Deformable Surfactant Vesicles Loading Ammonium Glycyrrhizinate: Characterization and *In Vitro* Permeation Studies

Luisa Di Marzio*[,1], Carlotta Marianecci[2], Federica Rinaldi[2], Sara Esposito[1] and Maria Carafa[2]

[1]*Dept. Scienze del Farmaco – University "G. d'Annunzio" – Chieti, Italy*

[2]*Dept. Chimica e Tecnologie del Farmaco – University of Rome "Sapienza" – Rome, Italy*

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Abstract: This study focuses on the physico-chemical characterization of surfactant nanovectors according to the evidence that the knowledge of nanocarrier properties is a necessary step to translate their potentiality to nanomedicine applications. In particular, in this investigation we have prepared deformable surfactant vesicles (dSV) and characterized them in terms of dimensions, zeta-potential, deformability index and stability. Also the potential application of analyzed carriers is evaluated in terms of ammonium glycyrrhizinate (AG) entrapment efficiency and *in vitro* permeation experiments. The obtained results suggest the potential application of dSV in dermal administration of AG, a useful drug for the treatment of various skin diseases, such as dermatitis, eczema and psoriasis.

Keywords: Ammonium glycyrrhizinate, Deformable surfactant vesicles, *In vitro* permeation, NSVs, Triterpene saponins.

INTRODUCTION

Medicinal plants have been used for millennia as one of the main sources of therapeutic agents for mankind. Some 10000 higher plants of approximately 270000 terrestrial plants that have been classified taxonomically, are used medicinally. This may be strongly supported by the relatively large number of plant-derived drugs that have been approved by the U.S. Food and Drug Administration (FDA) in the first decade of the 21st century.

There is today a very great deal of interest among members of the global natural products community in investigating new plant constituents. To give some support for this, a total of 1142 and 1369 natural products from all types of organisms were published in 2001 and 2010 in the Journal of Natural Products, respectively [1].

Glycyrrhiza glabra L. is a perennial, herbaceous shrub, belonging to the family of Leguminosae. The plant is endemic to Mediterranean countries, such as Spain, Greece and Southern Italy [2], and contains triterpene saponins (3–5%), mainly glycyrrhizic acid (a derivative of glycyrrhetic acid), and flavonoids (1–1.5%) [3, 4].

Triterpene saponins offer an anti-inflammatory activity, due to an indirect strengthening of the glucocorticoid activity [5, 6]. Recent studies demonstrate that liquorice extracts are useful for the treatment of dermatitis, eczema, and psoriasis, with an efficacy comparable to that of corticosteroids [7-8]. In particular, the ammonium salt of glycyrrhizic acid is characterized by an anti-inflammatory activity [9, 10]. It was recently reported that glycyrrhizin reduces the inflammatory events following spinal cord injury as edema, tissue damage, apoptosis, inducible nitric oxide synthases expression and nuclear factor-κB activation improving the recovery of limb function [11].

*Address correspondence to this author at the Department of "Scienze del Farmaco", Università "G. D'Annunzio" Chieti/Pescara, Via dei Vestini, 66100 Chieti – Italy; Tel: +3908713554705; Fax: +3908713554911;
E-mail: l.dimarzio@unich.it

The possibility of enhancing skin permeation is a great challenge in developing new drug delivery systems [12, 13].

From the above findings, the application of this compound as a potential anti-inflammatory drug can be further improved by using certain drug delivery systems, e.g., non-ionic surfactant vesicles (Niosomes®), which can enhance the permeation through the skin stratum corneum and hence promote the dermal pharmacological action [14, 15] or ethosomes, innovative vesicular systems that have appeared in the fields of pharmaceutical technology and drug delivery in recent years [16]. This carrier presents interesting features correlated with its ability to permeate intact through the human skin due to its high deformability.

In recent years, surfactant vesicles acquired growing scientific attention as an alternative potential drug delivery system to conventional liposomes. These kinds of vesicles formed by surfactants are known as Niosomes® or non-ionic surfactant vesicles (NSVs). NSVs are arisen from the self-assembly of non-ionic amphiphiles in aqueous media resulting in closed spheroidal structures. The self-assembly of non-ionic surfactants into vesicles was firstly reported in the seventies by researchers in the field of cosmetics [17]. Niosomes are analogous to liposomes, in fact the NSVs can be prepared following the same procedures, under a variety of conditions, leading to the formation of unilamellar or multilamellar vesicular structures [18]. When compared to phospholipid-based vesicles, the surfactant vesicles have several advantages such as greater stability, thus lesser care in handling, storage and lower cost. Those advantages make commercial and novel surfactant more attractive than phospholipids for industrial applications both in the field of pharmaceutics and cosmetics [19-23]. Moreover niosomes, like liposomes, are capable of encapsulating both hydrophilic and lypophilic drugs and be useful in miming biological membranes [24-26]. The encapsulation of drugs in niosomes can minimize drug degradation and inactivation after administration, prevent undesirable side effects, and increase drug bioavailability and targeting the pathological area.

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In this work, deformable niosomes (dSV) made up of surfactants (Tween 20 and Dicetyl phosphate) and cholesterol at various concentrations were prepared to investigate the potential application of dSV for dermal delivery of ammonium glycyrrhizinate, useful for the treatment of various inflammatory based diseases.

Vesicle characterization studies were carried out evaluating dimensions, zeta-potential (ζ), anisotropy and drug entrapment efficiency. Stability studies were also carried out.

Permeation profiles of ammonium glycyrrhizinate/dSV were evaluated *in vitro* using cellulose membranes.

2. MATERIALS AND METHODS

2.1. Materials

Tween 20 (Tw20) was a Merck product (Merck SPA. Milan, Italy). Dicetyl phosphate (DCP), cholesterol (Chol), Hepes salt {N-(2-idroxyethyl) piperazine-N-(2-ethanesulfonicacid)}, calcein, ethanol (99.8% v/v) were Sigma–Aldrich products (Sigma–Aldrich SRL, Milan, Italy).

Ammonium glycyrrhizinate (AG) was a kind gift of Bernett (Pavia, Italy). All other chemicals used throughout this investigation were of analytical grade and no additional purification was carried out. Double-distilled water was used throughout the study.

2.2. Vesicle Preparation and Purification

Two methods were used to prepare dSV mixing Tw20, DCP and Chol in different molar ratios (Table **1**): the thin layer evaporation method and the high speed stirring technique. Briefly, when the thin layer evaporation method was used, surfactants mixtures were dissolved in a round bottom flask using 2 ml of a chloroform/methanol mixture (3:1 v/v) [14, 27].

Table 1. Sample Composition (mM)

Sample	Tween 20	DCP	Chol
N	15	=	15
1	7.5	7.5	15
2	10	5	15
3	12.5	2.5	15

The solvent mixture was vacuum evaporated and the resulted dried film was hydrated with 5 ml 0.01 M pH 7.4 Hepes buffer solution, mixed with ethanol (25% v/v). The dispersion was vortexed for 5 min and then sonicated for 15 min at 25°C using a tapered microtip operating at 20 kHz at an amplitude of 16% (Vibracell-VCX 400-Sonics, U.S.A.), under N_2 stream.

In the high speed stirring technique, surfactant mixtures were dissolved in ethanol (20 ml) at 40°C, under magnetic stirring. Distilled water (50 ml) was added dropwise to the obtained solutions to form multilamellar dSV.

To obtain unilamellar dSV, dispersions were stirred at high speed by an Ultra-turrax T25 basic (IKA®-Werke GmbH & Co. KG, Staufen, Germany) at 15000 rpm for 1 min [9].

AG-loaded vesicles were also prepared by hydrating the dried film with an aqueous solution of AG at 1 % w/v.

2.3. Light Scattering Experiment

Dynamic light scattering (DLS) was used to determine mean size and size distribution of non-ionic surfactant vesicles. The vesicle dispersions were diluted 100 times with the same buffer used for their preparation to avoid multiscattering phenomena. Buffer solutions used for dynamic light scattering experiments were filtered through 0.45 μm cellulose filters to eliminate dust particles. Vesicle mean size and size distribution (polydispersity index, pdi) were measured at 25°C using a Malvern Nano ZS90 light scattering apparatus (Malvern Instruments Ltd., Worchestershire, UK) at a scattering angle of 90.0°. For experimental measurements, the instrumental parameters were: a medium refractive index of 1.330, a medium viscosity of 1.0 mPas and a dielectric constant of 80.4. A third-order cumulant fitting autocorrelation function was used to calculate sample mean size and polydispersity index.

The same apparatus was used for the evaluation of zeta potential of NSVs, which were appropriately diluted (1:10) in distilled water at 25°C. The laser doppler anemometry was used and hence the electrophoretic mobility of non-ionic surfactant vesicles was measured.

The ζ -potential value was calculated from the electrophoretic mobility using a Smoluchowsky constant F (Ka) of 1.5. Reported data represent mean of the ζ-potential (mV) and of the hydrodynamic diameter (nm) for the surfactant vesicles.

2.4. Freeze–Fracture

Vesicles were examined by means of the freeze–fracture microscopy technique.

The samples were impregnated in 30% glycerol and then frozen into partially solidified Freon 22, freeze–fractured in a freeze–fracture device (105°C, 10^{-6} mmHg) and replicated by evaporation from a platinum/carbon gun. The replicas were extensively washed with distilled water, picked up onto Formvar-coated grids and examined with a Philips CM 10 transmission electron microscope.

2.5. Measurement of Deformability Index

Comparative measurements of elasticity of the bilayers of the different liposome formulations were carried out by extrusion measurements [28, 29]. Briefly, the vesicles were extruded through a polycarbonate membrane with a pore size of 50 nm (Nucleopore, The Netherlands) at a constant pressure of 5 bars. The elasticity was expressed in terms of a deformability index $\boldsymbol{D}$, which is proportional to $\boldsymbol{j(rv/rp)^2}$, where $\boldsymbol{j}$ is the weight of the suspension, which was extruded in 5 min through the polycarbonate membrane, $\boldsymbol{rv}$ the diameter of the vesicles after extrusion and $\boldsymbol{rp}$ the pore size

of the membrane. Vesicle suspensions were diluted at the same surfactant concentration. The loss of surfactant during the extrusion was calculated. A corrected deformability index D_{cor} to the maximum deformability index for each formulation was calculated too.

$$D_{cor} = [(j(r_v / r_p)^2) / (j_0(r_{v0} / r_p)^2] \times 100 \quad \text{(Eq. 1)}$$

where rv_0 is the liposomes diameter before extrusion and j_0 is the weight of suspension used for extrusion.

2.6. Vesicle Physical Stability Evaluation

Physical stability studies of AG loaded and unloaded dSV were carried out to investigate if significant changes of the size and ζ-potential of surfactant vesicle dispersion occur during storage. For colloidal stability at different temperatures, the vesicle formulations were stored at 4, 25 and 40°C for a period of 90 days. Samples from each batch were withdrawn at definite time intervals (1, 30, 60 and 90 days) and the ζ-potential and the mean of hydrodynamic diameter of vesicles were determined as previously described in Section 2.3.

2.7. Determination of Drug Entrapment Efficiency

AG entrapment within non-ionic surfactant vesicles was determined on vesicles purified by ultracentrifugation (Beckman, Fullerton, CA), equipped with a fixed angle rotor Beckman mod. F1202. The centrifugation speed was 56,000 ***g*** for 45 min. The supernatant was removed from the dSV pellet and the drug amount was determined by spectrophotometric method.

Spectrophotometric analyses were carried out at 256 nm, by means of a spectrophotometer (Perkin-Elmer, lambda 3a, UV-Vis spectrometer) equipped by 1.0 cm path-length quartz cells. Drug entrapment efficiency (e.e.) was calculated as follows:

$$\text{e.e.} = \frac{\text{mass of incorporated drug}}{\text{mass used for vesicle preparation}} \times 100 \quad \text{(Eq.2)}$$

2.8. *In Vitro* Permeation Experiments

In vitro permeation experiments were performed in a two compartment diffusion cell; the donor compartment was separated from the acceptor one by a cellulose ester dialysis membrane (SpectrumLab USA) characterized by a 8000 MWCO (Dalton).

Permeation experiments were carried out for 24 h, at 32 ± 0.5°C, under continuous stirring, using purified vesicle preparations. The exact half cell volumes (7.0 ml) and surface areas (8.8 cm^2) were taken into account when analyzing permeation data. Before each experiment, the system was allowed to equilibrate for 1 h. The donor cell was filled with purified AG vesicle formulation, while acceptor cell was full of 0.01 M Hepes buffer pH 7.4. At fixed time intervals, 200µl samples were drawn from the acceptor compartment and AG concentration was determined by spectrophotometer analyses as described above, and an equivalent amount of buffer solution was added each time to maintain a constant volume in the receiver compartment. Dilution of the receiver medium was taken into account when processing the permeation data. For an appropriate comparison, a AG/Tw20/ethanol dispersion was used at the same drug concentrations. The drug diffusion rate through dialysis membrane using vesicular systems, was compared with the permeation profiles obtained using the other sample, i.e. drug/surfactant dispersions. The cumulative amount of AG that permeated through the dialysis membrane after the n^{th} sampling (Q_n) was estimated by Eq. (3):

$$Q_n = C_n V \sum_{i=1}^{n-1} V_s C_i \quad \text{(Eq. 3)}$$

where C_i and C_n are the various measured concentrations from 1 to n, V is the volume of the solution in the acceptor compartment and V_s is the sampling volume.

2.9. Statistical Analysis

Results are expressed as the mean of three independent experiments ± S.E. Two groups comparison was performed using paired-samples Student's t-test with a significance level (p-value) of 0.05 (*) and 0.01 (**).

CONCLUSION

In this study, we evaluated dSV as carriers for the topical application of a natural anti-inflammatory agent such as ammonium glycyrrhizinate. For this purpose, we prepared some formulations at different surfactant percentages (Table **1**), in absence and in presence of the anionic surfactant DCP to evaluate the effect of the composition on physico-chemical characteristics of dSV. All formulations were submitted to light scattering analysis to choose the most suitable formulation to be tested *in vitro*.

As shown in Table **2**, dSV showed a narrow particle size distribution; furthermore the presence of DCP plays a fundamental role in increasing ζ-potential value, in accordance with the evidence that this value is an indicator of the good physical stability of nanocarriers [30].

Table 2. Vesicle Characterization

Sample	Size (nm)	pdi	ζ-Potential (mV)	DI*	e.e.[#]
N	622±9	0.2	-3.28±0.07	1.73	13.2
1	363±6	0.3	-23.3±0.5	2.21	38.2
2	368±8	0.2	-23.7±0.7	1.67	39.7
3	520±2	0.2	-20.5±0.8	5.67	40.0

*DI value of sample N, in absence of ethanol = 0.52.
[#]AG loading dose 0.1% w/v.

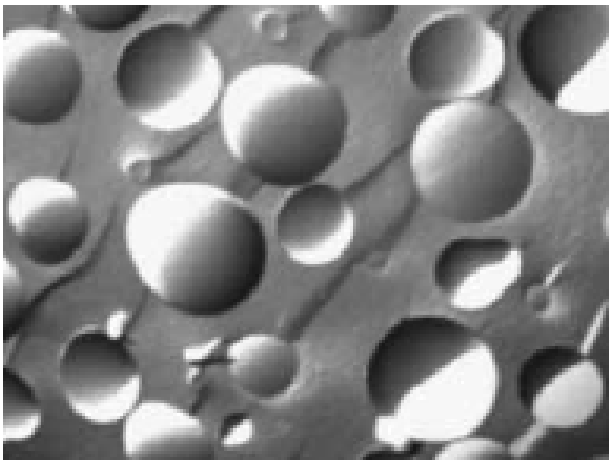

Fig. (1). Freeze-fracture electron micrographs. AG/dSV formulation 3.

Deformability of the non-elastic and elastic niosomes was shown in Table **2**. The DI values of the blank elastic niosomes (sample 3) were more than the blank non-elastic niosomes of at least 3 times, according to the previously reported data [9, 16, 28, 31].

DLS analysis was also carried out on ammonium glycyrrhizinate/dSV and no significant ($p < 0.05$) size variation with respect to unloaded dSV was observed, thus showing that the presence of the drug was not a determinant factor capable of influencing the mean size of the dSV colloidal suspensions. On the other hand, the presence of the drug led to a significant increase of ζ-potential values (up to -40 mV for all analyzed samples). This could be related to a partial adsorption of drug molecules on vesicle surface.

DI of ammonium glycyrrhizinate loaded dSV were not significantly different ($p < 0.05$) from unloaded vesicles.

Drug entrapment within a vesicular carrier is an important parameter to be defined to really evaluate the delivery potentiality of the system. For this reason, the entrapment efficiency of ammonium glycyrrhizinate within the formulations was evaluated in an attempt to investigate the influence of dSV composition, on the drug loading capacity.

As shown in Table **2**, DCP amount influenced the entrapment efficiency of the colloidal carrier. Namely, the lower the amount of DCP, the greater the ammonium glycyrrhizinate entrapment within the vesicles. That could be related to a possible electrostatic repulsion effect between the drug and the anionic surfactant.

In an agreement with previously reported findings [16, 31], the values of entrapment efficiency for ammonium glycyrrhizinate are higher than those expected for conventional vesicle formulations [32]. This fact can be explained by the presence of ethanol, which increases the ammonium glycyrrhizinate solubility in the polar phase of the colloidal formulations of ethosomes. It is reasonable to imagine that the higher DI of sample 3 could be related to higher content of ethanol, leading to higher e.e. value.

The transmission electron micrograph of ammonium glycyrrhizinate/dSV after freeze fracture, prepared according to the composition 3 (Table **1**), is reported in Fig. (**1**). The sample was the one with the highest drug e.e. and, as it is

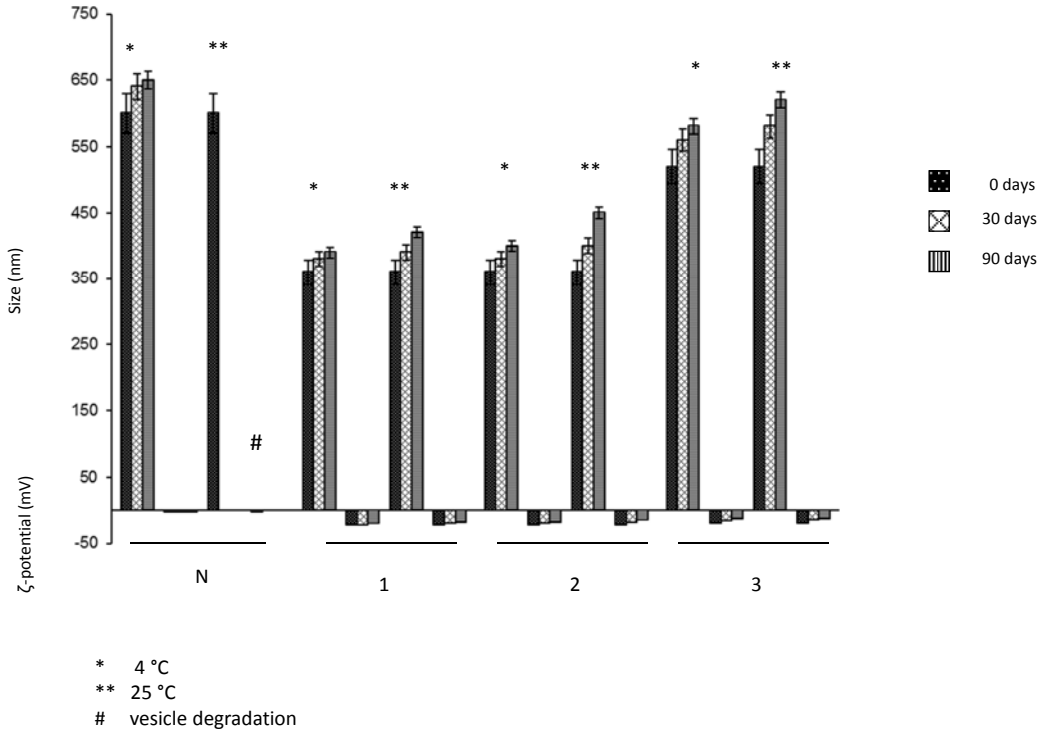

Fig. (2). Physical stability of dSV. Vesicle dimensions and ζ-potential values were evaluated by dynamic light scattering at definite time intervals at 4°C and 25°C. Reported data are the means of three experiments ± S.E.

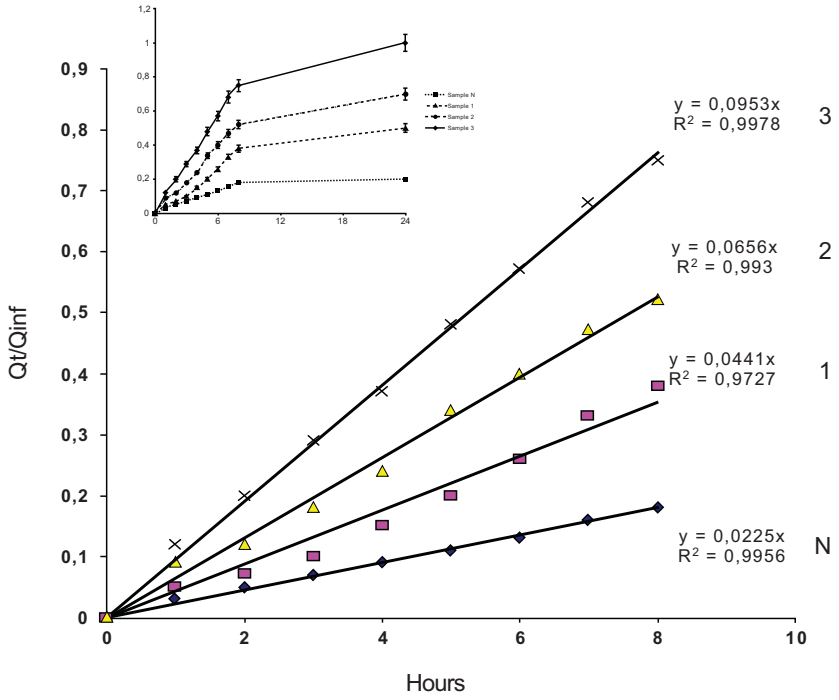

Fig. (3). ***In vitro*** **AG release.** Permeation experiments were carried out for 24 h, at 32 ± 0.5°C. Reported data are the means of three experiments ± S.E.

possible to observe, the vesicles show a monolayer structure and their size is quite uniform.

The physical stability studies of unloaded dSV are shown in Fig. (**2**). From the analysis of obtained results it is evident that dSV in 0.01 M HEPES buffer pH 7.4 are stable for at least six months when stored at 4°C (not reported data). At 25°C, sample N showed sample degradation after few days. Such reduced colloidal stability for sample N can be attributed to the absence of DPC, leading to very low value in ζ-potential. Samples prepared in presence of DCP showed good stability for at least 2 months when stored at 25°C. AG loaded-dSV, showing higher values in ζ-potential are stable for at least six months when stored at 4°C and 25°C (not reported data).

The permeation profile of AG-dSV was compared with those obtained from an aqueous solution and a water–ethanol solution of AG at the same percentage of ethanol used for dSV preparation to evaluate the influence of the organic solvent: in both case AG permeation was negligible.

Comparing permeation profile of dSV samples it is possible to observe that the higher the vesicle deformability (sample 3) the faster the drug permeation, according to previously reported data [9]; in addition, *in vitro* experiments showed that AG flux, calculated as the slope of the linear regression line in the time interval 1 to 8 hours, is linearly dependent on time and that ammonium glycyrrhizinate/dSV can ensure a sustained release of the drug (Fig. **3**).

The reported results are very encouraging and confirm that deformable vesicles are a very promising carrier for the topical administration thus prompting various opportunities for the development of suitable therapeutic strategies through the topical route.

CONFLICT OF INTEREST

None declared.

ACKNOWLEDGEMENTS

None declared.

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