germany), hydrogenated soybean lecithin (Phospholipon® 80H, Lipoid GmbH, Germany), ubichinon (Coenzyme Q_{10}, Gfn-Selco, Germany), carbomer (Ultrez 10® Polymer, Lubrizol, USA), phenoxyethanol (and) methylparaben (and) ethylparaben (and) propylparaben (and) butylparaben (Phenonip™, Clariant, Switzerland), diazolidinyl urea (Germall™II, Ashland, USA), propylene glycol (BASF, Germany), potassium dihydrogenphosphat (Sigma-Aldrich, USA), polysorbate 80 (Tween 80, Sigma-Aldrich, USA), sodium hydroxide (Sigma-Aldrich, USA), triethanolamine (TEA) (Sigma, USA), edetate disodium (Titriplex III, Merck Millipore, USA). All other chemicals were of analytical grade and the water used was double distilled.

Preparation of Q_{10}-loaded liposomes

Liposomes composed of Phospholipon® 80 (PL 80), labeled as LD1, were prepared by the following method: Q_{10} (0.5 % w/w) and Phospholipon® 80 (10 % w/w) were dissolved in ethanol (16 % w/w) at 50 ^{o}C. The obtained solution was added at room temperature to the phosphate buffer solution (PBS) pH 6.5 under agitate stirring (10 000 rpm, 15 minutes) using the Ultra-Turrax® T 25 mixer (Ika, Labortechnik, Germany). In the case of liposomes LD2 which were prepared from Phospholipon® 80 H (PL 80H), Q_{10} and Phospholipon® 80 H (10 % w/w) were dissolved in ethanol (16 % w/w) at 65 ^{o}C. Afterwards the obtained solution (warmed at 65 ^{o}C) was added to the previously warmed (at the same temperature) phosphate

buffer solution pH 6.5 under agitate stirring (10 000 rpm, 15 minutes) using the Ultra-Turrax® T 25 mixer (Ika, Labortechnik, Germany). The most important thing in the preparation procedure is the temperature in the second stage when dissolved phospholipid mixtures (PL 80H or PL 80) with dissolved Q_{10} are added to the water phase (PBS). The temperature must be above the phase transition temperature (Tm) of phospholipids, since phospholipids have to be in fluid thermodynamical state in order to form liposomes. Therefore, the preparation of Q_{10}-loaded liposomes LD2 from PL 80 H (Tm > 50 ^{o}C) was performed at 65^{o}C, while the preparation of Q_{10}-loaded liposomes LD1 from PL 80 was performed at room temperature, as phospholipids of PL80 are already at room temperature in fluid state. These spontaneously formed multilamellar vesicles (MLV) were pressed through 200 nm size pore polycarbonate membrane to obtain unilamellar liposomes with the help of a Mini Extruder Lipofast® extrusion device (Avestin Ottawa, Canada).

Preparation of Q_{10}-loaded liposome gels and conventional gel

As a vehicle for the incorporation of the liposome dispersions, carbomer (Ultrez® 10 Polymer) was used since the compatibility of liposomes with carbomer gels was demonstrated previously [25-26]. The gel was prepared by the following procedure: carbomer resin (0.8% w/w) was dispersed in distilled water in which propylene glycol (5% w/w), edetate disodium (0.1% w/w) and the preservatives (Phenonip® 0.2% w/w and Germal® II 0.3 % w/w) were previously added and left to wet for 30 minutes. The mixture was then neutralized by dropwise addition of 10% (w/w) triethanolamine under stirring (300 rpm, 5 minutes) using the Ultra-Turrax® T 25 mixer (Ika, Labortechnik, Germany), until a transparent gel appeared.

The liposome dispersions LD1 or LD2 were mixed into the gel by an electrical mixer (200 rpm, 5 min, Heidolph RZR 2020, Germany) and liposome gels G1 or G2, were obtained, respectively. The concentration of the colloidal carriers achieved in the gels was 10% (w/w,

liposome dispersion/total), while the concentration of pure Q_{10} was 0.05% (w/w) in the gels. The control gel (gel with free Q_{10}, G3) was prepared by solubilizing first Q_{10} (0.05% w/w) in water using Polysorbat 80 (Tween® 80, 0.25% w/w) as a solubilizer and then adding all other components. Afterwards the gel was prepared as described above.

Characterization of liposomes in Q_{10}-loaded liposome dispersions and liposome gels

The diameter of vesicles, polydispersity index (PDI) and zeta potential were determined by photon correlation spectroscopy (PCS) using the Zetamaster S (Malvern Instruments, UK). The particle size was calculated from the autocorrelation function of the intensity of light scattered from particles, assuming a spherical form of particles, a medium viscosity of 0.89 mPa.s, and refractory index of 1.33. Liposome dispersions were diluted with PBS (pH 7.4) prior to the measurements, that is, 5 µL of the vesicle dispersions were diluted with 495 µL of PBS (pH 7.4). To obtain the zeta potential values of vesicles, 10 µl of dispersions were diluted with 990 µl of PBS pH 7.4.

To obtain the particle size and the PDI of vesicles incorporated in the hydrogel, 0.2 g of the hydrogel was diluted (1:10 w/w) with PBS (pH 7.4), mixed until a clear dispersion was obtained, and, afterward, the dispersion was centrifuged at 3000 rpm. Then, 50 µL of the supernatant were further diluted with 450 µL of PBS (pH 7.4) and analyzed.

PDI was used as a value of a unimodal size distribution, which ranges from 0 (homogenous dispersion) to 1 (high heterogeneity). Each sample was measured three times and the mean value is represented.

Determination of pH value in Q_{10}-loaded liposome dispersions and gels

The pH value of samples was measured directly in samples at room temperature using the pH meter (HI 8417, Hanna Instruments, USA). These values served to evaluate the chemical

stability of samples. Three measurements were performed for each sample and the mean value was calculated.

Determination of oxidation index in Q_{10}-loaded liposome dispersions

The oxidation was monitored by determination of the oxidation index using the method introduced by Klein [27]. It was measured following the protocol of Zuidam et al. [28] using the spectrofotometer (Cintra 20 GBC Spectral, USA). According to that method, ethanol is added to an specific amount of the liposome dispersion, and afterwards the UV spectar is recorded in the range from 200-300 nm. Further, the absorbances of samples are measured at 215 and 233 nm, and corrected by subtracting the absorbances measured at 300 nm, while ethanol is used as control:

Oxidation index = $A_{233\ nm} - A_{300\ nm} / A_{215\ nm} - A_{300\ nm}$

Rheological evaluation of Q_{10}-loaded gels

The Rheometer (Rheolab MC 120, Paar Physica, Stuttgart, Germany) was used to determine flow properties of fresh (48 h after preparation) gels: gel with incorporated LD1 liposomes (G1), gel with LD2 liposomes (G2) and gel without liposomes (G3)-plain/conventional gel. In order to predict their physical stability, flow properties of these gels were determined after subjecting them to long term stability testing i.e. the gels were stored at room temperature (20 ± 2^oC) and at 40 ^oC for 6 months. Measurements were performed at 20 ± 0.1^oC by using the cone/plate MK 22 (radius of measuring cone 25 mm, angle of measuring cone 1^o) measuring system. Continuous flow tests were carried out by increasing the shear rate from 0

to 200 s^{-1} and decreasing it back to 0 s^{-1}, each stage lasting 200s. Three measurements were performed for each sample and the mean value was determined.

Determination of Q_{10}-content in the samples by HPLC assay

The amount of non-degraded Q_{10} was determined quantitatively in the samples by HPLC analysis, 24 hours after samples preparation and in predetermined time intervals during 6 months storage of samples at different temperatures (4 ^{o}C, RT and 40 ^{o}C). The samples were diluted with izopropanol prior determination of the Q_{10} -content (2 g of gels with isopropyl alcohol, 1: 500 w/v; 2 g of liposome dispersions with izopropanol, 1: 1000 w/v). The HPLC system consisted of a HPLC pump (Waters M 600E, Waters Corporation, USA), a sample injector (Rheodyne 7125i, Idex Health & Science, LLC, USA), an analytical column (Chromolith Performance RP-18e, 100 mm X 4,6 mm, 5 µm, Merck, Germany) and a detector (Spectral UV/VIS, PDA SPD - M10 $A_{VP,}$ Shimadzu, Japan). The used mobile phase (isocratically delivered) was isopropanol : methanol (25 : 75, v/v). The analysis was performed at room temperature. The flow rate was 2,1 ml/min, the injection volume was 20 µl and the UV detection was at λ = 275 nm. Three measurements were performed for each sample and the mean value was determined. Linearity was analyzed and confirmed though the calibration curve obtained using nine stock solutions of Q_{10} in the concentration range 0.05-10 µg/ml. The correlation coefficient was R^2 = 0. 9990 (y = 9.9864x + 0.1249).

Visualization of liposomes in the Q_{10}-loaded liposome dispersions and liposome gels

Cryo-electron microscopy for liposome dispersions

Liposomes in liposome dispersions LD1 and LD2 were visualized by cryo-electron microscopy after their preparation, and their shape and lamellarity were investigated. Five microliters of dispersions were put onto a perforated coated net of copper (Quantifoil R

1.2/1.3, 400 mesh). The excess of samples was removed with a sheet of filter paper. The samples were quickly frozen with liquid ethane (-170 to -180 °C) in a cryo-box (Carl Zeiss NTS GmbH). Excess ethane was removed by blotting the samples in the cold and the samples were placed with the help of a cryo-transfer device (Carl Zeiss NTS GmbH) in a precooled transmission-cryo-electron-microscope (Philips CM 120). Microscopy was performed at 120 kV.

Freeze fracture electron microscopy for liposome dispersions and liposome gels

Freeze fracture electron microscopy (FFEM) was used for the visualization of liposomes in liposome dispersions LD1 and LD2, as well as in liposome gels G1 and G2, after their preparation and after 6 months of storage at room temperature. Small amounts of the dispersion or gel were mounted on a gold specimen holder, which was placed between two copper preparation holders. The samples were then quickly frozen by plunge/freezing (Jet Freeze Device BAL/TEC, JFD 030, Lichtenstein) into liquid propane at -180^{o}C. The frozen specimens were kept in liquid nitrogen until mounting onto a sample holder. The sample holder was then placed into a freeze fracture device (Freeze Etching System, BAL-TEC, BAF 060, Lichtenstein). The samples were fractured and the fracture plane was replicated by evaporation of 2 nm of platinum at an angle of 45^{o} followed by 20 nm of carbon at an angle of 90^{o}. The replicas were removed from the freeze fracture device and subsequently cleaned in chloroform/methanol (1:1) mixture. The obtained replicas were mounted onto copper grids and visualized using a transmission electron microscope (Zeiss EM 900, Zeiss, Germany).

Statistical analysis

Statistical analysis was carried out using One-Way Analysis of Variance. Significant differences were determined at $p < 0.05$.

Results and discussion

Characterization and stability evaluation of the Q_{10}-loaded liposome dispersions

Investigation of the shape and lamellarity of liposomes

FFEM confirmed the presence of spherical vesicles i.e. liposomes in the Q_{10}-loaded liposome dispersion LD1 after its preparation and also after 6 months storage of the liposome dispersion at room temperature, i.e. the structure of vesicles did not change. However, after 6 months of storage, liposomes seemed to be larger compared to their size after preparation (Fig. 1 a, b). On the basis of the micrographs it was assumed that the vesicles were unilamellar. Further, cryo-electron microscopy was used to study the shape and lamellarity of liposomes of LD1, and it revealed that liposomes were of spherical shape, mostly unilamellar (Fig. 2 a, short arrow), but also bilamellar vesicles could be seen (Fig. 2 a, long arrow). As to the liposome dispersion LD2, FFEM also confirmed the presence of spherical liposomes in the liposome dispersion after its preparation and also after its 6 months storage at room temperature (Fig. 1 c, d). Their size increased after 6 months storage compared to the initial size. Cryo-electron microscopy showed that liposomes of the dispersion LD2 were of spherical shape, mostly unilamellar (Fig. 2 b, short arrow), but that bilamellar vesicles were also present (Fig. 2 b, long arrow).

INSERT FIGS. 1 and 2

Organoleptic characteristics, physical and chemical characterization of liposomes in Q_{10}-loaded liposome dispersions and their stability evaluation

Q_{10}-loaded liposome dispersion LD1, containing liposomes composed of unsaturated phospholipids (vesicles in fluid thermodynamic state) represented a clear liquid of orange

color, which did not change its appearance during storage at different temperatures. The results of the particle size analysis showed that the mean size of liposomes was 125.5 ± 0.2 nm, 24h after preparation, indicating that the Q_{10}-loaded liposomes were of small size (Table 1). Regarding the homogeneity, the low value of PDI i.e. 0.20 ± 0.001 indicated homogeneous population of liposomes (Table 2). This was in accordance with the studies of other authors who also achieved to develop Q_{10}-loaded liposomes of particle size less than 200 nm and of satisfactory homogeneity [29, 30]. The storage of liposomes LD1 at different temperatures (4 °C, RT and 40 °C) for 6 months revealed that their particle size did not change significantly when they were stored at 4 °C and RT, compared to their initial values. In contrast, the storage at 40 °C induced a not marked, but still statistically significant increase in particle size. The same trend was seen for the PDI of liposomes LD1 (Table 2). This observation that the stability of Q_{10}-loaded liposomes decreases with increasing the storage temperature was in accordance with the results of Çelik et al. [29] who reported high stability of Q_{10}-loaded liposomes when stored at 4 °C for 60 days. The authors explained the high stability of liposomes at low temperature by inhibited particle fusion due to decreased fluidity of the bilayer structure. They showed that increasing the temperature from 4 °C to even 25 °C decreases liposomes stability, inducing not only an increase in particle size and PDI, but also in the leakage of Q_{10} from liposomes. However, in their study, the stability decreased already at 25 °C, while in our study the stability was satisfactory at 20 °C (which can be explained by the different composition of investigated Q_{10}-loaded liposomes), but decreased significantly at 40 °C. Furthermore, Lee and Tsai [30] showed that particle size of Q_{10}-loaded liposomes, as well as Q_{10}-content, were stable at least for one month when liposomes were stored at 4 °C. Thus, both studies [29, 30] showed high stability of Q_{10}-loaded liposomes at 4 °C .

The physical stability of liposomes can also be predicted on the basis of the zeta potential value. As the liposome dispersion LD1 possessed a very high zeta potential of -63.9 ± 0.1

mV, it was classified according to the Riddick′s classification [35] into dispersions of high physical stability which do not show aggregation. Hence, the liposome dispersion LD1 was considered as a physically stable dispersion, which was confirmed by particle size and PDI measurements revealing that these liposomes were physically stable when stored at all temperatures (Tables 1 and 2).

As to the pH value, the results indicated that the liposome dispersion LD1 due to the use of buffer possessed a mild acidic pH value of 6.47 ± 0.03, being desirable for liposomes for topical application and also because they show around this pH value high chemical stability. Namely, it was found that chemical hydrolysis of liposomal lipids occurs at slowest rate at pH 6.5 [31]. Thus, it is preferred for a liposome dispersion to have a pH value around 6.5. The pH value should always be above pH 4.5 as this pH value represents the critical lower limit when the degradation process of the vesicles may occur [32]. In addition, the storage of the liposome dispersion LD1 at different temperatures for 6 months did not induce significant changes in pH values, i.e. the values were in the range 6.27 ± 0.05 – 6.47 ± 0.03, indicating that no hydrolysis of liposomal phospholipids occurred during storage.

Further, the oxidation index of the liposome dispersion LD1 was after its preparation 0.51 ± 0.02, indicating that no oxidation of phospholipids occurred. Furthermore, the storage of the liposome dispersion LD1 at 4 °C and RT for 6 months did not induce marked changes of the oxidation index, i.e. values obtained after 6 months were acceptable (0.55± 0.02 and 0.73± 0.02, respectively). However, the storage at 40 °C led to higher values of the oxidation index i.e. to significant oxidation of the liposomal phospholipids. The value of the oxidation index of the liposome dispersion LD1 stored at 40 °C for 6 months was 1. This result is in accordance with the results of other authors who investigated the influence of the incubation temperature on the oxidation index of liposomes, and found that the increase of temperature

from 4 °C to 45 °C, increases the oxidation process in liposomes [33, 34]. Thus, the liposome dispersion LD1 could be considered chemically stable (according to pH and oxidation index values) when stored at 4 °C and RT for 6 months.

As to the Q_{10}-content, it was after the preparation of the liposome dispersion LD1 not significantly different from the declared Q_{10}-content of 0.5% w/w (0.498 ± 0.003 % w/w). During 6 months storage at different temperatures the Q_{10}-content decreased significantly in the liposome dispersion LD1 (Table 3). However, the smallest decrease was at 4°C (Table 3).

Keeping in mind all characterization parameters (particle size, PDI, pH, oxidation index) and the Q_{10}-content, it was concluded that the liposome dispersion LD1 showed highest stability at 4 °C which was in accordance with the results of other authors [29, 30]. Thus, this liposome dispersion should be stored at 4 °C for maximum 90 days (due to Q_{10}-instability).

Regarding the liposome dispersion LD2, containing liposomes composed of saturated phospholipids (vesicles in gel thermodynamic state), it represented a clear liquid of orange color, which did not change its appearance during storage at 4 °C and RT. However, after 6 months storage at 40 °C it changed its appearance i.e. it became an unclear (turbid) orange liquid. Liposomes of LD2 were of significantly higher particle size than liposomes of the dispersion LD1, as their mean size was 222.9 ± 0.5 nm (Table 1), indicating that the type of phospholipids used had a marked influence on the size of vesicles. However, they were of higher homogeneity since their PDI value was 0.171 ± 0.003 (Table 2). As to their physical stability during 6 months storage, it decreased regardless of the storage temperature, i.e. the particle size and PDI increased significantly (Tables 1 and 2). The smallest increase in particle size and PDI was observed at 4 °C. However, during storage even at this low temperature, the particle size of liposomes LD2 was after 6 months 453.8 ± 2.1 nm, indicating aggregation or fusion of liposomes. The same trend was seen for PDI (Table 2). Thus, the liposome

dispersion LD2 could be considered physically stable only when stored at 4 °C for 30 days (Tables 1 and 2).

What was also not going in favor for the physical stability of the liposome dispersion LD2 was its zeta potential being -34.3 ± 0.2 mV (measured 24h after its preparation). According to the zeta potential value the liposome dispersion LD2 was assumed to be a dispersion of insufficient physical stability which could eventually lead to liposome aggregation and hence increase of their particle size during storage. This assumption was confirmed in the stability study by particle size and PDI measurements of these liposomes (Table 1 and 2) and by FFEM (Fig 1c,d). These results revealed that the type of phospholipids used for liposome preparation exerted a high influence on vesicles physical stability i.e. the use of saturated phospholipids was connected with lower physical stability of vesicles.

The pH of the liposome dispersion LD2 was after its preparation 7.17 ± 0.02, which was as in the case of the liposome dispersion LD1 desirable (for stability reasons). The storage at different temperatures for 6 months did not induce significant changes in pH values, i.e. the values were during storage in the range 6.27 ± 0.05 - 6.47 ± 0.03, indicating that no hydrolysis of liposomal phospholipids occurred during the whole storage time.

Furthermore, the oxidation index of the liposome dispersion measured after its preparation was 0.40 ± 0.01, and remained during storage around this value regardless of the storage temperature. The values of the oxidation index obtained after 6 months of storage at 4 °C, RT and 40 °C were 0.40 ± 0.04, 0.42 ± 0.02 and 0.46± 0.05, respectively, revealing that no oxidation of the phospholipids of the liposome dispersion LD2 occurred. This result was expected since this liposome dispersion was prepared from saturated phospholipids (PL 80H). However, the liposome dispersion LD2 could not be considered chemically stable during storage since its Q_{10}-content decreased during storage.

The Q_{10}-content in the liposome dispersion LD2 was after its preparation not significantly different from the declared Q_{10}-content of 0.5% w/w (0.495 ± 0.004). During storage of this liposome dispersion at different temperatures, the Q_{10}-content decreased significantly, regardless of the storage temperature (Table 3). However, the highest decrease was in samples stored at 40 °C, while the smallest decrease was at 4°C (Table 3). The Q_{10}-content decrease was significantly higher in the liposome dispersion LD2 than in the LD1. The instability of the liposome dispersion LD2 could also be explained by the structure of the molecule of Q_{10}. Namely, Q_{10} has a long unsaturated carbon chain which entangles around the phospholipid structure. Since LD1 is composed mainly of unsaturated phospholipids, one of the two chains remains slightly bended inside the liposome structure, which allows entanglement of Q_{10} and reduces the leakage from liposomes, which normally occurs in the liposome dispersion LD2. The Q_{10}-amount which leaked out from liposomes is more susceptible to degradation. Thus, Q_{10} is less stable in the liposome dispersion LD2.

In conclusion, keeping in mind all characterization parameters and the Q_{10}-content, the liposome dispersion LD2, could be considered physically and chemically stable only when stored at 4°C for a maximum of 30 days (Tables 1-3).

INSERT TABLE 1, 2 AND 3

Characterization and stability evaluation of Q_{10}-loaded liposome gels and conventional gel

Organoleptic characteristics of Q_{10}-loaded liposome gels and conventional gel

Liposome gels G1 and G2, as well as the conventional gel G3, were after their preparation of semisolid consistency as required for skin application. The gels were of a light orange color, with a smooth and glossy texture, and homogenous. During 6 months storage at 4 ºC, RT and 40 ºC, liposomal gels did not change their organoleptic characteristics.

Freeze-fracture electron microscopy of Q_{10}-loaded liposome gels

In order to confirm the integrity of liposomes of the dispersions LD1 and LD2 in gels G1 and G2, respectively, the gels were analyzed by FFEM after preparation and after 6 months storage at room temperature. As seen in the FFEM micrographs shown in Fig. 3 (a, b), the liposomes were identified in the gel structure of G1, both after preparation and after 6 months storage, indicating their compatibility with carbomer gel. The same was observed in the FFEM micrographs of the gel G2, shown in Fig. 3 (c, d), i.e. liposomes were seen in both the freshly prepared gel and also in the gel stored for 6 months at room temperature. However, there was a difference between FFEM micrographs of gels G1 and G2, especially after 6 months storage. Namely, in the micrograph of the gel G1, a large unilamellar liposome was seen, while in the micrograph of the gel G2, lamellar structures and a large multilamellar liposome (MLV) were observed. As after the preparation mostly unilamellar liposomes were seen in both liposome dispersions, which were further incorporated into gels, the aforementioned observation indicates that more intense changes (e.g. fusion or aggregation of vesicles) occurred in the gel G2 where the MLV was observed. Further, the observation of liposomes in gels indicates that the viscosity modifier carbomer does not destruct the liposome structure. Therefore, it is always advised to incorporate liposomes in a gel vehicle, especially gels prepared with carbomer resin.

These results were in accodance with the results of Thoma et al. [20], who showed that the incorporation of liposomes into a polyacrylate gel (carbomer gel) does not influence i.e. destroy the liposome structure, but enhances their physical stability.

INSERT FIG. 3

pH values of Q_{10}-loaded liposome gels and conventional gel

Regarding the pH values of gels G1, G2 and G3 after their preparation, they were 6.12 ± 0.03, 6.13 ± 0.02 and 6.00 ± 0.02, respectively. Thus, gels possessed mild acidic pH values, revealing their suitability for topical application onto the skin, which also possesses an acidic value. The storage of gels G1, G2 and G3 at 4°C, room temperature and elevated temperature of 40°C for 6 months did not lead to significant changes in the pH values, i.e. the values were during the storage at different temperatures in the range from 5.95 ± 0.03 to 6.19 ± 0.04. Thus, obtained pH values of gels were during the whole investigation time appropriate for formulations aimed for dermal application.

The pH value of formulations applied onto the skin is very important, i.e. **formulations should possess a mild acidic pH value** in order to not disturb the pH of the skin, which is normally acidic, ranging between 4 and 6 [36]. It is important to maintain this pH range, since acidic sphingomyelinase and ß-glucocerebrosidase, which are enzymes in the skin being involved in the synthesis of ceramides and are of crucial importance for the formation of the stratum corneum permeability barrier, require an acidic pH to exert their optimum effect [36]. Further, the formation of lamellar structures of the intercellular lipids in the stratum corneum requires an acidic pH. Additionally, acidic pH values of 4.5 - 6 are needed for free fatty acids to form by partial ionization of lamellar liquid crystals [37]. Moreover, maintenance of the skin pH is of crucial interest, as changes in the pH, play a role in the pathogenesis of several skin diseases, including atopic dermatitis, irritant contact dermatitis, ichthyosis, fungal infection, and acne [38].

The aforementioned small pH changes in samples G1-G3 indicated that total neutralization of carboxylic groups of polyacrylic acid (carbomer) occurred by the addition of the neutralizer TEA during the preparation of gels, as well as that in the case of liposome gels G1 and G2 no

chemical changes i.e. no hydrolysis of liposomal phospholipids took place during storage at 4 °C, room temperature and 40 °C. This was very important for the long-term stability of liposome hydrogels.

Q_{10}-content in Q_{10}-loaded liposome gels and conventional gel (stability of Q_{10} in gels)

The Q_{10}-content in liposome gels G1 and G2, and the liposome-free gel G3, after their preparation, did not vary significantly from the declared Q_{10}-content of 0.05 % w/w (0.049 ± 0.003, 0.050 ± 0.001 and 0.049 ± 0.003 % w/w, respectively). After 6 months of storage at 4 °C, RT and 40 °C, the Q_{10}-content was in the liposome gel G1, 85.73 ± 0.15, 68.73 ± 0.12 and 60.98 ± 0.05 % of its initial content, respectively (Table 3). As to gel G2, the Q_{10}-content was 70.12 ± 0.18, 36.56 ± 0.08 and 35.68 ± 0.08 % of its initial content, after 6 months of storage at 4 °C, RT and 40 °C, respectively (Table 3).

This significant decrease of Q_{10}-content in the liposome gels during storage at all temperatures indicated that the stability of Q_{10} in gels was not sufficiently high. The smallest decrease of Q_{10}-content was found in liposome gels G1 and G2 stored at 4 °C, while the highest decrease was observed in gels stored at 40 °C (Table 3). The decrease of Q_{10}-content was even more pronounced in the conventional gel G3, where the Q_{10}-content was 54.88 ± 0.14, 20.33 ± 0.15 and 20.13 ± 0.10 % of its initial content, after 6 months storage at 4 °C, RT and 40 °C (Table 3). Thus, Q_{10} was significantly more stable in the liposome gels G1 and G2, especially in gel G1, compared to the liposome free conventional gel G3. Regarding gels G1 and G2, Q_{10}-content decreased significantly less in the gel G1, indicating higher Q_{10} stability in the gel G1. Namely, gel G1 was obtained by the incorporation of the LD1 liposome dispersion (made of unsaturated phospholipids) being in a liquid thermodynamic state into the gel, in contrast to the gel G2 obtained by adding the LD2 liposome dispersion (made of

saturated phospholipids), which is in a gel thermodynamic state, into the carbomer gel. In conclusion, the flexible liquid-state liposomes of LD1 enabled higher protection of Q_{10} in the carbomer hydrogel G1 than the rigid gel-state liposomes of LD2 in the liposome gel G2. Q_{10} showed also a significantly higher ($p<0.05$) stability in the liposome gel G1 than in the liposome-free gel G3. Thus, the Q_{10}-content decreased in the following manner in the samples: G1>G2>G3 (G3 showed the smallest Q_{10}-content, regardless of storage temperature). Hence, it was shown that the encapsulation of Q_{10} into nanocarriers i.e. liposomes increased the stability of Q_{10}. However, the stability of Q_{10} in liposome dispersions LD1 and LD2 was higher than in gels G1 and G2 (Table 3). The reason for that could be the higher water content in the gels and hence the higher amount of dissolved oxygen which leads to oxidation of Q_{10}, since Q_{10} is highly sensitive to oxidation. Further, during the incorporation of the liposome dispersions into the gel using Ultra-Turrax®, additional aeration of the gels could occur. Q_{10} could show also incompatibility with an ingredient in the gel vehicle. In conclusion, the Q_{10}-content was highest in the gel G1 during storage at 4 °C. However, even at that temperature the decrease was significant after 6 months (85.73 ± 0.15% of the initial content), which is not acceptable. Further, as this formulation should be used as a cosmetic preparation, it is intended to be kept at room temperature, which has been shown in this study to induce even higher decrease in Q_{10}-content. Therefore, in our next formulation study, Q_{10} will be additionally protected in a semisolid formulation in order to prevent its rapid degradation.

Particle size and PDI of liposomes in liposome gels (stability of liposomes in gels)

As to the particle size and PDI value of liposomes in gels G1 and G2, they did not increase significantly after the liposome incorporation into the gel, compared to their values in the liposome dispersions LD1 and LD2 (Tables 1 and 2). As to the particle size and PDI of

liposomes in the gel G1 during storage at 4 °C, RT and 40 °C, no significant changes ($p>0.05$) were observed, regardless of the storage temperature, confirming liposomes stability at all temperatures and compatibility with the carbomer gel. Hence, since no increase of particle size was measured, no aggregation of liposomes in the liposome gel occurred, i.e. the liposomes were of adequate stability in the gel G1. In contrast, in the case of liposomes in the gel G2, they significantly increased after 6 months storage at all temperatures, from 224.1± 0.3 to 296.7 ± 1.8 nm at 4 °C, to 361.2 ± 0.4 nm at RT and to 475.4 ± 0.5 nm at 40 °C, which revealed their fusion or aggregation in the gel. This was confirmed by FFEM (Fig. 3c, d). In addition, the PDI of liposomes increased in the gel G2 reflecting a decrease of liposome homogeneity in the gel, at all temperatures (Table 2). One could conclude that the aggregation of vesicles was due to the instability of liposomes of the liposome dispersion LD2 in the carbomer gel, but this was not the case, as liposomes were even less stable in the liposome dispersion LD2 during storage, i.e. the increase of particle size and PDI was higher in the liposome dispersion LD2 than in the liposome gel G2 (Tables 1 and 2). Thus, the carbomer gel increased the liposome stability to some extent possibly due to the gel structure decreasing the liposome aggregation. In conclusion, only liposomes in the liposome gel G1 were physically stable during storage at all temperatures.

Stability evaluation of Q_{10}-loaded liposome gels and conventional gel by rheological measurements

The rheological behavior of the gels was studied since it plays an important role in the mixing and flow characteristics of materials, their packaging into containers, physical stability and consumers' acceptability. The flow curves and the rheological parameters (yield stress, minimal and maximal apparent viscosities) of the two examined liposome gels G1 and G2, as well as the liposome free gel G3, obtained 48 hours after their preparation are shown in Fig. 4 and Tables 4-6.

INSERT TABLES 4, 5 AND 6, AND FIGURE 4

All gels showed a non-Newtonian, shear-thinning flow behavior according to the Herschel-Bulkley model (excellent fitting $R > 0.999$). According to literature, shear-thinning behavior is characterized by continuously decreasing viscosity, thus indicating successive loss of polymer entanglement upon increasing shear stress [39]. Shear-thinning is a desirable property of semisolid dosage forms, since they should be "thin" during application and "thick" otherwise [40,41]. The flow curves (Fig. 4) also showed a plastic behaviour of the gels, since they possessed yield values, indicating that the gel network exhibited resistance to an external force before it started flowing [42]. In addition, the up and down flow curves of gels (Fig. 4) did not overlap completely, but they formed hysteresis loop areas. It is desirable for a pharmaceutical or cosmetic product to show some degree of thixotropy [42]. These gel samples showed marginal thixotropy, since most carbomer polymer gels exhibit little or no thixotropy, due to which viscosity immediately recovers upon cessation of shearing [43,44]. As to rheological parameters, the yield stress can be used to evaluate the quality of a formulation and according to some authors it is the most reliable parameter for describing the stability [45,46]. For topical preparations it is desirable to posses the yield stress not only in terms of good stability, but also because it describes the flow behavior at small shear rates i.e. before and after the application. The yield value of the fresh liposome gels G1 and G2 were 61 ± 0.8 Pa and 114 ± 9.1 Pa, respectively, while for the liposome-free gel G3 it was 51 ± 5.8 Pa (Table 4), indicating high stability of all gels. As to the minimal and maximal apparent viscosities, they describe different conditions of a structure, i.e. maximal apparent viscosity describes the system structure at rest, while the minimal apparent viscosity represents a measure of destruction of the gel structure. The values of these two parameters for gels G1-G3 are represented in Tables 5 and 6. All three gels possessed minimal and maximal apparent

viscosities appropriate for semisolids aimed for topical application onto the skin. Namely, other authors reported for carbomer gels (being investigated using the same procedure for rheological measurements as in our study) after their preparation, minimal apparent viscosity values in the range 2.77 ± 0.06 – 2.97 ± 0.06 Pas, and maximal apparent viscosity values in the range from 42.2 ± 0.9 – 49 ± 1 Pas [47]. However, the authors reported an increase of these two parameters during storage of the gels due to the structuration of the systems i.e. minimal apparent viscosity values increased to even 5 ± 0.06 Pas, while maximal apparent viscosity values increased to even 88 ± 1 Pas. As to the yield stress, the liposome free gel G3 possessed lowest yield stress, minimal and maximal apparent viscosities, as well as hysteresis loop area (1091 Pa/s), while the liposome gel G2 possessed highest yield stress, maximal apparent viscosity and hysteresis loop area (3479 Pa/s). The hysteresis loop area of G1 (2096 Pa/s) was between those of G2 and G3.

Besides performing rheological measurements of the gels after their preparation, also analysis of gels during their 6 months storage at RT and 40 °C was performed in order to determine the range of conditions under which the product will perform well i.e. remain physically stable.

On the basis of the flow curves of the gels stored at RT and 40 °C, shown in Fig. 4, it was concluded that the gels maintained plastic flow behavior during their storage at both temperatures, which is appropriate for topical use. In the case of gel G1 and G2, the flow curves did not change significantly, regardless of the storage temperature. In contrast, the flow curve of gel G3 changed when the gel was stored at 40 °C. As to rheological parameters (Tables 4-6), variations were observed in all gels during storage. The yield stress values of gel G1 significantly increased ($p<0.05$) during storage at both temperatures. This can be explained by an increasing number of bonds in the polymer network which yields a higher physical stability. The same trend was seen for the gel G3. Thus, their stability did not

decrease during storage, even at high temperature of 40 °C. Therefore, high stability of gels G1 and G3 was assumed. In contrast, gel G2 showed an initial high yield stress value, which during storage at both temperatures decreased, however, not significantly. Further, the minimal and maximal apparent viscosities of gels G1 did not change significantly ($p>0.05$) during storage at RT compared to their initial values, while minimal apparent viscosity increased significantly ($p<0.05$) when the gel was stored at 40 °C (Table 5 and 6). In the case of gel G2, the viscosity values did not change significantly ($p>0.05$) regardless of the storage temperature. In contrast, regarding gel G3, storage at 40 °C led to a significant increase ($p<0.05$) of the minimal apparent viscosities. However, the value of minimal apparent viscosity was higher only for 5 % compared to its initial values. This increase in viscosity values of gel G3 could be explained by changing, e.g. by strengthening the gel structure due to forming new bonds. In conclusion, liposome gel G1 exhibited less extensive changes in rheological parameters and hence in the structure than other gels (including having high yield stress which increases during storage).

On the basis of these results it was concluded that all gel samples G1-G3 remained physically stable during storage at RT and 40 °C and that they did not exhibit relevant changes in their rheological properties, which makes them suitable for topical application onto the skin. Thus, if they were stable regarding their rheological parameters at these temperatures, they would also be stable at 4 °C.

Furthermore, the addition of liposomes into the gel vehicle neither decreased the stability of the carbomer gel, as shown by rheology measurements of liposome gels G1 and G2, nor the stability of liposomes as shown by particle size measurements (performed by PCS), indicating liposomes' stability in the carbomer gel.

Conclusion

This study demonstrated the feasibility of preparing Q_{10}-loaded liposome dispersions, as well as of Q_{10}-loaded liposome gels by incorporating liposome dispersions into the carbomer gel vehicle. The incorporation of Q_{10}-loaded liposome dispersions into the gel did not lead to instability or degradation of liposomes. The obtained liposome gels, being semisolid formulations, were in contrast to the liquid liposome dispersion suitable for topical application onto the skin and their rheological properties were appropriate for this kind of application. In order to predict their long term stability, the liposome gels were stored for 6 months at 4 ºC, room temperature and 40 ºC (accelerated ageing). The liposome gels maintained the non-Newtonian plastic flow behavior without significant thixotropy during storage, even at high temperature of 40 ºC, and the rheological parameters did not change significantly, indicating their physical stability. Among the two investigated liposome gels G1 and G2, the **gel G1 containing unsaturated liquid-state liposomes, was the optimal formulation** as, in contrast to gel G2, it did not show an increase in liposome size and PDI (i.e. no agglomeration or aggregation of liposomes occurred), it provided significantly higher stability for Q_{10} than liposome gel G2 and liposome-free gel G3, and its pH value was adequate for topical application. The only limiting factor was the observed decrease of the Q_{10}-content in the gel G1 at room temperature and at elevated temperature of 40 ºC, indicating that extreme storage conditions should be avoided. Thus, the liposome gel G1 should be stored at 4 °C.

In conclusion, it was able to increase the stability of Q_{10} in the final formulation/product by incorporating it into liposomes prior to addition into the gel vehicle, as Q_{10} was significantly more stable in the liposome gel G1 than in the liposome-free gel G3. Hence, the Q_{10}-loaded liposome gel G1 could be considered a physically and chemically stable gel appropriate for

topical use. However, additional measures regarding further increase of Q_{10}-stability in the gel should be undertaken.

As to the application, the developed Q_{10}-loaded liposome gel G1 is aimed to be used as it is, or as an enriched formulation with additional cosmetic actives (emollients, moisturizing agents, etc.) - which should be investigated in future studies - as a cosmetic anti-ageing preparation. Not only Q_{10}, but also liposomal constituents (unsaturated phospholipids in liposome dispersion LD1 and gel G1) are beneficial for skin care [48-50]. Especially valuable is phosphatydilcholine (PC) from soya due to its high content of linoleic acid which has an important role in the function of the epidermal lipid barrier [51]. This study showed the feasibility of preparing a Q_{10}-loaded liposome gel serving as a delivery system of Q_{10} to the skin. It is proposed that this Q_{10}-loaded liposome gel would enhance the penetration of Q_{10} into the skin, since it was shown by Lee and Tsai [29,30] that the encapsulation of Q_{10} into liposomes enhanced its accumulation (at least twofold) in the skin compared to a suspension with free Q_{10}, as well as that the Q_{10}-content and treatment duration were the key factors determining the accumulation of in the skin. Thus, the next step in our research will be the *in vitro* evaluation of the penetration of Q_{10} from this liposome gel into the skin, as well as an *in vivo* investigation of its anti-ageing effect and its influence on the transepidermal water loss (TEWL) and hydration of the skin.

Disclosure of interest

The authors report no conflict of interest.

References

1. Kumar S, Rao R, Kumar A, Mahant S, Nanda S. Novel Carriers for Coenzyme Q10 Delivery. Curr Drug Deliv. 2016;13(8):1184–1204.
2. Shapiro SS, Saliou C. Role of Vitamins in Skin Care. Nutrition 2001;17:839–844.
3. Brunke RA. Coenzym Q_{10} (INCI: Ubichinon). Euro Cosmetics 2002;4:43–46.
4. Hoppe U, Bergemann J, Diembeck W, Ennen J, Gohla S, Harris I, et al. Coenzyme Q10, a cutaneous antioxidant and energizer. BioFactors 1999;9:371–378.
5. Wang J, Wang H, Zhou X, Tang Z, Liu G, Liu G, Xia Q. Physicochemical characterization, photo-stability and cytotoxicity of coenzyme Q10-loading nanostructured lipid carrier. J Nanosci Nanotechnol. 2012;12(3):2136–48.
6. Korkm E, Gokce EH, Ozer O. Development and evaluation of coenzyme Q10 loaded solid lipid nanoparticle hydrogel for enhanced dermal delivery. Acta Pharm. 2013;63(4):517–29.
7. Schwarz JC, Baisaeng N, Hoppel M, Löw M, Keck CM, Valenta C. Ultra-small NLC for improved dermal delivery of coenyzme Q10. Int J Pharm. 2013;447(1-2):213–217.
8. Chen S, Liu W, Wan J, Cheng X, Gu C, Zhou H, Chen S, Zhao X, Tang Y, Yang X. Preparation of Coenzyme Q10 nanostructured lipid carriers for epidermal targeting with high-pressure microfluidics technique. Drug Dev Ind Pharm. 2013;39(1):20–8.
9. Gavin PD, El-Tamimy M, Keah HH, Boyd BJ. Tocopheryl phosphate mixture (TPM) as a novel lipid-based transdermal drug delivery carrier: formulation and evaluation. Drug Deliv Transl Res. 2017;7(1):53–65.
10. Cevc G, Blume G. Hydrocortisone and dexamethasone in very deformable drug carriers have increased biological potency, prolonged effect, and reduced therapeutic dosage. Biochim Biophys Acta 2004; 1663(1-2):61–73.

11. Dragicevic-Curic N, Scheglmann D, Albrecht V, Fahr A Temoporfin-loaded invasomes: development, characterization and in vitro skin penetration studies. J Control Release 2008;127(1):59–69.
12. Ahmed TA. Preparation of transfersomes encapsulating sildenafil aimed for transdermal drug delivery: Plackett-Burman design and characterization. J Liposome Res. 2015;25(1):1–10.
13. Khan MA, Pandit J, Sultana Y, Sultana S, Ali A, Aqil M, Chauhan M. Novel carbopol-based transfersomal gel of 5-fluorouracil for skin cancer treatment: in vitro characterization and in vivo study. Drug Deliv. 2015;22(6):795–802.
14. Al Shuwaili AH, Rasool BK, Abdulrasool AA. Optimization of elastic transfersomes formulations for transdermal delivery of pentoxifylline. Eur J Pharm Biopharm. 2016;102:101–14.
15. Kamran M, Ahad A, Aqil M, Imam SS, Sultana Y, Ali A. Design, formulation and optimization of novel soft nano-carriers for transdermal olmesartanmedoxomil delivery: In vitro characterization and in vivo pharmacokinetic assessment. Int J Pharm. 2016;505(1-2):147–158.
16. Pleguezuelos-Villa M, Mir-Palomo S, Díez-Sales O, Buso MAOV, Sauri AR, Nácher A. A novel ultradeformable liposomes of Naringin for anti-inflammatory therapy. Colloids Surf B Biointerfaces. 2018;162:265–270.
17. Franzé S, Marengo A, Stella B, Minghetti P, Arpicco S, Cilurzo F. Hyaluronan-decorated liposomes as drug delivery systems for cutaneous administration. Int J Pharm. 2018;535(1-2):333–339.
18. Raschke T, Eckert J Düsing H-J, Kallmayer V, Wittern K-P. Encapsulation Technologies in Cosmetics. SÖFW-Journal 2003;10:62–68.

19. Schreier H, Bouwstra J. Liposomes and niosomes as drug carriers: dermal and transdermal drug delivery. J. Control. Release 1994;30:1–15.
20. Thoma K, Jocham UE, Liposome Dermatics: Assessment of Long-Term Stability. In: Braun-Falco O, Korting HC, Maibach HI, editors. Liposome Dermatics, Berlin: Springer-Verlag; 1992, p. 150-166.
21. Arsic I, Vidovic S. Protection of encapsulated vitamin A from UV radiation in aqueous dispersions and gel systems, Conference Proceedings, Int. Conference of IFSCC, Budapest, 1997, p. 452–455.
22. Blume G. Liposomes a Dermal System for Active Ingredients, Conference Proceedings of Cosmetic and Household Ingredients, Warsaw, 1999, p. 34-43.
23. Blume G, Teichmüller E, Verma DD, Fahr A. Stability of liposomes containing retinol. Euro Cosmetics 2000;11/12:38–40.
24. Kristl J, Volk B, Gašperlin M, Šentjurc M, Jurkovič P. Effect of colloidal carriers on ascorbyl palmitate stability. Eur J Pharm Sci. 2003;19:181–189.
25. Dragicevic-Curic N, Winter S, Krajisnik D, Stupar M, Milic J, Graefe S, Fahr A. Stability evaluation of temoporfin-loaded liposomal gels for topical application. J Liposome Res. 2010;20(1):38–48.
26. Dragicevic-Curic N, Winter S, Stupar M, Milic J, Krajisnik D, Gitter B, Fahr A. Temoporfin-loaded liposomal gels: viscoelastic properties and in vitro skin penetration. Int J Pharm. 2009;373(1-2):77–84.
27. Klein RA. The detection of oxidation in liposome preparations. Biochim Biophys Acta 1970;210:483–486.
28. Zuidam NJ, de Vrueh R, Crommelin DJA. Characterization of liposomes. In: Torchilin V, Weissig V, editors. Liposome: A Practical Approach, Oxford, UK: Oxford University Press; 2003, p. 31-78.

29. Çelik B, Sagıroglu AA, Özdemir S. Design, optimization and characterization of coenzyme Q10- and D-panthenyl triacetateloaded liposomes. International Journal of Nanomedicine 2017;12:4869–4878.
30. Lee WC, Tsai TH. Preparation and characterization of liposomal coenzyme Q10 for in vivo topical application. Int J Pharm. 2010;395(1-2):78-83.
31. Grit M, de Smidt JH, Struijke A, Crommelin DJA. Hydrolysis of phosphatidylcholine in aqueous liposome dispersions. Int J Pharm. 1989;50:1-6.
32. Frokjaer S, Hjorth EL, Worts O. Stability testing of liposomes during storage. In: Gregoriadis G, editors. Liposome Technology, Vol.1, Florida: CRC Press Inc., Boca Raton; 1984, p. 235.
33. Zaharijev J. Dobijanje lipozoma od fosfolipida soje i ispitivanje njihove stabilnosti (Preparation of liposomes from soya phospholipids and investigation of their stability). Master thesis, Faculty of technology and metallurgy, University Belgrade, 1997.
34. Konings AWT. Lipid peroxidation in liposomes. In: Gregoriadis G, editor. Liposome Technology, CRC Press, Boca Raton, Florida; 1984, p. 139-161.
35. Müller RH. Zetapotential-Theorie.In: Müller RH, editor. Zetapotential und Partikelladung in der Laberpraxis, Stuttgart: Wissenschaftliche Verlagsgesellschaft mbH; 1996, p. 19–99.
36. Rippke F, Schreiner V, Schwanitz HJ. The acidic milieu of the horny layer: new findings on the physiology and pathophysiology of skin pH. Am J Clin Dermatol. 2002;3(4):261-72.
37. Bouwstra JA, Gooris GS, Dubbelaar FE, Weerheim AM, Ponec M. pH, cholesterol sulfate, and fatty acids affect the stratum corneum lipid organization. J Investig Dermatol Symp Proc. 1998;3(2):69-74.

38. Schmid-Wendtner MH, Korting HC. The pH of the skin surface and its impact on the barrier function.Skin Pharmacol Physiol. 2006;19(6):296-302.
39. Marriott C. Rheology. In: Aulton ME, Taylor KMG, editors. Aulton's Pharmaceutics: The Design and Manufacture of Medicines. 4th ed. Elsevier Ltd.; 2013. p.94–114.
40. Pena LE, Lee BL, Sterns JF. Structural rheology of model ointment. Pharm Research 1994;11:875–881.
41. Florence AT, Attwood D. Physicochemical principles of pharmacy: in manufacture, formulation, and clinical use. London: Pharmaceutical Press; 2016. p. 313-314.
42. Briceno MI. Rheology of Suspensions and Emulsions. In: Nielloud F, Marti-Mestres G, editors. Pharmaceutical Emulsions and Suspensions, New York: Marcel Dekker, Inc.; 2000, p.557–607.
43. Carvalho FC, Calixto G, Hatakeyama IN, Luz GM, Gremião MP, Chorilli M. Rheological, mechanical, and bioadhesive behavior of hydrogels to optimize skin delivery systems. Drug Dev Ind Pharm. 2013;39(11):1750–7.
44. Islam MT, Rodríguez-Hornedo N, Ciotti S, Ackermann C. Rheological characterization of topical carbomer gels neutralized to different pH. Pharm Res. 2004;21(7):1192–9.
45. Brummer R. Rheology Essentials of Cosmetics and Food Emulsions. Berlin Heidelberg: Springer-Verlag; 2006.
46. Tamburić S, Craig DQM. Rheological Evaluation of Polyacrilic Acid Hydrogels. Pharm Sci. 1995;1:107–109.

47. Djekic Lj, Krajišnik D, Mićic Z, Čalija B. Formulation and physicochemical characterization of hydrogels with 18β-glycyrrhetinic acid/phospholipid complex phytosomes. J Drug Deliv Sci Tech 2016;35:81-90.

48. Gooris GS, Kamran M, Kros A, Moore DJ, Bouwstra JA. Interactions of dipalmitoylphosphatidylcholine with ceramide-based mixtures. Biochim Biophys Acta. 2018;1860(6):1272-1281.

49. Lautenschläger H. Strong effects - phospholipids in cosmetics. Kosmetik International 2003;2:38-40.

50. van Hoogevest P, Prusseit B, Wajda R. Phospholipids: Natural Functional Ingredients and Actives for Cosmetic Products. SOFW-Journal 2013;8:9-14.

51. Rhodes LE, Essential fatty acids. In: Loden M, Maibach HI, editors. Dry skin and moisturizers, chemistry and function, Boca Raton London, New York, 2000, 311-32.

Table 1. Mean size of Q_{10}-loaded liposomes in liposome dispersions and liposome gels, during storage at 4 ^{o}C, 20 ± 2 ^{o}C and 40 ^{o}C for 180 days. Each data represents the mean±SD (n=3).

Sample	**Mean size of liposomes (nm)**								
	After preparation	4 ^{o}C			20 ± 2 ^{o}C			40 ^{o}C	
		30days	90days	180days	30days	90days	180days	30days	90days	180days
LD1	125.5±0.2	125.0±0.6	125.7±0.9	125.3±0.5	124.8±0.5	127.9±1.5	124.5±0.7	128.8±0.4	132.6±0.8	131.5±1.1
LD2	222.9±0.5	231.6±0.4	320.7±1.7	453.8±2.1	254.7±0.4	496.6±0.7	666.8±0.6	313.9±0.4	523.8±1.4	779.0±1.8
G1[a]	125.9±0.4	126.2±0.5	127.5±0.9	126.8±0.4	126.0±0.2	126.5±0.4	127.1±0.5	126.8±0.3	127.9±0.5	129.5±0.6
G2[b]	224.1±0.3	230.2±0.9	244.8±0.7	296.7±1.8	230.2±0.5	244.8±0.6	361.2±0.4	256.6±0.6	304.8±0.4	475.4±0.5

[a]G1 gel containing the Q_{10}-liposome dispersion LD1;[b]G2 gel containing the Q_{10}-liposome dispersion LD2

Table 2. PDI of Q_{10}-loaded liposomes in liposome dispersions and liposome gels, during storage at 4 ^{o}C, 20 ± 2 ^{o}C and 40 ^{o}C for 180 days. Each data represents the mean±SD (n=3).

Sample	PDI									
	After preparation	4 ^{o}C			20 ± 2 ^{o}C			40 ^{o}C		
		30days	90days	180days	30days	90days	180days	30days	90days	180days
LD1	0.201±0.006	0.202±0.009	0.209±0.008	0.211±0.011	0.193±0.005	0.241±0.009	0.252±0.006	0.224±0.009	0.233±0.013	0.270±0.006
LD2	0.171±0.003	0.175±0.009	0.201±0.007	0.273±0.011	0.180±0.006	0.214±0.005	0.301±0.014	0.222±0.008	0.263±0.007	0.414±0.009
G1[a]	0.220±0.005	0.222±0.005	0.225±0.010	0.230±0.004	0.222±0.006	0.241±0.008	0.243±0.005	0.223±0.005	0.232±0.006	0.254±0.016
G2[b]	0.182±0.004	0.183±0.008	0.185±0.011	0.211±0.009	0.180±0.003	0.189±0.005	0.218±0.004	0.198±0.004	0.218±0.005	0.290±0.010

[a] G1 gel containing the Q_{10}-liposome dispersion LD1;[b] G2 gel containing the Q_{10}- liposome dispersion LD2

Table 3. Q_{10}-content in liposome dispersions, liposome gels and plain gel during storage at 4 °C, 20 ± 2 °C and 40 °C for 180 days. The initial Q_{10}-content measured after 48 h was taken as 100%. Each data represents the mean±SD (n=3).

Sample	Q_{10}-content (%)								
	After preparation	4 °C			20 ± 2 °C			40 °C	
		30days	90days	180days	30days	90days	180days	30days	90days	180days
LD1	100%	99.97±0.03	98.67±0.08	90.91±0.17	98.18±0.13	84.32±0.09	75.01±0.10	93.89±0.15	79.09±0.11	66.12±0.09
LD2	100%	98.18±0.10	92.98±0.11	75.09±0.16	95.98±0.16	72.22±0.16	61.00±0.11	90.13±0.19	66.37±0.08	56.89±0.17
G1[a]	100%	99.07±0.08	90.67±0.13	85.73±0.15	96.98±0.09	78.18±0.06	68.73±0.12	92.90±0.09	75.10±0.07	60.98±0.05
G2[b]	100%	98.14±0.18	85.78±0.12	70.12±0.018	90.34±0.11	62.78±0.09	36.56±0.08	88.64±0.13	59.48±0.09	35.68±0.08
G3[c]	100%	95.67±0.09	80.89±0.07	65.88±0.14	90.12±0.08	60.52±0.05	20.33±0.15	87.19±0.15	60.22±0.08	20.13±0.10

[a]G1 gel containing the Q_{10}-liposome dispersion LD1;[b]G2 gel containing the Q_{10}- liposome dispersion LD2; [c]G3 gel containing free Q_{10}

Table 4. Yield stress values of Q_{10}-loaded liposome gels and Q_{10}- conventional gel (G1-G3), during storage at 20 ± 2 ^{o}C and 40 ^{o}C for 180 days. Each data represents the mean±SD (n=3).

Sample	**Yield stress (Pa)**						
	48 h	20 ± 2 ^{o}C			40 ^{o}C		
		30days	90days	180days	30days	90days	180days
G1[a]	61±0.8	56±8.8	70±13.4	71±12.6	67±6.9	61±3.8	66±7.6
G2[b]	114±9.1	111±4.8	117±1.4	106±10.0	100±5.5	105±8.8	112±3.0
G3[c]	51±5.8	51±1.0	58±3.2	59±5.0	55±8.6	53±12.0	58±1.3

[a]G1 gel containing the Q_{10}-liposome dispersion LD1, [b]G2 gel containing the Q_{10}-liposome dispersion LD2, [c]G3 gel containing the free Q_{10}

Table 5. Minimal apparent viscosity values of Q_{10}-loaded liposome gels and Q_{10}-conventional gel (G1-G3), during storage at 20 ± 2 ^{o}C and 40 ^{o}C for 180 days. Each data represents the mean±SD (n=3).

Sample	**Minimal apparent viscosity η_{min} (Pas)** $D = 200\ s^{-1}$						
	48 h	20 ± 2 ^{o}C			40 ^{o}C		
		30days	90days	180days	30days	90days	180days
G1[a]	2.93±0.08	2.97±0.04	2.89±0.02	2.95±0.06	3.00±0.01	3.38±0.02	3.34±0.07
G2[b]	2.85±0.06	2.82±0.11	2.79±0.04	2.77±0.11	2.92±0.01	2.95±0.01	2.94±0.10
G3[c]	2.26±0.01	2.47±0.01	2.33±0.02	2.22±0.05	2.38±0.05	2.44±0.02	2.38±0.09

[a]G1 gel containing the Q_{10}-liposome dispersion LD1, [b]G2 gel containing the Q_{10}-liposome dispersion LD2, [c]G3 gel containing the free Q_{10}

Table 6. Maximal apparent viscosity of Q_{10}-loaded liposome gels and Q_{10}-conventional gel (G1-G3), during storage at 20 ± 2 ^{o}C and 40 ^{o}C for 180 days. Each data represents the mean±SD (n=3).

Sample	**Maximal apparent viscosity η_{min} (Pas)** $D = 4.08\ s^{-1}$						
	48 h	20 ± 2 ^{o}C			40 ^{o}C		
		30days	90days	180days	30days	90days	180days
G1[a]	50.8±1.3	50.1±2.5	50.7±1.1	51.6± 0.2	50.4±1.9	60.4±1.5	49.5±0.6
G2[b]	55.4±1.3	53.1±1.8	55.1±1.7	52.2± 4.0	55.2± .6	57.2±0.8	54.6±3.6
G3[c]	33.6±0.8	37.6±0.6	36.4±0.4	33.2±0.07	35.5±1.1	36.6±0.1	34.8±1.5

[a]G1 gel containing the Q_{10}-liposome dispersion LD1, [b]G2 gel containing the Q_{10}-liposome dispersion LD2, [c]G3 gel containing the free Q_{10}

Figure captions

Fig. 1. Identification of liposomes by FFEM in the Q_{10}-loaded liposome dispersion LD1 and LD2. a) Liposome dispersion LD1, after preparation; b) liposome dispersion LD1, after 6 months storage at room temperature; c) liposome dispersion LD2, after preparation; d) liposome dispersion LD2, after 6 months storage at room temperature. Scale bar = 200 nm in all micrographs.

Fig. 2. Identification of liposomes in the Q_{10}-loaded liposome dispersions LD1 and LD2 by cryo-electron microscopy after their preparation. a) Liposome dispersion LD1, b) liposome dispersion LD2. Short arrows depict spherical unilamellar vesicles, while the long arrows depict spherical bilamellar vesicles. Scale bar = 200 nm in all micrographs.

Fig. 3. FFEM micrographs of gel G1 containing liposome dispersion LD1 and gel G2 containing liposome dispersion LD2. a) Gel G1, after preparation (scale bar = 100 nm); b) gel G1, after 6 months storage at room temperature (scale bar = 100 nm); c) gel G2, after preparation (scale bar = 200 nm); d) gel G2, after 6 months storage at room temperature (scale bar = 200 nm).

Fig. 4. Flow curves of gels, 48 h after preparation, after 30, 90 and 180 days of storage at room temperature (RT) and 40 ^{o}C. a) Gel G1; b) gel G2; c) gel G3.