1 **Glycerosomes: investigation of role of 1,2-dimyristoyl-*sn*-glycero-3-**
2 **phosphatidycholine (DMPC) on the assembling and skin delivery performances**

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19 **Abstract**
20 Glycerosomes were formulated using 1,2-dimyristoyl-sn-glycero-3-phosphatidycholine
21 (DMPC), diclofenac sodium salt and 10, 20 or 30% glycerol in the water phase, while
22 corresponding liposomes were prepared with the same amount of DMPC and
23 diclofenac, without glycerol. The aim of the present work was to evaluate the effect of
24 the used phospholipid on vesicle features and ability to favour diclofenac skin
25 deposition by comparing these results with those found in previous works performed

using hydrogenated soy phosphatidylcholine (P90H) and dipalmitoylphosphatidylcholine (DPPC). Liposomes and glycerosomes were multilamellar, liposomes being smaller (72±6 nm). Interactions among glycerol, phospholipids and drug led to the formation of a non-rigid bilayer structure and a variation of the main transition temperature, which shifted to lower temperature. The addition of glycerol led to the formation of more viscous systems (from ~2.5 mPa/s for basic liposomes to ~5 mPa/s for glycerosomes), which improved spreadability of the formulations on the skin. Results obtained in vitro were promising using glycerosomes, irrespective of the amount of glycerol used: the amount of drug, which accumulated into and permeated through the different skin strata, was high and comparable with that obtained using P90H, suggesting that glycerosomes may represent an efficient carrier for both local effect or systemic absorption.

**Key words:** Glycerosomes; DSC; Rehological Studies; SAXS; Skin Delivery

## 1. Introduction

The skin, which consists of dermis (the deepest layer), and epidermis (the external layer), behaves as a difficult to penetrate barrier for active compounds. In particular, the stratum corneum, the outermost epidermis layer, acts as an effective barrier limiting the passage of exogenous and endogenous molecules, due to its "brick and mortar" structure. To avoid the lack of bioavailability associated with topical application of drugs, liposomes have been proposed and considered as one of the most suitable topical drug delivery systems thanks to their ability to increase drug accumulation at the site where its activity is desired, as firstly suggested by Mezei et al. in 1980 (Mezei and Gulasekharam, 1980). However, the countless studies carried out over the past decades

51 evidence that basic liposomes are not very efficient carriers for transdermal drug
52 delivery because they do not penetrate the skin but rather remain confined into the upper
53 layers of the stratum corneum due to their inability to pass through the narrow (≤ 30
54 nm) intercellular passages (virtual pores) of the skin. To improve performances of
55 liposomes as carriers for topical delivery of active molecules, at first, their chemical and
56 physical properties were modified by varying the preparation methods and/or by
57 including additional molecules in the formulation such as cholesterol, which may
58 increase the stability of the lipidic bilayer, or charged lipid molecules, which modify the
59 electrical charge of vesicular surfaces and reduce liposome aggregation (Christina R.
60 Miller et al., 1998; Gillet et al., 2011; Hsia et al., 1970; Kirby et al., 1980; Manconi et
61 al., 2008; Montenegro et al., 2006). Successively, a new generation of phospholipid
62 vesicles has been developed by adding co-solvents (i.e. ethanol) or edge activators (able
63 to enhance deformability or flexibility of the vesicles) to basic formulation, thus,
64 obtaining ethosomes or transfersomes (Ainbinder et al., 2010; Cevc et al., 1998; Cevc
65 and Blume, 2001; Touitou et al., 2000). In the last years, glycerosomes, penetration
66 enhancer containing vesicles (PEVs), and hyalurosomes have been proposed as the
67 newest and most efficient carriers for skin delivery (Castangia et al., 2015, 2013, Manca
68 et al., 2015, 2013a). Glycerosomes were firstly designed in 2012 (Manca et al., 2013b;
69 Zaru et al., 2012) using DPPC and high concentrations of glycerol (10-30%) in the
70 water phase. Glycerosomes containing 20 and 30% glycerol were able to ameliorate
71 both accumulation and transdermal delivery of diclofenac sodium salt, one of the most
72 potent non-steroidal anti-inflammatory compound, in comparison with basic liposomes.
73 Following studies highlighted the important role played by the phospholipids in
74 modifying the performances of glycerosomes. Indeed, using P90H in the glycerosome

75 formulations, the most efficient delivery of diclofenac into and through the skin was
76 detected (Manca et al., 2016).
77 In the light of the previous results, the aim of the present study was to investigate the
78 influence of the phospholipid used to obtain glycerosomes on topical delivery of
79 diclofenac. To this purpose, glycerosomes (10, 20 and 30% glycerol) were prepared and
80 studied using DMPC, a phospholipid with a lower main transition temperature (~24°C)
81 than DPPC and P90H (~43 and 51°C respectively), as previously reported (Leonenko et
82 al., 2004; Mabrey and Sturtevant, 1976; Sinico et al., 2005).
83 Glycerosomes were prepared and fully characterized by transmission electron
84 microscopy (TEM), photon correlation spectroscopy, differential scanning calorimetry
85 (DSC), small and wide-angle X-ray diffraction (SAXS), and rheology to obtain detailed
86 information regarding their structure and properties. *In vitro* studies were carried out to
87 evaluate the vesicle influence on diclofenac delivery into and through new born pig
88 skin. Corresponding liposomes (without glycerol) were also prepared and used as
89 reference together with a commercial gel (Diclofenac Sandoz 1%®).
90

91 **2. Materials and methods**

92 **2.1. Materials**

93 1,2-dimyristoyl-sn-glycero-3-phosphatidycholine (DMPC) was purchased from Avanti
94 Polar Lipids (Alabaster, USA). Phosphate buffer solution (PBS, pH 7) was purchased
95 from Carlo Erba Reagents (Rodano, Italy). Diclofenac sodium salt, cholesterol, glycerol
96 and all the other products were of analytical grade and were purchased from Sigma-
97 Aldrich (Milan, Italy). Diclofenac Sandoz® 1% was bought in a drug store.

98 **2.2. Vesicle preparation**

99 Liposomes and glycerosomes, empty or loaded with diclofenac sodium salt (0.5%),
100 were prepared according to the thin film hydration method, mixing DMPC (30 mg/ml)
101 and cholesterol (1mg/ml) in chloroform and hydrating the obtained lipid film in two
102 steps with PBS or glycerol/PBS solution containing diclofenac sodium salt (5 mg/ml).
103 At first, an aliquot of PBS (to obtain liposomes) or glycerol/PBS solution (to obtain
104 glycerosomes) was added to the flask and the concentrated dispersion was mechanically
105 shaken for 1 hour at room temperature. Then, a second aliquot of the same solution was
106 added, the dispersion was shaken for another hour and finally sonicated with a Soniprep
107 150 ultrasonic disintegrator (MSE Crowley, UK). The obtained dispersions were
108 purified from the non-incorporated drug by dialysis. Dispersions (2 ml) were loaded
109 into dialysis tubing (Spectra/Pore® membranes, 12–14 kDa MW cut-off, Spectrum
110 Laboratories Inc., USA) and dialyzed against distilled water (2 l) at 25°C for 2 h, to
111 remove the non-encapsulated drug. Drug encapsulation efficiency (EE%), expressed as
112 the percentage of the amount of drug initially used, was determined by high
113 performance liquid chromatography (HPLC) after disruption of vesicles with methanol
114 (1:100 dilution). Analyses were performed at 227 nm using a chromatograph Alliance
115 2690 (Waters, Milano, Italy) equipped with a photodiode array detector and a computer
116 integrating apparatus (Empower™ 3) using a column Symmetry C18 (3.5 μ, 4.6×100
117 mm, Waters). The mobile phase was a mixture of water and acetonitrile (30/70v/v), at a
118 flow rate of 0.5 ml/min.

119 **2.3. Vesicle characterization**

120 To observe the vesicles by TEM, samples were stained with phosphotungstic acid (1%)
121 and examined with a JEM-1010 (Jeol Europe, France) transmission electron microscope
122 equipped with a digital camera MegaView III and Software "AnalySIS", at an
123 accelerating voltage of 80 kV.

124 The average diameter and polydispersity index of the samples were determined by
125 photon correlation spectroscopy using a Zetasizernano (Malvern Instrument, UK). Zeta
126 potential was estimated using the Zetasizernano by means of the M3-PALS (Phase
127 Analysis Light Scattering) technique. Just before analysis, the samples (100µl) were
128 diluted with water or glycerol/water blend (10 ml). A stability study was performed as
129 well by monitoring the vesicle average size and zeta potential over 90 days at room
130 temperature (25°C).

131 **2.4. Differential scanning calorimetry (DSC)**

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

137 **2.5. Small angle X-ray diffraction (SAXS)**

138 Vesicle structure was evaluated by SAXS using two linear, one-dimensional, position-
139 sensitive detectors (PSD 50 M; Hecus X-Ray Systems) containing 1024 channels of
140 width 54.0 mm. Cu Ka radiation of wavelength 1.542 Å was provided by a GeniX X-
141 ray generator, operating at 50 kV and 1 mA (Castangia et al., 2013). The diffraction
142 patterns were recorded at 25 °C. All scattering curves were reproduced twice with
143 subsequent calculation of the electron distance distribution, and yielded identical
144 results. For the figures, a representative curve was selected, plotting the scattering
145 intensity (I) as a function of the scattering vector (q). SAXS patterns were analyzed
146 using a global analysis program (GAP) developed by Pabst (Pabst et al., 2003, 2000).
147 The GAP allows fitting the SAXS pattern of bilayer-based structures, i.e., vesicles and
148 lamellar phases, as previously reported (Castangia et al., 2013).

149 **2.6. Rheological study**

150 Measurements were carried out using a controlled stress rheometer (Kinexus pro,
151 Malvern Instrument, Worcestershire, UK) equipped with an rSpace data acquisition
152 software. The viscosity was measured in a shear range of 0.01–10 Pa. Steady shear and
153 dynamic oscillatory tests were performed using a cone-plate geometry (CP 1/60). All
154 samples were subjected to an initial amplitude sweep to determine the linear viscoelastic
155 region. Subsequent, frequency sweep tests were performed from 0.01 to 10 Hz. The
156 oscillatory parameters used to compare the viscoelastic properties of the different
157 dispersions were the storage modulus (G′), or elastic part of the response, and the loss
158 modulus (G″) or viscous response. All measurements were carried out in triplicate, at a
159 constant temperature of 25±1 °C (Manca et al., 2016, 2014).

160 **2.7. Ex vivo skin penetration and permeation studies**

161 Non-occlusively experiments were performed using Franz vertical cells with a diffusion
162 area of 0.785 $cm^2$ and new born pig skin. One-day-old Goland–Pietrain hybrid pigs
163 (~1.2 kg) were provided by a local slaughterhouse. The skin, stored at −80°C, was pre-
164 equilibrated in PBS solution at 37°C, 12 h before the experiments. Skin specimens (n=6
165 per formulation) were sandwiched between donor and receptor compartments of cells.
166 The receptor compartment was filled with PBS solution, which was continuously stirred
167 with a magnetic bar and thermostated at 37°C to reach the physiological skin
168 temperature (~32°C). Dispersions (100 µl) were placed onto the skin surface and at
169 regular intervals (2, 4, 6, 8 h) the receiving solution was withdrawn, replaced with fresh
170 solution and lyophilized. After 8 h, the skin surface of specimens was washed and the
171 stratum corneum was removed by stripping with adhesive tape Tesa® AG (Hamburg,
172 Germany). The epidermis was separated from the dermis with a surgical sterile scalpel.
173 Tape strips, epidermis, dermis and lyophilized content of receptor compartments were

174 dispersed in methanol (2 ml), sonicated to extract the drug and then assayed for drug
175 content by HPLC (Section 2.2) (Manca et al. 2014).

176 **2.8. Statistical analysis of data**

177 Results are expressed as means±standard deviation. Significant differences were
178 evaluated by one-way ANOVA and post hoc, unpaired t-test by using XLStatistics for
179 Windows. Differences were considered significant at the 0.05 level of probability (p).

180

181 **3. Results and Discussion**

182 Several approaches have been proposed to improve dermal and transdermal drug
183 bioavailability using vesicular carriers. Among these, association of phospholipids with
184 different penetration enhancers (i.e. ethanol, propylene glycol, transcutol, etc.) has
185 attracted a great attention in the last decades. Liposomes are still considered optimal
186 candidates for cutaneous drug delivery and their derived vesicles, obtained modifying
187 their basic composition with co-solvents, surfactants or polymers can improve this
188 ability. To this purpose, glycerosomes, obtained by adding high amounts of glycerol to
189 liposomes, appeared as promising carriers for the topical delivery of synthetic and
190 natural compounds (Zaru et al., 2012). Here, the used phospholipid seems to
191 significantly affect the structure and effectiveness of the lamellar vesicles. Indeed,
192 previous studies underlined the positive effect of hydrogenated soy phosphatidylcholine
193 (P90H) glycerosomes in comparison with DPPC glycerosomes on diclofenac skin
194 accumulation and permeation. To go deeper in the evaluation of the effect of different
195 phospholipids on glycerosome features, a phospholipid with a low transition
196 temperature, DMPC, was selected to prepare and study glycerosomes containing 10, 20
197 and 30% glycerol and corresponding basic liposomes. As a further reference, a
198 commercial gel (Diclofenac Sandoz® 1%), was also tested.

### 3.1. Vesicle characterization and stability evaluation

DMPC glycerosomes were almost spherical but irregularly shaped and multilamellar, irrespective of the amount of glycerol used, as depicted in Figure 1 (Manca et al., 2016, 2013b).

To evaluate the effect of the drug on the physico-chemical properties of the vesicles, empty and diclofenac loaded liposomes and glycerosomes were prepared (Table 1). Empty liposomes were larger in size than the drug loaded counterparts (~128 nm versus ~72 nm), thus, suggesting an important involvement of diclofenac in the bilayer assembling and liposome features because the drug may intercalate into the bilayer thanks to its amphipatic nature, as already proposed (Manca et al., 2016, 2013b). Indeed, when glycerosomes were prepared with DPPC or P90H, the encapsulation of diclofenac sodium salt caused a size reduction of all vesicles (liposomes and glycerosomes) compared to the corresponding empty ones, indicating that the drug intercalated in the bilayer changing the packing of the vesicular membrane and the curvature radius of the vesicles (El Maghraby et al., 2000; Manca et al., 2016, 2013b). Using DMPC, the effect of diclofenac was evident for liposomes (Table 1) and negligible for glycerosomes, probably because when DMPC is spread on water/glycerol solution an expansion of monolayer films is detected, as previously found (Crowe et al., 1984).

Diclofenac loaded glycerosomes were small in size (~106 nm, $p>0.05$) and homogeneously dispersed ($PI \leq 0.237$) regardless of the amount of glycerol used, while diclofenac loaded liposomes were less homogeneously dispersed ($PI \geq 0.311$) but smaller (~72 nm).

Zeta potential of empty formulations was slightly negative (~-12 mV), while diclofenac-loaded vesicles were high negatively charged (~-45 mV) due to the distribution of

224 diclofenac sodium salt on the vesicle surface. The drug showed a highly negative zeta
225 potential (-69 mV) when dissolved in PBS and a slightly less negative charge when
226 dissolved in glycerol/PBS (30%) blend, -43 mV (Table 1).
227 The high negative zeta potential values of the loaded vesicles can ensure their stability
228 in dispersion. Indeed, stability studies performed monitoring size, size distribution and
229 zeta potential (data not shown) during 90 days of storage at room temperature (~25°C)
230 confirmed the high stability of 10, 20 and 30% glycerosomes in comparison with
231 liposomes. No significant changes on the above mentioned parameters were detected for
232 glycerosomes during the storage period, while liposome mean diameter increased up to
233 ~170 nm (Figure 2). As previously found, the glycerol seems to stabilize the vesicle
234 dispersion avoiding aggregation or fusion phenomena, which occurred instead for
235 liposome dispersions (Manca et al., 2016, 2013b).
236 Liposomes and glycerosomes showed a great ability to encapsulate diclofenac
237 (EE~75%, $p>0.05$ among all). Actually, the EE measured for DMPC liposomes and
238 glycerosomes was similar to that previously reported for formulations obtained using
239 DPPC and P90H, denoting the negligible effect of the used phospholipids on this
240 parameter.

241 **3.2. DSC studies**

242 Aiming to better evaluate the possible interactions of glycerol and diclofenac with
243 DMPC bilayer, DSC studies were performed (Figure 3). The empty liposomes showed a
244 main transition temperature from gel to liquid crystalline phase ($T_m$) at ~24.98°C (Ali et
245 al., 2000). It is important to highlight that the presence of cholesterol inhibited the pre-
246 transition and enlarged the main transition peak, thus, confirming its ability to modify
247 the bilayer packing, as previously found for DPPC and P90H liposomes (Manca et al.,
248 2016, 2013b). The addition of increasing amount of glycerol caused little shifts of $T_m$ to

249 lower temperatures up to ~24.47°C for 30% glycerosomes (Figure 3), suggesting a
250 potential interaction of glycerol with the phospholipid membrane. A different behaviour
251 was observed when diclofenac was added to the formulations. In particular, the main
252 transition peak of liposomes disappeared and $T_m$ was almost undectable, indicating a
253 strong interaction of diclofenac with the bilayer that probably caused the observed
254 reduction of the vesicle mean diameter. Differently, diclofenac addition to the
255 glycerosomes led to the appearance of two peaks related to pre-transition and main
256 transition temperatures of the bilayer from gel to liquid crystalline phase. Moreover, the
257 main transition peak shifted to lower temperature and both peak intensity and $T_m$ shifts
258 were directly proportional to the glycerol concentration. This was especially evident for
259 20 and 30% glycerosomes. Both shifts of the pre- and main transition peaks seem to be
260 related to a higher dispersion of both cholesterol and diclofenac in the PBS/glycerol
261 blend with a consequent reduction of their distribution on the bilayer surface (as
262 observed for liposomes) as well as to a simultaneous interaction of glycerol with the
263 bilayer. Indeed, calorimetric data from other works reported that glycerol and other
264 alcohols mixed with DMPC expand monolayer films. It is believed that the expansion
265 of the monolayer increases the fluidity of the hydrocarbon chains, thus, decreasing the
266 main transition temperature (Eliasz et al., 1976). These effects were much more evident
267 when the highest glycerol concentrations, 20 and 30%, were tested.

268 **3.3. SAXS studies**

269 More detailed information on vesicle assembling and bilayer structure were obtained by
270 SAXS analysis. Relate scattering profiles were obtained plotting intensity versus wave
271 vector (q, $Å^{-1}$) (Figure 4). Liposomes and glycerosomes showed similar diffraction
272 patterns characterized by the presence of a main band centred at q ~0.1 $Å^{-1}$ for
273 liposomes and 10% glycerosomes and shifted to lower q values for 20 and 30%

274 glycerosomes. For all samples, the repetition bands of multilamellar structure could be
275 appreciated to higher q values. The shifted main band to lower q values corresponds to
276 an enlargement of the repetition distance between the lamellae (d), which increased
277 from ~68-63 Å for empty and diclofenac loaded liposomes and 10% glycerosomes to
278 ~73 Å for empty and diclofenac 20 and 30% glycerosomes (Table 2). Empty liposomes
279 showed a higher value of lamellar width (d ~67 Å) than the diclofenac loaded liposomes
280 (~63 Å) that could be related to the above mentioned decrease of the vesicle mean
281 diameter and the disappearance of the main transition peak, probably due to the
282 diclofenac intercalation into the bilayer. In glycerosomes, the drug encapsulation did not
283 lead to a similar decrease of the lamellar width as well as to any change of the vesicular
284 mean diameter. The polar head distance to the center of the bilayer ($Z_H$) and the bilayer
285 thickness ($d_B$) were almost equal for all the empty vesicles (liposomes and
286 glycerosomes) while they increased in diclofenac loaded 20 and 30% glycerosomes
287 with respect to liposomes and 10% glycerosomes, thus, indicating an enlargment effect
288 of glycerol on the bilayer, according to DSC results.

289 **3.4. Rheological properties of liposomes and glycerosomes**

290 Liposomes and glycerosomes showed Newtonian behaviour as well as the adopted
291 solvent, probably because of the high dilution of the dispersions (Manconi et al., 2003).
292 Glycerol improved the viscosity as a function of its concentration: samples at higher
293 glycerol concentrations showed the highest viscosity (Figure 5, upper panel).

294 More information on vesicle interactions and bilayer nature were obtained plotting
295 rheological parameters, shear storage modulus (G′) and shear loss modulus (G″), versus
296 the oscillation frequency (Figure 5, lower panel). Oscillatory measurements obtained as
297 a function of frequency showed for all the samples the viscous modulus higher than the
298 elastic one up to 1 Hz, where a sharp cross-over point appeared. Moreover, at higher

299 frequencies, the storage modulus (G′) started to become higher than the loss modulus
300 (G″) and, in addition, the G′ and G″ profiles showed by all the samples were almost
301 completely superimposed, with the exception of the highest glycerol concentration.
302 Although, as above described, glycerol affects dimensions and internal structure of the
303 liposomes, collected data indicate that, from the rheological point of view, the
304 interactions with the unloaded liposomes and glycerosomes are not influenced by the
305 solvent itself, in the chosen samples and shear flow experimental conditions. The
306 overall behaviour is similar to that of a dilute solution.

307 **3.5. *In vitro* penetration and permeation studies**

308 *In vitro* experiments, performed using Franz diffusion cells, were carried out to
309 investigate the potential ability of glycerosomes to enhance diclofenac skin delivery in
310 comparison with the references (the corresponding liposomes and the commercial gel,
311 Diclofenac Sandoz 1%®). Previous works demonstrated the superior ability of
312 glycerosomes, prepared with DPPC and P90H, to improve the diclofenac deposition in
313 the different skin strata and permeation into the receptor compartment in comparison
314 with the corresponding liposomes and gel formulation. Specifically, 20 and 30%
315 glycerosomes obtained using P90H allowed the highest diclofenac permeation and
316 accumulation in the skin doubling the amount provided by the corresponding DPPC
317 glycerosomes and reaching the highest values, i.e. ~5% of diclofenac deposition in the
318 stratum corneum and ~10% in the whole skin (Manca et al., 2016).
319 In the present study, similarly to P90H glycerosomes, 10, 20 and 30% glycerosomes
320 prepared with DMPC provided the same diclofenac accumulation in the whole skin
321 (~10%). However, using DMPC the accumulation was lower in the stratum corneum
322 (~3%) and higher in the deeper skin strata, i.e.. ~4.5% in epidermis and ~2% in dermis
323 (Figure 5, left panel) if compared to that provided by 20 and 30% glycerosomes

324 prepared with P90H. The lowest amount of diclofenac in the whole skin was found
325 using the commercial gel, Diclofenac Sandoz 1%® (~2,5%), which provided a total drug
326 amount 4-fold smaller than that of glycerosomes prepared with DMPC, DPPC and
327 P90H.
328 Transdermal diclofenac delivery was studied: Figure 5 (right panel) shows the amount
329 of drug permeated (μg/cm$^2$) up to 8h. The highest drug permeation was found using
330 20% glycerosomes (~26 μg/cm$^2$), which allowed a permeation 6 times higher than that
331 obtained applying the commercial gel (~4.2 μg/cm$^2$), followed by 10% glycerosomes
332 (~19 μg/cm$^2$). Using 30% glycerosomes and basic liposomes the drug permeation
333 slightly decreased (~16 and 14 μg/cm$^2$, respectively) but it was still significantly higher
334 (~4 times) than that obtained from the commercial gel. Summarizing overall results,
335 both DMPC and P90H glycerosomes effectively improve the dermal and transdermal
336 delivery of diclofenac sodium salt to a greater extent and in a similar way than DPPC
337 glycerosomes. However, whatever the phospholipid used, the addition of glycerol to
338 liposomes favours the accumulation and permeation of diclofenac sodium salt probably
339 because of their penetration enhancer effect. Indeed, they act as a moisturizing agent,
340 which perturb the ordered structure of the skin (Barichello et al., 2008; Westh, 2003).
341 Results of this work have shown that when DMPC is used, a 20% glycerol is enough to
342 lead to the best results in diclofenac delivery into and through the skin. In particular,
343 using this phospholipid with a low $T_m$, the drug was mainly localized in deeper skin
344 strata, differently from the corresponding glycerosomes previously prepared with P90H
345 or DPPC.
346
347 **4. Conclusions**

348 DMPC glycerosomes confirmed the effective ability of these carriers to improve the
349 topical delivery of diclofenac. In particular, the combination of phospholipid, glycerol
350 and drug allowed the formation of stable, multilamellar and non-rigid structure,
351 probably thanks to the interaction of glycerol with the phospholipidic membrane. The
352 ability of the prepared vesicles to improve the skin delivery of diclofenac was
353 comparable to that of the previously studied P90H glycerosomes. However, DMPC
354 glycerosomes showed to improve the drug accumulation in the deepest skin strata,
355 confirming the key role of the phospholipid in influencing the performances of
356 glycerosomes. In the light of overall results, glycerosomes, whatever the phospholipid
357 used, may represent a promising and suitable dermal delivery system for different
358 drugs.
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478 **Figure Captions**

479 **Figure 1.** TEM images of diclofenac loaded liposomes (A), 10% glycerosomes (B),
480 20% glycerosomes (C), and 30% glycerosomes (D).

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

484 **Figure 3.** Representative DSC curves of empty (left panel) and diclofenac loaded
485 liposomes and glycerosomes (right panel). The mean values±standard deviation of
486 pretransition and transition temperature from gel to liquid-crystalline phase were
487 reported for each peak.

488 **Figure 4.** Representative SAXS patterns of empty (left panel) and diclofenac loaded
489 (right panel) liposomes and 10, 20 and 30% glycerosomes.

490 **Figure 5.** Representative flow curves of water, diclofenac loaded liposomes and
491 glycerosomes (upper panel). Storage modulus (G′) and loss modulus (G″) as a function
492 of the frequency of diclofenac loaded liposomes and glycerosomes (lower panel).

493 **Figure 6.** Amount of diclofenac accumulated in stratum corneum (SC), epidermis (EP),
494 and dermis (D, left panel) or permeated in the receptor compartment (RC, right panel)
495 after 8 h application of drug in commercial gel or loaded in liposomes and 10, 20 and
496 30% glycerosomes. Data represent the means±standard deviations (error bars) of at least
497 six experimental determinations.

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