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Formulation, characterization and in vitro skin penetration of charged flexible liposomes containing carboxyfluorscein as hydrophilic model drug

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ABSTRACT

The aim of this work was to evaluate the charged liposomes containing sodium cholate to produce positively and negatively flexible liposomes in comparison to conventional liposomes. The effect of these charged flexible liposomes on the *in vitro* transdermal delivery of carboxyfluorscein (CF) was investigated. All of the tested liposomes were characterized in terms of size distribution, zeta potential and vesicle stability. The influence of these liposomes on the in vitro rat skin deposition of CF was studied by in vitro Franz diffusion cell experiment. The results showed that all of the investigated liposomes had almost small particle size range from 170 to 193 nm with low polydispersity index (PDI<0.3). All liposomal formulations exhibited negative zeta potential except cationic liposomes had positive zeta potential with proper drug entrapment efficiency and stability. *In vitro* skin deposition data showed that, flexible liposomes gave significant deposition of CF on the skin compared to conventional liposomes. Moreover, the cationic charged flexible liposomes, prepared with stearyl amine (SA), were also able to deliver a higher amount of CF. These results suggested that the hydrophilic CF delivery to the skin was strictly correlated to the vesicle composition.

INTRODUCTION

The liposomes have been considered as a system for dermal and transdermal delivery.[1] Many studies had reported that the skin delivery of numerous drugs was enhanced following application of liposomes. For instance, tretinoin for the treatment of acne, glucocorticoids for the treatment of atopic eczema, lignocaine and tetracaine as anesthetics.[2-4] The possible mechanisms by which conventional liposomes could increase skin delivery of drugs have been appraised. These include the vesicles intact the skin surface and its components enter the intercellular lipid matrix of the stratum corneum (SC), modifying the lipid lamellae.[5] Another mechanism reported that intact vesicles might penetrate the SC, due to the effect of transepidermal osmotic pressure.[6] Touitou et al reported that the conventional liposomes could not deeply penetrate skin, because they remain restricted to upper layers of the stratum corneum.[7] Therefore, only localized or rarely transdermal effects of conventional liposomes have been observed.

Recently, several approaches have confirmed that flexible membrane of the liposomes could enhance the skin penetration. Therefore, the flexible liposomes could result in improved drug transport across the skin as compared to vesicles with rigid membrane.[8] As a result, a sequence of vesicles with flexible membranes were developed in order to enhance the dermal or transdermal delivery of the drugs. For example, the liposomes with edge activators such as sodium cholate, span 80 and tween 80.[9] An edge activators destabilize lipid bilayers of the vesicles and increases flexibility of the membrane.[10] These vesicles had ability to penetrate intact skin under-occlusive application efficiently.[10]

Furthermore, the thermodynamical state of the liposomal membrane can be affected by changing the surface charge of liposomes.[11] The lipid layer in the SC has a high content of negatively charged lipids and it is well known that the skin may act as a negatively charged membrane.[12-13] It has been declared that the existence of charges at the vesicle membrane may affect the transdermal deposition of drugs. Negatively charged liposomes give a higher permeated drug than positively charge one, which in turn can improve drug deposition in skin layers.[12] However, results in the literature are opposing.

The authors suggested that the presence of negative charge with edge activator in the membrane might allow for a better efficiency of penetration. Manosroi et al. showed that the transdermal absorption of amphotericin B was higher when entrapped in charged liposomes than in non-charged ones and that positive liposomes produced a higher absorption through the stratum corneum than the negatively charged ones.[14]

Therefore, the purpose of this study was to evaluate the flexible liposomal systems containing sodium cholate, which gave a flexible bilayer for skin drug delivery of hydrophilic model drug, carboxyfluorscein (CF). Furthermore, two types of positively and negatively flexible liposomes containing stearylamine (SA) and dicetyl phosphate (DCP) were prepared, respectively. The obtained liposomes were characterized in terms of particles size, zeta potential and stability. Penetration experiments were conducted in order to investigate the penetration ability of these vesicles.

MATERIALS AND METHOD

Soybean lecithin Lipoid S100 was purchased from Lipoid KG (Germany). Sodium cholate was purchased from Serva (Germany). Dicetyl phosphate (DCP) and stearylamine (SA) were purchased from Sigma (Germany). 5(6)-Carboxy-fluorescein was purchased from Sigma-Aldrich, (Steinheim, Germany). Methanol (HPLC grade) was purchased from Carl Roth GmbH&Co. (Germany). Ethanol, purity ≥ 99.8%, was purchased from Carl Roth GmbH&Co. (Germany). Phosphate buffered saline pH = 7.4 was composed of 136.89 mM NaCl, 2.68 mM KCl, , 1.47 mM KH_2PO_4 and 8.20 mM Na_2HPO_4 x 2 H_2O (all from Merck, Darmstadt, Germany).

Preparation of liposomes

The liposomes were prepared by a conventional rotary evaporation method.[15-16] The lipoids S100, cholesterol or sodium cholate were used (to get the flexibility of the liposomes, the ratio of surfactant and lipids should be equal to 0.2).[17] The prepared liposomes and their composition are represented in table (1). Briefly, the lipoid S100 and other components were dissolved in organic solvent, chloroform/methanol (1:2) in a round bottom flask. The flask was connected to a rotor evaporator under vacuum (Rotavapor, Büchi, Germany). The mixture was dried under vacuum at the transition temperature of in case of lipoid S100 is about 55 °C. A thin film of the lipid formed on the wall of the flask. The lipid film was then flashed with nitrogen gas for removal of possible traces of organic solvent. The liposomal dispersion was formed after film rehydration with PBS pH 7.4 containing 20 mM CF. The liposomal dispersion was sonicated by means of the probe sonicator to get liposomes of the smaller size. All liposomal dispersions were stored at 4°C until further investigation.

Physicochemical Characterization of lipid dispersions

Particle size measurements

Photon correlation spectroscopy (PCS): Dynamic light scattering was measured at 25 °C. The particle size, the polydispersity index (PDI), zeta-potential (mV) were measured using a Zeta plus instrument (Brookhaven Instruments, Brookhaven, USA). The liposomes were appropriately diluted with the aqueous phase of the formulations prior to the measurements. The particle size values given are the averages of 4 measurements and are expressed as z-average. PDI measures the size distribution of the liposomes. Zeta-potential (mV) was measured by the instrument from electrophoretic mobility of the particles.[16]

Entrapment efficiency (EE%)

The free CF was separated from entrapped CF by using the ultracentrifugation (Optima™ Max-E, Ultra Centrifuge, Beckman Coulter, Pasadena, CA) at 30 000 rpm at 4 °C, for 60 min. Purified deposit was then diluted to the initial volume using PBS (pH = 7.4) in order to used directly for in vitro permeation study.

Entrapment efficiency of CF was calculated indirectly from the amount of free drug, according to the following equation:

$$\text{Entrapment efficiency (\%)} = (CF_t - CF_f / CF_t) \times 100$$

Where CF_f was the amount of free CF and CF_t was the total amount of CF.

Storage stability studies

In order to determine the physical stability of the liposomes, the vesicles were stored at 4 °C for up to 2 months under light protection.[18] In predetermined time intervals, the particle sizes of the vesicles and PDI were measured.

In-vitro skin penetration studies

It is difficult to get human skin, thus an abdominal rat skin was used. Previously, rat skin membranes model were well established for *in vitro* testing, as it is comparable to human skin in stratum corneum (SC) thickness as well as water permeability.[19] The abdominal hair of male rat skin was removed with an electric clipper carefully. After the rats were scarified, the subcutaneous fat tissue was carefully removed from the skin by means of a scalpel and surgical scissors. Afterwards the skin was wrapped into aluminum foil and stored at -20 °C until use less than 4 weeks.[20] All procedures were approved by Institutional Animal Care and Use Committee and were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. Prior to the experiments, the skin samples were taken from the freezer and let thaw at room temperature for about 30 min. After thawing, the skin surface was carefully wiped with cotton wool balls wetted with PBS buffer.

The samples of skin were mounted onto Franz diffusion cells (FDC) with a nominal area for diffusion of 3.14 cm^2 and a receptor volume of about 12 ml. The epidermal side of the skin was exposed to ambient conditions while the dermal side was bathed with BPS buffer pH 7.4. The receptor fluid was kept at 37 1°C throughout the experiments to reach the physiological skin temperature (i.e.32 1°C). The constant stirring was maintained by magnetic stirring at 500 rpm. Care was taken to remove all air bubbles between the underside of the skin (dermis) and the receptor solution throughout the experiment. After equilibration for 30 min, 200 µL of liposomal dispersions containing CF were applied to the skin surface. Samples were taken from the receptor fluid (500µL) every hour and the withdrawn volume was replaced by the same volume of fresh PBS pH 7.4 to maintain a constant volume. After 6 h, the formulations were wiped off by the cotton wool pads wetted with PBS buffer 3-4 times.

For determinations of the drug deposition in the different skin layers, the skin was fixed onto cork plates and stretched using small pins. The SC was then subsequently removed by tape stripping. Transpore tape (3M TransporeTM tape, St. Paul, MN, USA) with a surface area of approximately 4 cm^2 was applied on the SC surface of the skin. The tape was firmly pressed on skin surface and pulled off immediately with one smooth stroke. Each

Table 1: Composition of the different types of charged flexible liposomes.

Composition	Formulations (molar ratios)			
	CF-FL1	CF-FL2	CF-FL3	CF-CL
Lipoid-S100	10	10	10	10
Cholesterol				5
Sodium cholate	2	2	2	
Stearylamine		1		
Dicetylphosphate				1

CF-CL: conventional liposomes; CF-FL1: flexible liposomes containing sodium cholate; CF-FL2: flexible liposomes containing sodium cholate and dicetyphosphate; CF- FL3: flexible liposomes containing sodium cholate and stearyl amine.

Table 2: Physical characterization of the different types of charged flexible liposomes.

Formulations	Particle size (nm)	PDI	Zeta potential
CF-CL	193±1.1	0.241±0.030	- 3.7±0.45
CF-FL1	187±4.6	0.211±0.014	- 4.3±0.27
CF-FL2	174±2.8	0.261±0.014	-12.19±0.76
CF-FL3	170±1.2	0.265±0.011	8.13±0.34

Table 3: Amounts of CF (expressed as cumulative % of dose applied, n = 3) in the different layers of rat skin after 6 hours of non-occlusive incubation.

Code	SC	Stripped Skin	Receptor	Total
CF-CL	0.989 ± 0.096	0.435 ± 0.087	0.181 ± 0.098	1.605 ± 0.064
CF-FL1	1.449 ± 0.217	0.728 ± 0.199	0.212 ± 0.078	2.389 ± 0.131
CF-FL2	5.155 ± 1.302	1.913 ± 0.121	1.188 ± 0.204	7.979 ± 0.542
CF-FL3	2.968 ± 0.513	1.637 ± 0.248	0.350 ± 0.092	4.231 ± 0.285

skin sample was stripped with 10 pieces of adhesive tape to confirm the removal of the SC.[21] The amount of CF in the stripped skin was determined by cutting into small pieces. The tapes, stripped skin were placed each in PBS pH=7.4:ethanol(2:1) overnight following by 5 min vortexing and 5 min sonication for complete extraction of CF following by filtration. The tapes, stripped skin and receptor fluid were assayed for the content of CF by fluorescence spectroscopy, excitation 485 nm, emission 520 nm.[16] All experiments were done in triplicate.

Statistical data analysis

Data analysis was carried out with the software package Microsoft Excel, Version 2003 and origin software, version 6. Results are expressed as mean ± standard error (n = 3 independent samples).

RESULTS AND DISCUSSION

Particle size investigations

Different types of charged flexible liposomes containing sodium cholate were prepared and studied the skin permeation properties. The additions of such surfactants sodium cholate in vesicle membranes lead to flexibility of the vesicles membrane, which was called flexible liposomes.[21-23] The efficiency of these flexible vesicles on delivering CF was investigated and compared with conventional liposomes. The compositions of these different vesicular systems, their particle size distribution, PDI and zeta potential are presented in table (1).

The size distribution of the vesicles was determined by dynamic light scattering. The frequency distribution curves of particle size data were unimodal in shape. The particle sizes of flexible liposomes (CF-FL1, CF-FL2 and CF-FL3) were 187 ± 4.6, 174 ± 2.8 and 170 ± 1.2 nm, respectively. The particle size of conventional liposomes (CF-CL) was 193 ± 1.1 nm. As expected a decrease in the particle size was detected in the case the flexible liposomes, this result was in agreement with literature.[24]

Being charged flexible liposomes, charging agents would be expected to decrease the vesicle size. This result could be attributed to the existence of charging agents in liposomal bilayer, which increase its affinity to be curved, hence reducing the size of the vesicles. [25-26]

All liposomal dispersions investigated in this study showed a best polydispersity index (PDI) below 0.3, which indicate the good homogeneous of the prepared liposomes.[27] The PDI of the investigated formulations was in the range from0.211±0.014 (CF-FL1) to 0.265±0.011 (CF-FL3).

Regarding the zeta potential table (1), CF-CL and CF-FL1 possessed a small negative surface charge, considering that the formulations are neutral.[24] In case of CF-FL2, a negatively charged vesicle was produced due to presence of dicetyl phosphate, while, CF-FL3 gave positively charged vesicles due to presence of stearyl amine.[13, 28]

Entrapment efficiency (EE %)

The entrapment efficiency of CF in flexible liposomes CF-FL1, CF-FL2, and CF-FL3 reported, 41.7, 55.7, and 58.4% respectively, compared with 25.3 % reported for conventional liposomes CF-CL fig. (1). The lower EE% is to be predictable for hydrophilic drugs. It is obvious that charged flexible liposomes represented the highest EE% which is accompanied with high zeta potential of the vesicles. The reason for that was attributed to the high value of zeta potential which frequently lead to increase the repulsion forces of the bilayer structure of the vesicles which consequently increasing the size of the inner aqueous core of the liposomes. Being CF, hydrophilic compound, increasing the size of aqueous core compartment contributes in increasing the amount of CF in the vesicles as it is observed in this study.[29]

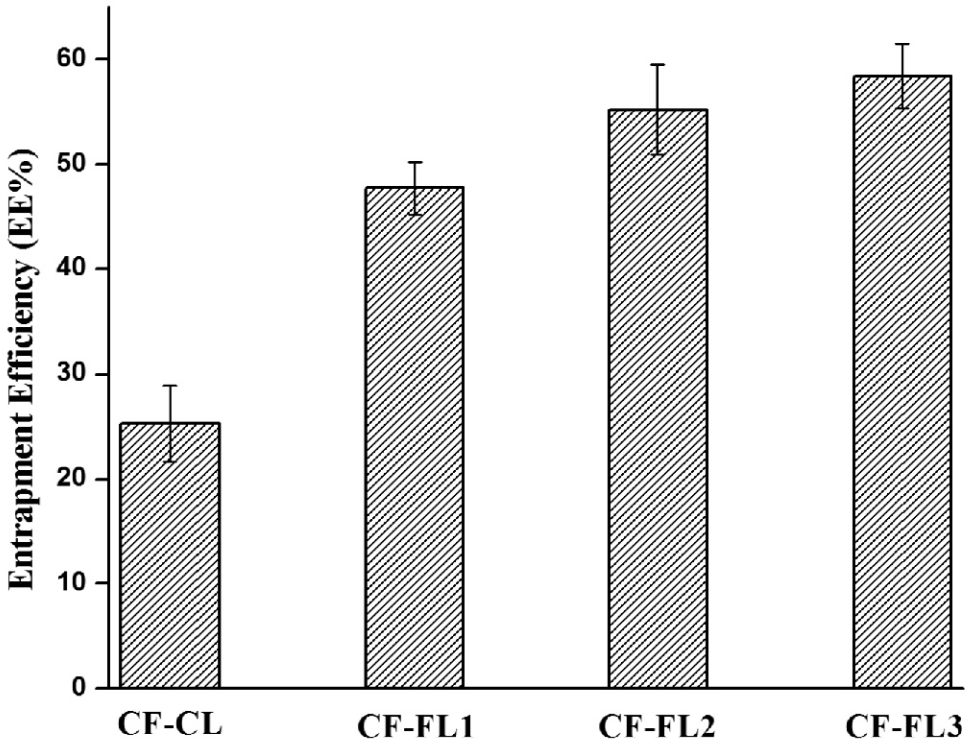

Figure 1: The entrapment efficiency of CF loaded different liposomes.

Storage stability

In the different liposomal dispersions, the storage temperature had low effect on the physical stability of the vesicles, i.e. the particle size and PDI values showed a smaller change during storage at 4°C. The formulated liposomes showed a small change during the storage at 4°C and they could be considered physically stable when stored at 4°C for two months. The particle size and the PDI of different liposomal dispersion almost did not alter significantly during storage, regardless of the storage time (Fig. 2 A and B).

In vitro penetration studies

In order to assess the ability of charged liposomes to deliver CF into the different rat skin layers using a standardized skin stripping technique with a Franz diffusion cell. CF was selected as hydrophilic model drug because a poor penetration through the skin by passive diffusion of such hydrophilic compound can be expected.[30]

Fig. 3 and table (3) show the amount of carboxyfluorscein (CF) in the stratum corneum (SC), dermis and receptor fluid of

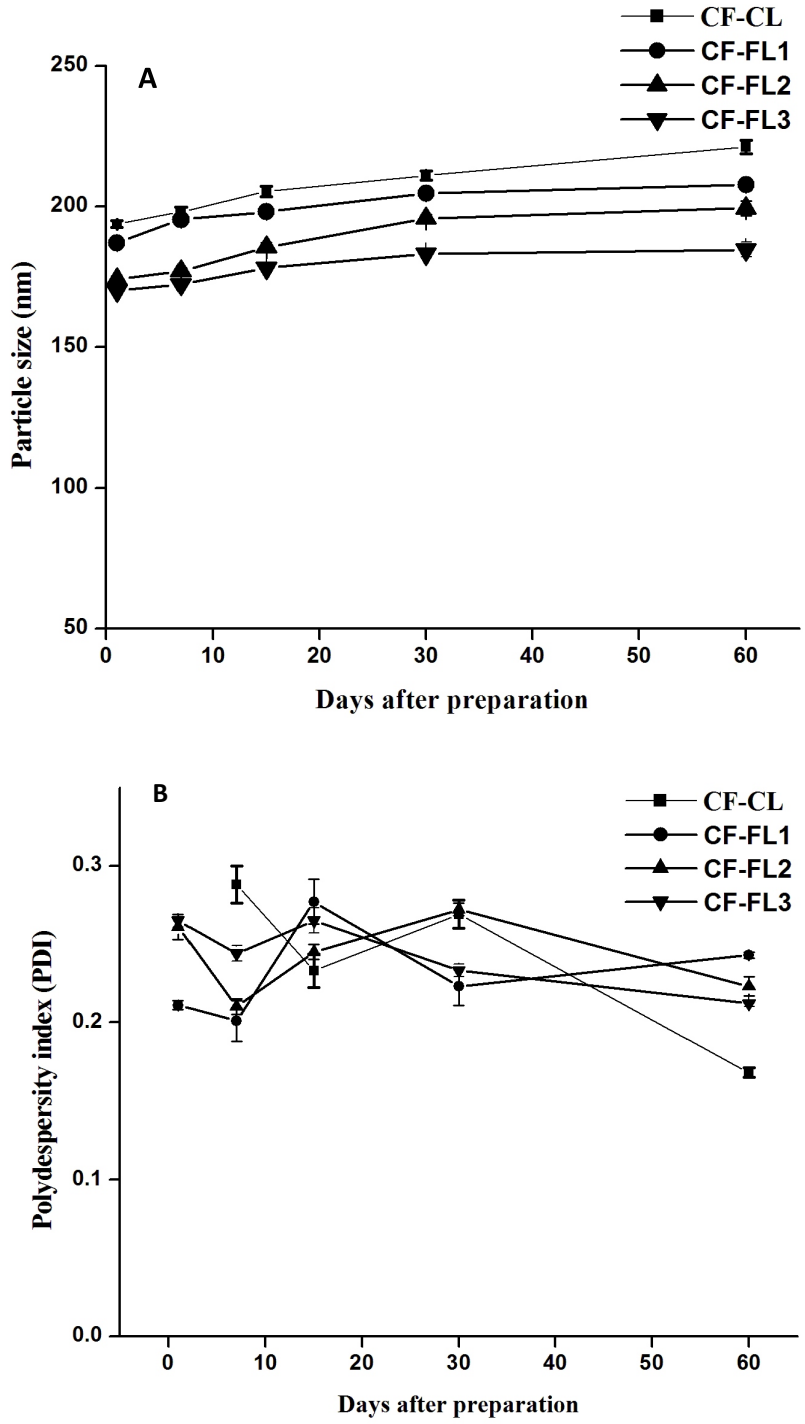

Figure 1: Stability study of CF loaded different liposomes stored at 4 oC over 2 months duration. (A) Change of particle size (z-average), (B) change of the polydispersity index (PDI).

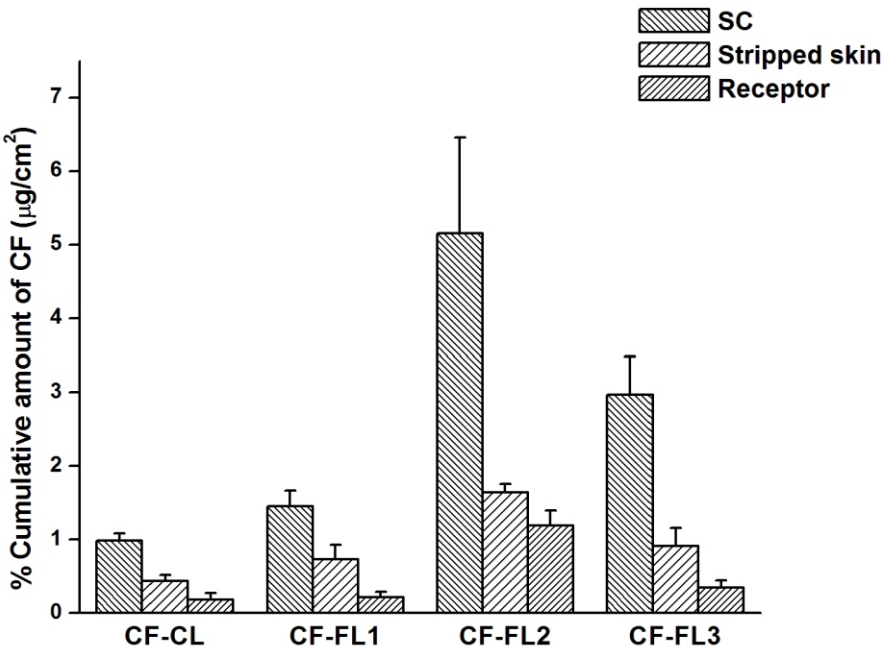

Figure 1: Amount of CF delivered into the SC, Stripped skin and the receptor fluid after 6 h of incubation with rat skin (CF-CL: conventional liposomes; CF-FL1: flexible liposomes containing sodium cholate; CF-FL2: flexible liposomes containing sodium cholate and SA and CF-FL3: flexible liposomes containing sodium cholate and DCP.

Franz type diffusion cells. Concerning the CF amount in the SC, the entrapped CF in flexible liposomes significantly enhanced the deposition of CF after 6 h compared with the conventional liposomes. The significant variation in penetration is observed between neutral liposomes (CF-FL1), positively charged liposomes (CF-FL2), and negatively charged liposomes (CF-FL3).

Namely, the amount of CF delivered by CF-FL2 was 3.6-fold higher than the amount delivered by CF-FL1 and 1.7-fold higher than the amount delivered by CF-FL3 in the SC (Fig. 3 and Table 3). In addition, CF-FL2 also showed a 5.2-fold higher deposition of CF in the SC compared to CF-CL. These data revealed the synergistic effect of charged liposomes containing sodium cholate and its higher penetration enhancing ability compared to neutral liposomes. Furthermore, the amount of CF in the deeper skin was 1.2, 2.6 and 4.4-fold higher for CF-FL2 as compared to CF-FL3, CF-FL1 and CF-CL, respectively. As well, the amount of CF recovered in the receptor compartment was 3.4, 5.6 and 6.6-fold higher for CF-FL2 (Fig. 3 and Table 3). Therefore, CF-FL2 showed highest accumulation of CF in the SC, deeper skin layers, and in the receptor compartment of the Franz diffusion cell, as compared to other formulations. As shown in Fig. 3, the adding of DCP enhanced the penetration of CF at the same molar ratio as SA, representing that the existence of a negative charge in the liposomal bilayer is enough to enhance the deposition of the entrapped CF.

The possible explanation of these results for these liposomes depends in the addition of sodium cholate, which makes the phospholipid bilayer greatly elastic vesicles. These results were in agreement with other studies using flexible vesicles containing sodium cholate for transdermal carrier systems.[21-22]

Due to the flexibility of the liposomal bilayer, penetration into the SC may be facilitated particularly under non-occlusive conditions, and driven by the existence of the transepidermal osmotic gradient allowing vesicles to squeeze and migrate as intact into the SC.[15-23]

These proposed mechanisms include the ability of liposome to work as drug carrier systems, in which intact vesicles transported the stratum corneum carrying drug into the skin. Another mechanism stated that vesicles could act as penetration promoters, in which vesicle bilayers interact with the stratum corneum causing modification in the intercellular lipid lamellae. This effect will facilitate the partitioning the drug molecule into and penetration through the stratum corneum.[31-32] These results were in agreement with other studies using ultradeformable vesicles containing sodium cholate for transdermal carrier systems.[16, 31]

Concerning the influence of charged liposomes on the skin deposition of CF, the skin positively charged liposomes was higher compared to negatively charged liposomes. The data obtained is in agreement with other studies.[33-34] The proposed mechanism include that the skin act as a negatively charged membrane, hence the positive charges on the bilayer of liposomes could bind to negative charges of the SC enhancing the drug penetration through the skin.[24, 35]

Song and Kim explored a higher drug penetration from positively charged flexible liposomes compared to neutral and negatively charged flexible liposomes.[36] All data presented revealed that CF deposition in the different skin layers was dependent on the type of charging agent incorporated on flexible liposomes. Skin incubation with positively charged liposomes (SA) led to the highest drug deposition in the SC compared to negatively charged liposomes (DCP).

CONCLUSION

In the present study, two types of charged flexible vesicular systems containing CF in comparison to conventional liposomes were developed and characterized. CF could be delivered into the

stratum corneum (SC) and deeper skin layers. Hence, the deposition of CF was enhanced after skin incubated with flexible liposomes particularly when containing sodium cholate compared to conventional liposomes. The results of the penetration study revealed that cationic flexible liposomes were most effective in delivering the CF into the SC and skin layers. The flexible vesicles incorporating sodium cholate and SA when applied onto rat skin, significantly increased distribution of CF in the SC, stripped skin and receptor fluid compared with conventional liposomes.

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