Glycerosomes: use of hydrogenated soy phosphatidylcholine mixture and its effect on vesicle features and diclofenac skin penetration

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Abstract

In this work, diclofenac was encapsulated, as sodium salt, in glycerosomes containing 10, 20 or 30% of glycerol in the water phase with the aim to ameliorate its topical efficacy. Taking into account previous findings, glycerosome formulation was modified, in terms of economic suitability, using a cheap and commercially available mixture of hydrogenated soy phosphatidylcholine (P90H). P90H glycerosomes were spherical and multilamellar; photon correlation spectroscopy showed that

obtained vesicles were ~131 nm, slightly larger and more polydispersed than those made with dipalmitoylphosphatidylcholine (DPPC) but, surprisingly, they were able to ameliorate the local delivery of diclofenac, which was improved with respect to previous findings, in particular using glycerosomes containing high amount of glycerol (20 and 30%). Finally, this drug delivery system showed a high in vitro biocompatibility toward human keratinocytes.

Key words: (trans)dermal drug delivery, hydrogenated phospholipid vesicles, pig skin, keratinocytes, DSC, rheology.

Introduction

Phospholipid vesicles took a great deal of interest, particularly to improve dermal and transdermal delivery of several drugs, avoiding degradation in gastrointestinal tract, first pass hepatic metabolism and ameliorating patient compliance. Unfortunately, the skin, which consists of three different layers, prevent the passage of most external molecules and in particular, the stratum corneum is the effective barrier limiting the passage of exogenous substances thus reducing the bioavailability of topically applied drugs. Liposomes has been firstly suggested in 1980 by Mezei et al. (Mezei and Gulasekharam, 1980; Mezei, 1988) as innovative delivery systems for the topical administration of drugs. Successively, many authors studied their possible exploitation for cutaneous application of different bioactive molecules (Schreier and Bouwstra, 1994). In most cases, liposomes do not penetrate the skin but remain almost confined in its upper layers providing a limited drug penetration. In the last decades, new classes of phospholipidic vesicles have been developed using specific additives such as ethanol and surfactants able to modify the physico-chemical and functional properties of liposomes, enabling a more efficient skin delivery of drugs (Ainbinder et al., 2010; Cevc and Blume, 1992; Touitou et al., 2000). More recently, the use of penetration enhancers, cosolvents or polymers has been proposed as innovative approach to promote phospholipid vesicle performances as skin carriers (Manca et al., 2015, 2014a, 2014b, 2013a, 2013b; Manconi et al., 2012). Glycerosomes are phospholipid vesicles containing glycerol (10–30%) in the water phase, they have been previously suggested for topical application of diclofenac and quercetin (Manca et al., 2014a, 2013b). Diclofenac is considered one of the most potent non steroidal anti-inflammatory agent and it is commercially available in different formulations for parenteral, oral and topical administration (Cevc and Blume, 2001; Todd and Sorkin, 1988). Systemic treatments are often associated to serious adverse effects mainly due to the poor diclofenac specificity, especially in the gastrointestinal tract, where causes bleeding and stomach ulcerations. To avoid these problems, achieving the therapeutic drug concentration in the target tissue, topical formulations of diclofenac are commercially available and used in clinical practice (Cevc and Blume, 2001; Singh and Roberts, 1994). Actually, these

formulations are not completely effective and are frequently associated to systemic treatments to ensure the desired therapeutic effect.

In this work, with the aim to ameliorate topical performances, diclofenac sodium salt was encapsulated in glycerosomes. It was previously loaded in glycerosomes, prepared with dipalmitoylphosphatidylcholine (DPPH), a pure and expensive phospholipid, and glycerol at different concentrations (10-30%). The glycerol effect on fluidity of vesicle membranes and its naturally occurring hydration of the skin was described (Manca et al., 2013b).

At the present, glycerosomes were alternatively obtained using a commercial mixture of hydrogenated soy phosphatidylcholine (Phospholipon® 90H, P90H), which is cheap and easily commercially available, and different amounts of glycerol (10-30%) in the water phase. Diclofenac was encapsulated into glycerosomes as sodium salt and formulations were deeply characterized by transmission electron microscopy, photon correlation spectroscopy, differential scanning calorimetry and rheology. Thus in vitro permeation performances of vesicles were evaluated and compared with that of conventional liposomes and glycerosomes made using dipalmitoylphosphatidylcholine. Moreover, their in vitro biocompatibility was assessed using human keratinocytes.

2. Materials and methods

2.1. Materials

Hydrogenated soy phosphatidylcholine (Phospholipon® 90H, P90H) was purchased by AVG S.r.l. (Garbagnate Milanese, Italy). Phosphate buffer solution (PBS, pH 7) was purchased from Carlo Erba Reagents (Rodano, Italy). Diclofenac sodium salt (DCF_{Na}), cholesterol, glycerol and all the other products were of analytical grade and were purchased from Sigma-Aldrich (Milan, Italy). Cell medium, foetal bovine serum, penicillin and streptomycin were purchased from Life Technologies Europe (Monza, Italy).

2.2. Vesicle preparation

Empty or diclofenac loaded (0.5% w/v) liposomes and glycerosomes were prepared by hydration of lipid film in two steps. In a typical preparation P90H (300 mg), cholesterol (10 mg) and diclofenac

sodium (50 mg) when appropriate were hydrated with 5 ml of water (to obtain liposomes) or glycerol/water (10, 20, 30% v/v) solution (to obtain glycerosomes) and the concentrated dispersion was mechanically shaken for 1 hour at room temperature (Manconi et al., 2003). Then, a further 5 ml of water or glycerol/water (10, 20, 30% v/v) solution were added and the dispersion was shaken for a further one hour. The obtained suspensions were then sonicated (20 cycles 5 sec ON and 2 sec OFF) using a Soniprep 150 ultrasonic disintegrator (MSE Crowley, UK).

The dispersions were purified from the non-incorporated drug by exhaustive dialysis. Each sample (2 ml) was placed into a membrane Spectra/Por® (12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, The Netherlands) and dialysed against distilled water (2 l) at 25°C for 2 hours, which were enough to remove the non-encapsulated drug. Drug encapsulation efficiency (EE%) was expressed as the percentage of the amount of drug initially used versus that found after dialysis. The drug amount was determined by high performance liquid chromatography (HPLC) after disruption of vesicles with methanol (1:100 dilution). Analysis was performed at 227 nm using a chromatograph Alliance 2690 (Waters, Milano, Italy) equipped with a photodiode array detector and a computer integrating apparatus (Millennium 32) using a column Symmetry C18 (3.5 μ, 4.6 × 100 mm, Waters). The mobile phase was a mixture of water and acetonitrile (30/70 v/v), at a flow rate of 0.5 ml/min.

2.3. Vesicle characterization

Vesicle formation and morphology were confirmed by transmission electron microscopy (TEM). Samples, stained with a 1% phosphotungstic acid, were examined with a JEM-1010 (Jeol Europe, Paris, France) transmission electron microscope equipped with a digital camera MegaView III and Software "AnalySIS", at an accelerating voltage of 80 kV.

The average diameter and polydispersity index (PI) of the samples were determined by photon correlation spectroscopy using a Zetasizer nano (Malvern Instrument, Worcestershire, United Kingdom). Zeta potential was estimated using the Zetasizer nano by means of the M3-PALS (Phase Analysis Light Scattering) technique. Just before analysis, the samples (100 μl) were diluted with

water or glycerol/water blend (10 ml). A stability study was performed by monitoring the vesicle average size and zeta potential over 90 days at 25±1°C.

2.4. Rheological characterization

The rheological study was performed in the oscillation mode using a Kinexus Nano controlled stress rheometer (Malvern Instruments, UK) equipped with data acquisition and elaboration software rSpace. Samples were laid within the gap of the measuring system and allowed to rest for 300 seconds before running analyses. In all cases, the exposed edges of the samples were covered with a silicone oil layer to prevent water evaporation during the measurement time span.

Rheological experiments were carried out using a cone-plate geometry (CP 1/60). Oscillatory frequency sweep tests were performed (f=0.01-10 Hz) in the range of linear viscoelasticity, preliminary determined by means of amplitude sweep tests. The oscillatory parameters used to compare the viscoelastic properties of the various dispersions were the storage modulus (G'), which describes the elasticity of the material, and the loss modulus (G″), which is related to the dissipated energy (viscous behavior). Flow curves were performed in a shear rate range of 10^{-3}-10^{3} s^{-1}: a stepwise increase of the stress was applied, with an equilibration time of 30 s. All the measurements were carried out in triplicate, at 25°C (Castangia et al., 2013; Manca et al., 2014b).

2.5. Differential Scanning Calorimetry (DSC)

Studies were performed by using a DSC 821e Mettler Toledo, scanning the samples (empty and drug loaded vesicles), in sealed aluminum pans under nitrogen atmosphere. Analysis were performed from 25 to 60°C, heating at a constant rate of 7°C/h. Sample runs were repeated at least 3 times to ensure reproducibility.

2.6. Ex vivo skin penetration and permeation studies

Non-occlusively experiments were performed using Franz vertical cells with a diffusion area of 0.785 cm^2 and new born pig skin. One-day-old Goland–Pietrain hybrid pigs (~1.2 kg) were provided by a local slaughterhouse. The skin, stored at −80°C, was pre-equilibrated in PBS solution at 37°C, 12 h before the experiments. Skin specimens (n=6 per formulation) were sandwiched between donor and

receptor compartments of cells. The receptor compartment was filled with PBS solution, which was continuously stirred with a magnetic bar and thermostated at 37±1°C to reach the physiological skin temperature (~32°C). Diclofenac formulations (100 µl) were placed onto the skin surface; at regular intervals (2, 4, 6, 8 h) the receiving solution was withdrawn, replaced with fresh solution and lyophilized. After 8 h, the skin surface of specimens was washed and the stratum corneum was removed by stripping with adhesive tape Tesa® AG (Hamburg, Germany). The epidermis was separated from the dermis with a surgical sterile scalpel. Tape strips, epidermis, dermis and lyophilized content of receptor compartments were dispersed in methanol (2 ml), sonicated to extract the drug and then assayed for drug content by HPLC (Section 2.2).

2.7. Cell culture studies

Human keratinocytes were grown as monolayer in 35 mm tissue culture dishes incubated in 100% humidity and 5% CO_2 at 37°C. Iscove's Modified Dulbecco's Medium high glucose, containing l-glutamine, supplemented with 20% foetal bovine serum, penicillin/streptomycin and fungizone, was used as growth medium (Manca et al., 2014c).

2.8. Cytotoxicity assessment, MTT assay

The keratinocytes (7500 cells/well) were seeded in 96-well plates with 250 µl of culture medium. After 24 h the cells were treated for 2, 4, 6, 8, 24 and 48 h with the empty and drug loaded vesicles or drug solution. Each sample was tested in triplicate for at least 3 times. The untreated cells were used as negative control. At the end of the experiments, the cells were washed three times with fresh medium and their viability was determined by the MTT [3(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] colorimetric assay, adding 200 µl of MTT reagent (0.5 mg/ml in PBS) to each well. After 2-3 h, the formed formazan crystals were dissolved in dimethyl sulfoxide and their concentration was spectrophotometrically quantified at 570 nm with a microplate reader (Synergy 4, Reader BioTek Instruments, AHSI S.P.A, Bernareggio, Italy). Results are shown as percent of cell viability in comparison with non-treated cells (100% viability).

2.9. Statistical analysis of data

Data analysis was carried out with the software package R, version 2.10.1. Results are expressed as the means±standard deviation. Multiple comparisons of means were used to substantiate statistical differences between groups, while Tukey and Student's t-test were used for comparison between two samples. Significance was tested at the 0.05 level of probability (p).

3. Results and discussion.

In the previous work, was found that glycerosomes prepared using DPPH were able to improve the skin deposition and permeation of diclofenac, in comparison with a) the corresponding liposomes, b) the aqueous solution and c) the commercial gel (Diclofenac Sandoz® 1%). Here, considering the high costs of pure phospholipids, as an alternative, the same glycerosomes containing 10, 20, 30% of glycerol and encapsulating diclofenac sodium salt, were prepared with P90H, which is a low cost, unpurified commercial mixture of different hydrogenated phospholipids (Aisha et al., 2014).

3.1. Morphology, characteristics, properties and stability of vesicles

Taking into account the above mentioned considerations and aimed at improving diclofenac skin delivery and economical suitability of formulations, physico-chemical characteristics and rheological properties of dispersions were evaluated. Morphology and structure of liposomes and glycerosomes, were observed by TEM, which revealed the presence of multilamellar vesicles, small in size and with spherical shape regardless of the presence of glycerol (Figure 1) as previously reported for DPPH glycerosomes (Manca et al., 2013b).

Glycerosomes prepared using P90H were slightly larger (~130 nm versus ~105 nm) and more polydispersed (~0.346 versus ~0.293) than the vesicles prepared with DPPH (Manca et al., 2013b), the diclofenac encapsulation caused a reduction of size of all vesicles (liposomes and glycerosomes) with respect to the corresponding empty ones ($p<0.05$), indicating that the drug, thanks to its amphipathic nature, can be intercalated in the bilayer as well, changing the packing of the vesicular membrane thus reducing the curvature radius (El Maghraby et al., 2000). This reduction was more evident in liposomes, as their mean diameter shifted from ~131 to ~81 nm ($p<0.01$) but, was observed also in glycerosomes, regardless of the glycerol concentration. As a consequence, diclofenac loaded

glycerosomes showed all the same mean diameter (~103 nm, p>0.05 among glycerosomes), which was significantly higher respect to that of the corresponding liposomes (p<0.05). The diclofenac effect was evident in the surface charge as well, since its encapsulation produced highly negatively charged vesicles irrespective to the glycerol concentration used, ensuring the vesicle stability on dispersion and denoting a drug partitioning in the bilayer surface. Diclofenac loaded glycerosomes were slightly polydispersed (PI~0.293) but the mean diameter results were always repeatable as confirmed by the low standard deviation values obtained from six different data. Glycerosomes showed a higher encapsulation efficiency (~80%, p<0.05 versus liposomes) than that of liposomes probably thanks to their higher size and thus, the greater encapsulation volume (Castangia et al., 2013; Manca et al., 2014b, 2013b).

The effect of glycerol was evaluated also during long-term stability of vesicles, monitoring their size and size distribution for 90 days at room temperature (~25°C) (Figure 2) (El-Samaligy et al., 2006). Drug loaded glycerosomes were highly stable and any change on vesicle size or polydispersity was registered during the study, indicating that no aggregation or fusion of vesicles, occurred. Differently, liposomes underwent a significant increase of mean diameter which was ~110 nm after 7 days (p<0.01 versus starting size) and was doubled in one month (~180 nm), remaining than constant up to 90 days. The glycerol seemed to stabilize the vesicle dispersion avoiding the aggregation or fusion phenomena, which occurred instead for liposome dispersion.

3.2. Rheological properties of liposomes and glycerosomes

Glycerol is a water miscible solvent much more viscous than water and its addition in phospholipid vesicles is expect to modify the system viscosity. In order to evaluate its effect, the rheological behavior of empty and diclofenac sodium loaded liposomes and glycerosomes were studied. In Figure 3, a representative flow curve of each sample was reported (n=3).

At low shear rates (below 10 s^{-1}), glycerol did not affect the rheological behavior of the vesicles, and the flow curves of liposomes and glycerosomes were almost superimposable. At higher shear rates the viscosities of glicerosome suspensions were higher than those of liposome suspensions.

The presence of diclofenac reduced the viscosity values with respect to the corresponding empty vesicles. This behavior can be due to the reduction of the size of DCF containing vesicles, as described above (Table 1). In this case, the reduction of the vesicle size, at the same concentration, i.e. the same volume fraction occupancy, leads an increases of the overall particle surface, thus improving the frictional effects.

The vesicles viscoelastic properties are closely related to the structure and flexibility of their membranes: in Figure 4, the values of G' (obtained at 1 Hz from the mechanical spectra measurements) are reported. Glycerosome G' decreased as the concentration of glycerol increased, confirming an interaction between glycerol and phospholipid membrane (as observed in DSC tests) (Hasan et al., 2016).

Moreover, the mechanical spectra revealed that the presence of the drug in the vesicle layers, due to the strong interaction with the phospholipids, is able to improve the fluidity of the vesicle membrane, thus reducing the elastic properties of the overall system.

3.3. DSC studies

The transition temperature from gel to liquid-crystalline state (T_m) of vesicles, has been measured by DSC. It depends on the length of the hydrocarbon chains and on the nature of the phospholipid head groups and can be affected by interactions with external molecules, ionic strength and pH of aqueous medium (Blume, 1979). In previous works, the addition of glycerol or glycols induced a shift of the T_m in a concentration depending manner (Castangia et al., 2013; Manca et al., 2013b). P90H is a mixture of phospholipids containing several phosphatidylcholines, differing in length of acyl chains, and free fatty acids, it has a T_m ~52,8°C (Manconi et al., 2008). The presence of cholesterol inhibited the pre-transition peak enlarging the main transition peak, thus confirming its ability to modify the bilayer packing (Figure 5). Glycerosomes T_m decreased as the concentration of glycerol increased reaching ~52.3°C for 30% glycerosomes denoting a glycerol interaction with phospholipid membrane. The encapsulation of diclofenac sodium salt allowed an enlarging and decrease of transition peak which displaced toward lower temperatures, ~47.0°C for liposomes and even lower

for glycerosomes up to ~46.6°C for 30% glycerosomes, confirming a strong interaction of drug with phospholipids.

3.4. *In vitro* penetration and permeation studies

The skin represents the main barrier between our body and the external environment and it inhibits the drug intake. In our previous work, DPPH glycerosomes loading diclofenac sodium salt, were prepared and their skin carrier ability was compared with that of corresponding liposomes and a commercial gel (Manca et al., 2013b). Actually, they were able to improve the diclofenac deposition in the different skin strata and its permeation in the receptor compartment, which simulated the biological fluids of the deeper tissues. Here, with the aim to further improve diclofenac penetration and permeation into and through the skin and also ameliorate the vesicle exploitation using a less pure and more economic phospholipid, diclofenac sodium was encapsulated in the same glycerosomes however obtained using P90H and the penetration behavior of the drug in and through the skin was assessed.

Using DPPH glycerosomes the highest amount of diclofenac deposition was provided by 20% glycerosomes and was ~5% in the whole skin. Here, 20% glycerosomes made with P90H, allowed the highest drug accumulation in the skin as well, but reaching ~5% only in the stratum corneum and ~11% in the whole skin, thus doubling the amount provided by the corresponding DPPH glycerosomes (Figure 6, left panel). The same behavior was observed using 30% glycerosomes while, 10% glycerosomes delivered, in the whole skin, a drug amount 4-folds (~8%) higher than that of DPPH vesicles. The lowest amount of diclofenac in the whole skin was found using the commercial gel, Diclofenac Sandoz 1%® (~2,6 %) and liposomes (~3.1%, $p>0.05$ between them). For all the studied formulations the largest amount of drug was accumulated in stratum corneum and epidermis while the lowest was found in dermis. The amount of drug permeated ($\mu g/cm^2$) was measured at different times up to 8 h and plotted against time (Figure 6, right panel). The highest amount of drug permeated was found using 30% glycerosomes (~22 $\mu g/cm^2$) followed by 20% glycerosomes (~20 $\mu g/cm^2$), which allowed permeation 3-4 times higher than that obtained applying the commercial gel

(~6 μg/cm^2). Using 10% glycerosomes and liposomes the drug permeated drastically decreased (~7 and ~2 μg/cm^2 respectively), Figure 6. These findings confirmed that the addition of glycerol to the phospholipid vesicles favoured the accumulation and permeation of diclofenac probably because it acts as a moisturizing agent, which perturbs the ordered structure of the skin. In addition, the glycerol effect was dependent to the used phospholipid and better results were obtained using 20 or 30% glycerosomes made with P90H.

3.5. *In vitro* biocompatibility (toxicity) studies

Topical formulations must necessary make contact with the keratinocytes since they are the main cells of epidermis. For this reason, *in vitro* toxicity of formulations against keratinocytes was performed (Figure 6). Studies were carried out as a function of time (2, 4, 8, 6, 24 and 48 h), incubating cells with diclofenac loaded vesicles and using the diclofenac water solution as reference. The toxicity was evaluated measuring the cell viability by the MTT assay (Figure 7). Diclofenac sodium water solution was slightly toxic up to 8 hours (viability ~85%) and toxicity increased at longer time exposure addressing ~63% of mortality after 48 hours of experiment. Liposomes and glycerosomes were highly biocompatible, the viability was always around 100% at each time tested. The low toxicity of diclofenac when loaded in vesicles may be related to the vesicle ability to be internalized inside the cells and modulate drug release and distribution avoiding its cytoplasmatic accumulation (Safra, 2003)

4. Conclusions

In the present work we demonstrated that glycerosomes represent a viable carrier for the topical administration of diclofenac. In particular, these vesicles can be easily fabricated using a cheap and unpurified hydrogenated soy posphatidylcholine forming stable and fluid vesicles, thanks to the combination of phospholipids and glycerol but also to the contribution of the drug, which is partitionned in the aqueous compartment and in the bilayer. In vitro (trans)dermal delivery studies, confirmed the high capacity of glycerosomes (especially 20% and 30% glycerosomes) to favor the

accumulation and permeation of the drug into and through the skin. Moreover, the high biocompatibility of these systems makes them as promising dermal delivery system.

Figure captions.

Figure 1. TEM micrographs of diclofenac sodium loaded liposomes (A), 10% glycerosomes (B), 20% glycerosomes (C) and 30% glycerosomes (D).

Figure 2. Variation of mean diameter (bars) and polidispersity index (square) of diclofenac sodium loaded liposomes and glycerosomes during 90 days of storage at room temperature (~25°C). Mean values±standard deviation (error bars) are reported (n=6).

Figure 3. Flow curves of empty vesicles (right panel) and sodium diclofenac (DCF_{Na}) loaded vesicles (left panel). Liposomes (black diamond); 10% glycerosomes (red square); 20% glycerosomes (green triangle); 30% glycerosomes (blu circle).

Figure 4. G' (at 1 Hz) of the different systems, obtained by means of mechanical spectra. The data are reported as mean value (n=3), and standard deviations always lay within 10% of the mean.

Figure 5. Representative DSC curves of empty (left panel, A) and diclofenac sodium (right panel, B) of liposomes and glycerosomes and mean values±standard deviation of transition temperature (T_m) from gel to liquid-crystalline phase.

Figure 6. Amount of diclofenac sodium accumulated in stratum corneum (SC), epidermis (EP), and dermis (D, left panel) or permeated in the receptor compartment (RC, right panel) after 8 h application of drug in commercial gel or loaded in liposomes and 10-30% glycerosomes. Data represent the means±standard deviations (error bars) of at least six experimental determinations.

Figure 7. In vitro cytotoxic effect of diclofenac sodium loaded conventional liposomes and 10, 20 and 30% glycerosomes (10–30% Gly) on human keratinocyte at different incubation times. Data represent the means±standard deviations of cell viability expressed as the percentage of control (100% of viability).

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