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Anti-inflammatory activity of novel ammonium glycyrrhizinate/niosomes delivery system: Human and murine models

Carlotta Marianecci [a], Federica Rinaldi [a], Marica Mastriota [b], Stefano Pieretti [b], Elena Trapasso [c], Donatella Paolino [c], Maria Carafa [a,*]

[a] Department Drug Chemistry and Technologies – University of Rome "Sapienza" – Rome, Italy
[b] Department Therapeutic Research and Medicine Evaluation – Istituto Superiore di Sanità – Rome, Italy
[c] Department of Health Sciences – University Magna Graecia of Catanzaro – Catanzaro, Italy

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ABSTRACT

Today there is a very great deal of interest among members of the global natural products community in investigating new plant constituents. Recent studies demonstrate that liquorice extracts are useful in the treatment of dermatitis, eczema, and psoriasis, with an efficacy comparable to that of corticosteroids. In this work, niosomes made up of surfactants (Tween 85 and Span 20) and cholesterol at various concentrations were prepared to investigate the potential application of niosomes for the delivery of ammonium glycyrrhizinate (AG), useful for the treatment of various inflammatory based diseases.

Vesicles were characterized evaluating dimensions, ζ potential, anisotropy, drug entrapment efficiency, stability, cytotoxicity evaluation and skin tolerability.

Release profiles of ammonium glycyrrhizinate/niosomes were evaluated *in vitro* using cellulose membranes. The best formulation was used to evaluate the *in vitro*/*in vivo* efficacy of the ammonium glycyrrhizinate/niosomes in murine and human models of inflammation.

The AG-loaded non-ionic surfactant vesicles showed no toxicity, good skin tolerability and were able to improve the drug anti-inflammatory activity in mice. Furthermore, an improvement of the anti-inflammatory activity of the niosome delivered drug was observed on chemically induced skin erythema in humans.

1. Introduction

Medicinal plants have been used for millennia as one of the main sources of therapeutic agents for mankind. Of the approximately 270,000 terrestrial plants that have been classified taxonomically, some 10,000 higher plants are used medicinally. From 210 small-molecule therapeutic agents included in a recent World Health Organization Model List of Essential Medicines, 17 of these are of plant origin. Therefore, there remains much interest in the constituents of plants as therapeutic agents. 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.

In spite of the above-mentioned complications in terms of drug discovery, 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 [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 present an anti-inflammatory activity, due to an indirect strengthening of the glucocorticoid activity [5,6]. Recent studies demonstrate that liquorice extracts are useful in 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 (Fig. 1) is characterized by an anti-inflammatory activity [9,10]. It was recently reported that glycyrrhizin reduced the inflammatory events following spinal cord injury as edema, tissue damage, apoptosis, iNOS expression, and NFκB activation improving the recovery of limb function [11]. 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 [12,13].

In recent years, surfactant vesicles acquired growing scientific attention as an alternative potential drug delivery system to conventional liposomes. This kind of vesicles formed by surfactants is known as Niosomes®

* Corresponding author at: Department of Drug Chemistry and Technologies, Sapienza Università di Roma, Piazzale A. Moro, 5-00185 Roma—Italy. Tel.: +39 0649913603; fax: +39 0649913133.
E-mail address: maria.carafa@uniroma1.it (M. Carafa).

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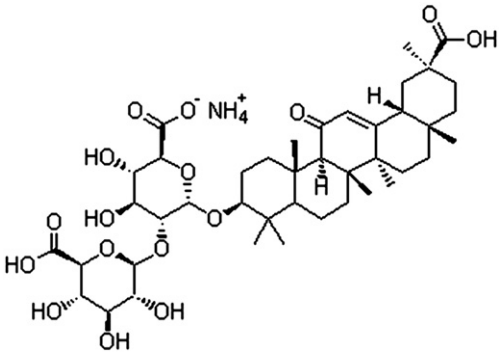


Fig. 1. Ammonium glycyrrhizinate.

or non-ionic surfactant vesicles (NSVs). The self-assembly of non-ionic surfactants into vesicles was firstly reported in the seventies by researchers in the field of cosmetics [14]. 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 [15]. When compared to phospholipid-based vesicles, the surfactant vesicles have several advantages such as greater stability, thus lesser care in handling and 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 [16–20]. Moreover niosomes, like liposomes, are capable of encapsulating both hydrophilic and lypophilic drugs [21,22]. The encapsulation of drugs in niosomes can minimize drug degradation and inactivation after administration, prevent undesirable side effects, and increase drug bioavailability and targeting to the pathological area.

In this work, niosomes made up of surfactants (Tween 85 and Span 20) and cholesterol at various concentrations were prepared to investigate the potential application of niosomes for delivery of ammonium glycyrrhizinate, useful for the treatment of various inflammatory based diseases.

Tween 85 and Span 20 were selected to evaluate the effect of combining two surfactant with different characteristics (HLB Span 20 = 8.6; insoluble in water—HLB Tween 85 = 11, CMC = 0.023 g/l, water 20 °C); furthermore Tween 85 (Polyoxyethylene sorbitan trioleate) was chosen according to its properties for microemulsion formation, protein solubilisation [23] and transport of materials across membranes or skin [24]. Tween 85 was chosen even because the oleic acid, present in this surfactant, is shown to inhibit endothelial cell activation and reduces expression of inflammatory molecules [25].

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

Release experiments, *in vitro* permeation profiles and toxicity of ammonium glycyrrhizinate/niosomes were performed. Experiments to evaluate the *in vitro*/*in vivo* efficacy of the ammonium glycyrrhizinate/niosomes were carried out in murine and human models of inflammation.

2. Materials and methods

2.1. Materials

Tween 85 (Tw85), Span 20 (Sp20), cholesterol (Chol), Sephadex G75, Hepes salt {N-(2-idroxyethyl) piperazine-N-(2-ethanesulfonicacid)}, calcein, and zymosan A were Sigma–Aldrich products (Sigma–Aldrich SRL, Milan, Italy). Diphenylhexatriene (DPH) was obtained from Acros Organics (Acros Organics BVBA, Geel, Belgium). 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

The thin layer evaporation method was used to prepare non-ionic surfactant vesicles from Tween 85, Span 20 and cholesterol in different molar ratios (Table 1) as elsewhere reported [12,26] (see S1).

The non-ionic surfactant vesicles were purified from un-entrapped substances by gel permeation chromatography using Sephadex G75 (glass column 50 × 1.2 cm) as the stationary phase and an isotonic pH 7.4 Hepes buffer solution as the eluent. The chromatographic purification was carried out at room temperature. At the end of the procedure 20 ml of the purified NSVs dispersion were collected.

2.3. Light scattering experiment

Dynamic light scattering 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., Worcestershire, UK) at a scattering angle of 90.0°. For the experimental measurements the instrumental parameters were as follows: a medium refractive index of 1.330, a medium viscosity of 1.0 mPa s 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. Results of light scattering experiments are given as the average values obtained using samples from three different batches ± standard deviation.

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. Vesicle physical stability evaluation and serum stability

Physical stability studies of AG loaded and unloaded NSVs, composed of Tw85 and Sp20 with Chol in different ratios, were carried out to investigate if significant changes of the size and ζ-potential of

Table 1
Sample composition.

Sample	TWEEN 85 mM	SPAN 20 mM	CHOL mM
1	15	15	–
2	7.5	7.5	15
3	7.5	7.5	7.5

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.

The effect of plasma on vesicle stability was studied. Calcein-loaded vesicle suspensions (250 μl) were added to 2.25 ml buffer solution in absence (0% FBS) or in presence of 10% of FBS [27,28]. Calcein leakage was evaluated after 3 h and compared to calcein fluorescence intensity after complete leakage by addition of isopropanol (1/1 v/v). Calcein fluorescence was detected at Ex/Em 492/520 nm by means of a Perkin-Elmer LS50B spectrofluorometer. Vesicle size determinations were also performed, after incubation.

2.6. Vesicle fluidity determination

DPH was added to vesicle composition at 2×10^{-4} M. DPH-labeled vesicles (prepared as above reported) were stored at least 3 h at room temperature before the fluorescence anisotropy experiments to anneal the vesicle bilayer structure and hence to avoid any possible experimental artifact. The fluidity of surfactant vesicular membranes was determined by fluorescence anisotropy using DPH as fluorescence probe, as previously reported [12] (see S2).

2.7. Determination of drug entrapment efficiency (e.e.)

AG entrapment within non-ionic surfactant vesicles was determined using HPLC [9] (see S3) on purified vesicles following their disruption with isopropanol (vesicle dispersion/isopropanol 1:1 v/v final ratio).

Drug e.e. was calculated as follows:

$$\text{e.e.} = (\text{mass of incorporated drug}/\text{mass of drug used for vesicle preparation}) \times 100 \quad (1)$$

Results are the average of three different batches ± SD.

2.8. In vitro skin permeation experiments

Samples of healthy adult human skin (mean age 34 ± 11 years) were obtained from abdominal reduction surgery. Membranes of the stratum corneum and viable epidermis (SCE) were isolated as previously reported [29]. Subcutaneous fat was surgically removed by means of a scalpel and the skin was immersed in distilled water at 60 °C for 2 min. Then SCE was peeled off and immediately used for the various permeation experiments. The barrier integrity of SCE was checked by determining the tritiated water permeability coefficient ($K_p = 1.5\times0.3\times10^{-3}$ cm h^{-1}), that resulted consistent with values previously reported. Skin permeation studies were carried out by using continuous flow Franz-type diffusion cells. The cell had a diffusional surface area of 0.75 cm^2 and a volume of 4.75 ml. Human skin was mounted horizontally with the stratum corneum side up, dividing the cell into two compartments: i.e., the donor and the receptor compartments. The receptor was filled with pH 7.4 isotonic phosphate-buffered solution. The receptor fluid was constantly stirred with a small magnetic stirring bar in order to ensure its homogeneity. Each formulation (200 μl at 0.5% w/v) was applied in the donor compartment and the amount was sufficient to maintain steady state conditions. Steady state conditions were maintained during experimental investigations. The experiments were carried out in non occlusive conditions for 24 h at a thermostated temperature of 35° ± 1 °C. The duration of experiments was 24 h. One milliliter of each sample was withdrawn every 1 h up to 24 h of incubation by using an FC 204 fraction collector (Gilson Italia S.r.l., Cinisello Balsamo (MI), Italy) connected to a Minipuls 3 peristaltic pump (Gilson Italia S.r.l., Cinisello Balsamo (MI), Italy). The volume withdrawn was replaced by the same volume of fresh receptor phase. Samples collected from the receiving compartment were analyzed by HPLC. Six different experiments were carried out for each formulation and the results are expressed as the mean value ± standard deviation.

2.9. Evaluation of carrier toxicity

2.9.1. NCTC 2544 cell cultures

Human keratinocyte cells (NCTC 2544) were transferred from cryovial tubes to plastic culture dishes (100 mm diameter) and seeded (5% CO2) for 3 days at 37 °C using DMEM supplemented with penicillin (100 UI/ml), streptomycin (100 μg/ml), amphotericin B solution (1% v/v) and FBS solution (10% v/v) as the culture medium throughout all in vitro experiments. After the incubation time, NCTC 2544 cells reached a confluence of 80% and were treated with 2 ml of trypsin solution, washed with 2 ml PBS, transferred into 10 ml plastic centrifuge tubes and harvested. Four milliliters of culture medium were added to human keratinocytes thus achieving a final volume of 8 ml. Samples thus obtained were centrifuged using a Megafuge 1.0 (Heraeus Sepatech) at 1200 rpm for 10 min at room temperature. Pellets were separated from the supernatant and resuspended with 6 ml of culture medium to obtain 1×10^6 cells/ml. The cell suspension was diluted up to a final concentration of 3×10^4 cells/ml and 1 ml of the diluted suspension was seeded into 12 well plastic culture dishes before in vitro investigations.

2.9.2. Cytotoxicity evaluation

Cytotoxic effects of empty niosomes on NCTC 2544 cells were evaluated with the trypan blue dye exclusion assay (cell mortality) and the MTT dye test (cell viability). NCTC 2544 were seeded at a density of 3×10^4 cells/ml in 12-well plastic culture dishes for the trypan blue dye exclusion test and at a density of 1.8×10^3 cells/100 μl in 96-well tissue culture plates for the MTT test. After 24 h of incubation the culture medium was replaced for each well by fresh culture medium and niosomes were added at final scalar dilutions (0.01, 0.1, 1, 10 μM in Tw85 concentration).

The toxicity experiments were carried out at three different incubation times, i.e. 24, 48 and 72 h. Untreated NCTC2544 cells were used as the control to perform the trypan blue dye exclusion assay; NCTC2544 were harvested using trypsin/EDTA (1×) solution (2 ml), washed twofold with phosphate buffer solution (2 ml) and transferred into 5 ml plastic centrifuge tubes. Culture medium (4 ml) was added to plastic centrifuge tubes up to a final volume of 8 ml. Samples were then centrifuged with a Megafuge 1.0 (Heraeus Sepatech) at 1200 rpm at room temperature for 10 min. The supernatant was withdrawn and the pellet was suspended in 200 μl of trypan blue buffer, re-suspended for 30 s and the amount of dead cells (blue stained cells) was observed with a hematocytometer chamber using an optical microscope (Labophot-2, Nikon, Japan). The percentage of cell mortality was calculated using the following equation:

$$\%\,\text{Mortality} = (D_c/T_c)\times 100 \quad (2)$$

where D_c is the number of dead cells and T_c the total number of cells.

Cell viability assay was carried out following the MTT dye test. NCTC2544 cells were seeded at a density of 1.8×10^3 cells/100 μl in a 96-well culture plates for 24 h at 37 °C and 5% CO^2 to allow the adhesion of culture cells. After 24 h of incubation, the culture medium was removed and substituted with fresh medium, human keratinocyte cells were then treated with niosomes. Ten microliters of MTT tetrazolium salt (5 mg/ml dissolved in PBS buffer) were added to each well at the end of incubation time and NCTC2544 cells were incubated for an additional 3 h, thus allowing the formation of violet formazan crystals. A mixture (200 μl) of dimethylsulfoxide/ethanol (1:1 v/v) was used to dissolve the formazan crystals and the 96-well culture plates were gently shaken at 230 rpm (KS 130 Control, IKA® Werke

GMBH & Co., Staufen, Germany) for 20 min. Cell viability of different samples was determined by an ELISA microplate reader (Labsystem mod. Multiskan MS, Midland, ON, Canada) set at a wavelength of 570 nm in absorption and 670 nm in emission. The percentage of viable cells, directly proportional to the amount of formazan crystals formed, was calculated using the following equation:

$$\%\ \text{Cell viability} = (\text{Abs}_T/\text{Abs}_U) \times 100 \quad (3)$$

where Abs_T is the absorbance of treated cells and Abs_U is the absorbance of untreated cells. Values of cell mortality and cell viability are expressed as the mean of six different experiments ± standard deviation.

2.9.3. In vivo evaluation of skin tolerability

Skin tolerability of NSVs was evaluated on human volunteers by means of the non invasive technique of reflectance spectrophotometry. The reflectance spectrophotometer, SP60 (X-Rite Incorporated, USA), correlated at a 0° illumination and viewing angle, was calibrated with the supplied white standard traceable to the National Bureau of Standard's perfect white diffuser. The spectrophotometer was connected to a personal computer, which performed different color determinations from the spectral data using the X-Rite acquisition and evaluation software. Reflectance spectra were observed over a wavelength range 400–700 nm using an illuminant C and 2° standard observer.

The investigation was performed on ten healthy volunteers who gave their written informed consent before entering the study after receiving full details of the procedure and what was involved. Subjects did not suffer from any ailments and they were not under any pharmacological therapy for at least 1 week before this experiment. Human volunteers rested for 30 min at room temperature (22 ± 2 °C and 40 ± 5% relative humidity) before the experiment. Six sites on the ventral surface of each forearm were defined using a circular template (1 cm^2). The distance between sites was at least 2 cm in order to avoid any possible interference. Erythema index (EI) baseline values were recorded at each designated site before application of formulations and then NSVs were applied on two sites using Hill Top chambers (Hill Top Research, Inc., Cincinnati, Ohio, USA).

Before reflectance spectrophotometric readings, the chambers were removed and the skin surface was gently washed with water to remove the applied formulation and the skin was allowed to dry for 15 min. The possible induced erythema was monitored at various times, 6, 24, and 48 h and was calculated according to the following equation:

$$\text{EI} = 100[\log 1/R_{560} + 1.5(\log 1/R_{540} + \log 1/R_{580}) - 2(\log 1/R_{510} + \log 1/R_{610})] \quad (4)$$

where $1/R$ is the inverse reflectance at a specific wavelength (510, 540, 560, 580, 610). The results are expressed as differences between the EI values measured after the application of various formulations and the EI baseline values measured at the beginning of the experiment (ΔEI).

2.10. In vivo evaluation of anti-inflammatory activity

2.10.1. Evaluation of anti-erythema activity

The efficacy of ammonium glycyrrhizinate-loaded niosomes to reduce the chemically induced erythema in comparison with aqueous solution of ammonium glycyrrhizinate was evaluated *in vivo* on healthy volunteers. Eight sites on the ventral surface of each forearm were randomly defined using a circular template (1 cm^2) and demarcated with permanent ink. The distance between sites was at least 2 cm to avoid any possible interference. For each volunteer (a total of six), all the sites were treated with an aqueous solution (100 μl) of methyl-nicotinate (0.2% w/v) for 15 min using Hill Top Chambers. At the end of this period the chambers were removed and the skin surface was gently washed with water to remove the applied formulation and then 200 μl of niosomes or the simple aqueous solution containing ammonium glycyrrhizinate were applied.

A saline solution (NaCl 0.9% w/v) (200 μl) was used as the blank. The induced erythema was monitored until its disappearance. The reduction of erythema was directly proportional to the amount of ammonium glycyrrhizinate permeated through the skin from the examined formulations [9].

2.10.2. Evaluation of anti-inflammatory activity

The following experiments were performed according to the guidelines of the European Community Council (86/609/EEC) for animal care and use. Due to its best behavior *in vitro* permeation experiments, sample 1 was chosen to carry out the *in vivo* experiments. Purified formulation of sample 1 was compared to the unstructured surfactant formulation with the same AG concentration (1.12 mg/ml) (sample 1U) and AG solution at the concentration of 1.12 mg/ml.

2.10.3. Edema induced by zymosan

Male CD-1 mice (Harlan, Italy) weighing 25–30 g were used for all experiments. Mice were housed for at least 1 week before the experimental sessions in colony cages (7 mice in each cage) under standard light (light on from 7.00 a.m. to 7.00 p.m.), temperature (21 ± 1 °C), relative humidity (60 ± 10%) with food and water available *ad libitum*. Edema was induced by a subcutaneous injection of 2.5% w/v zymosan A in saline in the dorsal surface of the right hind paw (20 μl/paw). Paw volume was measured 3 times before the injections and at 1, 2, 3, 4, 24, 48 and 72 h thereafter using a hydroplethysmometer specially modified for small volumes (Ugo Basile, Italy). Ammonium glycyrrhizinate/niosomes, ammonium glycyrrhizinate and vehicles (Hepes buffer or blank vesicles) were administered subcutaneously in the dorsal surface of mice paw 120 min before zymosan A in a volume of 40 μl/paw. The increase in paw volume was evaluated as percentage difference between the paw volume at each time point and the basal paw volume [30].

In the preliminary experiments, AG formulations were administered at different times before zymosan. It was found that AG formulation was effective in reducing zymosan-induced edema when administered 1–2 h before zymosan. Then only the effects recorded after the administration of AG formulation 2 h before zymosan were reported.

2.10.4. Nociception induced by formalin

The procedure used has been previously described [31]. Subcutaneous injection of a dilute solution of formalin (1%, 20 μl/paw) into the mice hind paw evokes nociceptive behavioral responses, such as licking, biting the injected paw or both, which are considered indices of pain. The nociceptive response shows a biphasic trend, consisting of an early phase occurring from 0 to 10 min after the formalin injection, due to the direct stimulation of peripheral nociceptors, followed by a late prolonged phase occurring from 20 to 40 min, that reflects the response to inflammatory pain. During the test, the mouse was placed in a Plexiglas observation cage (30 × 14 × 12 cm), 1 h before the formalin administration to allow it to acclimatize to its surroundings. The total time (s) that the animal spent licking or biting its paw during the formalin-induced early and late phase of nociception was recorded. AG formulations were administered 2 h before formalin, in the same way as in the edema experiments in mice.

2.11. Data analysis and statistics

Experimental data were expressed as mean ± S.E.M. For *in vivo* studies the significance among the groups was evaluated with the analysis of variance (two-way ANOVA) followed by Bonferroni's post-hoc comparisons.

Table 2
Sample characterization.

Sample	Size (nm)	pdi	ζ-Potential (mV)	Anisotropy	e.e. (%)
1	139.01 ± 1.65	0.13	−22.6 ± 1.5	0.10	–
1AG	79.0 ± 1.3	0.31	−18.6 ± 1.5	0.19	28.8
2	150.10 ± 2.63	0.30	−37.9 ± 1.4	0.28	–
2AG	98.1 ± 2.6	0.32	−34.9 ± 1.4	0.25	6.7
3	153.09 ± 1.90	0.35	−30.5 ± 1.4	0.20	–
3AG	109.1 ± 1.9	0.38	−26.5 ± 1.4	0.26	7.2

Statistical significance was assumed at $P<0.05$ ($^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$).

3. Results and discussion

3.1. Vesicle characterization and physical stability

The surfactant vesicles, prepared with the mixture of the amphiphilic substances and CHOL at different molar ratio (Table 1), were obtained by the "film" method.

The measurements carried out by DLS indicate that selected surfactants in presence of CHOL form vesicular structures with no significantly different sizes and ζ-potential (Table 2). ζ-Potential values indicate the stability of the prepared formulations. The high negative ζ-potential is important in preventing aggregation. It has been reported that a physically stable nanosuspension solely stabilized by electrostatic repulsion will have a minimum ζ-potential of −30 mV [32].

Apart from providing the hydrodynamic diameter and ζ-potential value, DLS provides also valuable information on the homogeneity of the suspension. A single sharp peak in the DLS profile implies the existence of a single population of scattering particles.

DLS and PDI measurements indicated that, among the non-ionic surfactant formulations, only sample 1 showed a significant yield of homogeneous vesicular structures. On the other hand, anisotropy values are remarkably influenced by CHOL presence (Table 2).

It can be underlined that the addition of CHOL plays a fundamental role in vesicle formation [33,34] increasing the cohesion among the apolar portion of bilayer, by the combination of the single chain of surfactant with steroid molecule: sample 1, prepared in absence of CHOL, shows a high fluid vesicle bilayer.

Difference in bilayer fluidity does not affect vesicle stability while calcein release is affected by this parameter. From the analysis of reported results it is evident that vesicles in 10 mM HEPES buffer pH 7.4 are stable for at least 6 months when stored at 4 °C (not reported data) and at least 3 months when stored at 25 °C (Fig. 2). Sample 1, with high bilayer fluidity, shows higher calcein release compared to samples 2 and 3; this probably related to dye diffusion through vesicle bilayer rather than to vesicle disruption (see S4).

The potential *in vivo* use of vesicle formulations is also related to serum stability [35].

To better simulate *in vivo* conditions, all tested samples were analyzed at pH 7.4 in the presence of 0%, and 10% FBS. The addition of serum to surfactant vesicular formulations at pH 7.4 did not modify (i.e. the percentage of calcein released after 3 h) vesicle stability (Fig. 3).

3.2. AG entrapment and in vitro release

Three different surfactant vesicle formulations were characterized evaluating the entrapment efficiency. Encapsulation efficiency of the vesicles was affected by the initial ammonium glycyrrhizinate concentration (0.5, 0.75, 1 % w/v): the increase of ammonium glycyrrhizinate concentration led to a decrease of encapsulation efficiency (not reported data).

These data are in agreement with those reported in literature [36] and for this reason only samples containing 0.5% AG were used for further studies (Table 2).

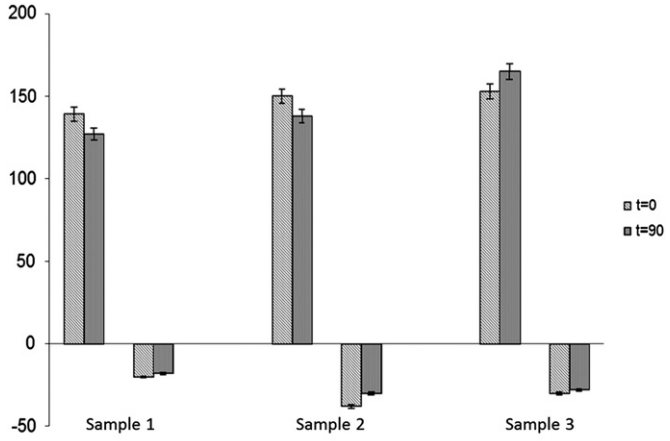


Fig. 2. Physical stability of surfactant vesicle. Vesicle dimensions and ζ-potential values were evaluated by dynamic light scattering at definite time intervals (days) at 25 °C. Reported data are the means of three experiments ± SD.

The interaction of AG with vesicles led to a decrease in vesicle dimensions and to a slight decrease in ζ-potential values (Table 2), but did not modify the vesicular structure and did not increase vesicle lamellarity (Fig. 4), regardless of the cholesterol content in the bilayer.

Loading colloidal carriers with an amphiphilic drug leads to a strong change in physicochemical properties [37]: although the exact mechanisms still need further elucidation, the reduction in particle size as a function of AG content in surfactant bilayers might be attributable to reduced surface tension of the vesicles [38].

AG is water-soluble and the collected data are in agreement with a preferential "partition" of the drug in the aqueous core of vesicles respect to the adsorption on the outer surface. On the other hand the increase in fluorescence anisotropy (Table 2) could be related to a partial "partition" of the drug in vesicle bilayer, due to the chemical structure of AG similar to CHOL, leading to lower fluidity. The variation in anisotropy value is more significant for sample 1, prepared in absence of CHOL, that is able to entrap higher amounts of AG (Table 2).

The decrease in vesicle dimensions led to higher instable systems compared to empty vesicles (Fig. 5), when stored at 25 °C. Only sample 1 shows a constant permeation rate (Fig. 6), that significantly increases after 8 h, in agreement with data of anisotropy and calcein release profiles (see S4). For these reasons *in vivo* experiments were performed only on sample 1.

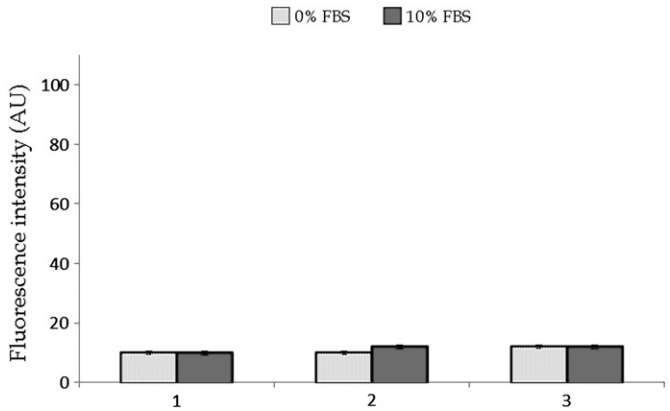
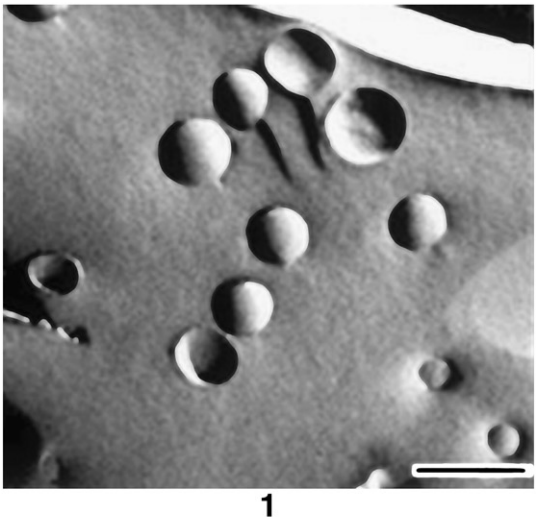
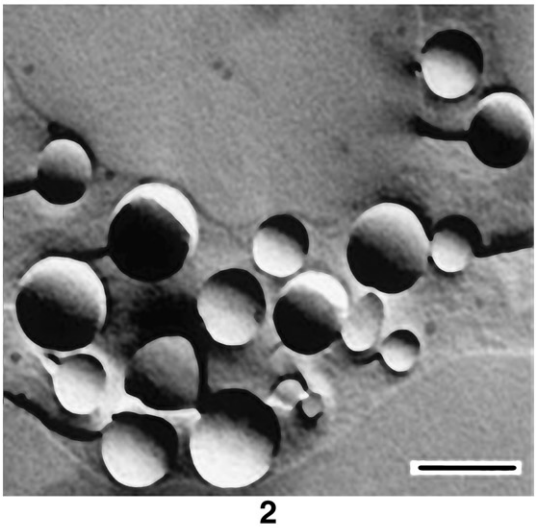


Fig. 3. Influence of FBS different concentrations on the in vitro stability of surfactant vesicles (samples 1, 2 and 3) at 37 °C. The vesicles were incubated at pH 7.4 in absence (0%) and in presence (10 %) of FBS. Samples were collected after 3 h and calcein leakage was measured fluorimetrically. Reported data represent the mean of three experiments; error bars = ± S.D.





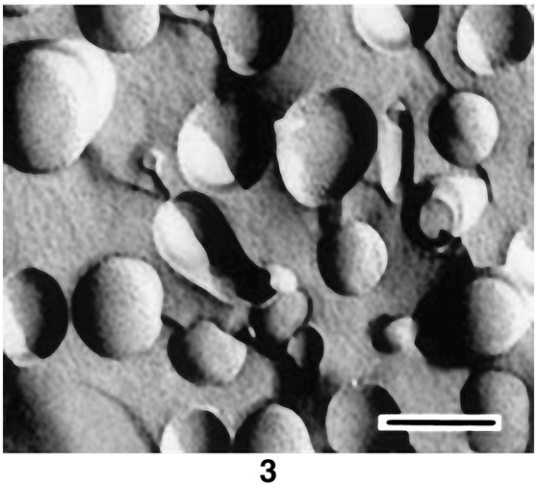


Fig. 4. Transmission electron micrographs of drug-loaded vesicles after freeze-fracture. The bar corresponds to 100 nm.

3.3. In vitro/in vivo experiments

3.3.1. In vitro permeation experiments through human skin

The permeation profiles of ammonium glycyrrhizinate through human SCE are reported in Fig. 7. Ammonium glycyrrhizinate permeated the SCE membranes both when loaded into niosomes and in aqueous solution, but in the first case the permeated amount at the end of the experiment was 23.2 % (23.2 μg) of the applied dose while in the case of the aqueous solution the permeated amount was 7.1% (7.1 μg) of the applied dose.

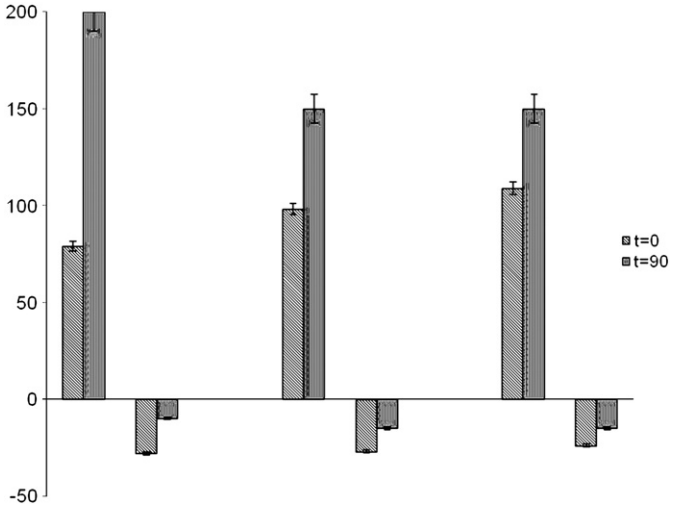


Fig. 5. Physical stability of drug-loaded surfactant vesicle. Vesicle dimensions and ζ-potential values were evaluated by dynamic light scattering at definite time intervals (days) at 25 °C. Reported data are the means of three experiments ± SE $^{**}P<0.01$ when compared with vesicle dimension determined at $t=0$.

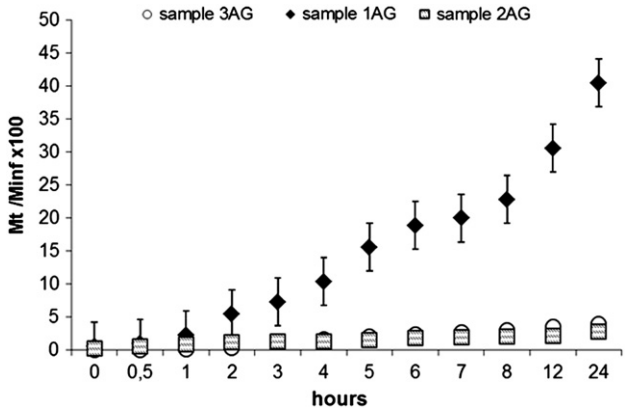


Fig. 6. *In vitro* AG release. Permeation experiments were carried out for 24 h, at 32 ± 0.5 °C. Reported data are the means of three experiments ± SD.

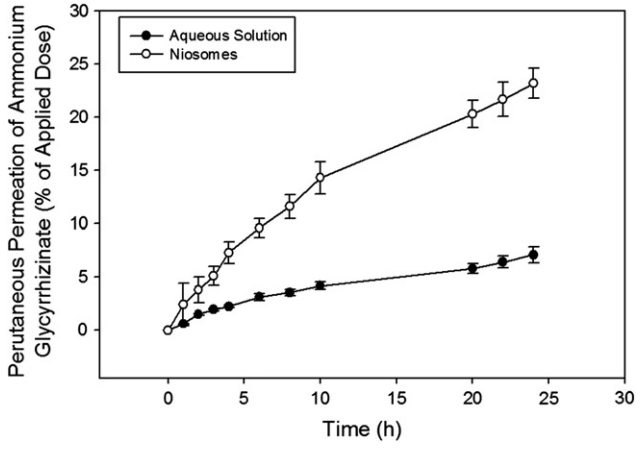


Fig. 7. *In vitro* percutaneous permeation through SCE membrane of ammonium glycyrrhizinate from niosomes or aqueous solution. Each value is the average of six different experiments ± standard deviation.

3.3.2. In vitro evaluation of toxicity

To verify the compatibility of proposed surfactant vesicles with a topical application the *in vitro* cytotoxic effect of niosomes was evaluated on NCTC2544 cells by using the trypan blue dye exclusion assay and MTT viability test. The niosomes were assayed *in vitro* in terms of both dose-dependent effect and incubation-time effect.

As shown in Fig. 7, niosomes showed no significant effect on NCT C2544 cell mortality with respect to the control (untreated cells) for concentrations up to 1 μM at exposition times of 24 h and 48 h. The findings regarding cell mortality were supported by viability data (Fig. 7). When the incubation time was of 72 h, niosomes caused a slight but significant ($P<0.001$) increase of cellular mortality. Also in this case cellular mortality data were supported by cellular viability findings.

At the highest concentrations investigated, i.e. 10 μM, niosomes caused a noticeable cytotoxic effect on human keratinocyte NCTC2 544 cells at all the investigated exposition times, namely, a cellular mortality higher than 70% was observed for incubation times ≥48 h (Fig. 7). The viability and morality data were in good agreement.

3.3.3. In vivo evaluation of skin tolerability

The tolerability of niosomes was further investigated *in vivo*, on human volunteers with a non-invasive technique, reflectance spectrophotometry. Human volunteers were tested for a topical treatment with formulations containing a surfactant concentration of 10 mg/ml, up to a skin exposition of 6, 24 and 48 h.

The results of skin tolerability are expressed as the variation of the erythema index (ΔEI) with respect to baseline value. The effect of the niosomes on human skin was compared with that induced by a 0.9% (w/v) NaCl solution (Fig. 8). ΔEI data show that unloaded niosomes are not able to elicit the erythema induction with respect to the control (aqueous saline solution) at all the exposition times investigated in this paper (6–48 h). These data were in good agreement with *in vitro* toxicity experiments, thus showing that niosomes are very well tolerated also *in vivo* in human volunteers (Figs. 8 and 9).

3.3.4. In vivo evaluation of anti-inflammatory activity

The results of *in vivo* experiments are reported in Fig. 10. In the panel A of Fig. 10, the effects of prepared formulations on edema development induced by zymosan are reported. In the panel B of Fig. 10, the effects of same formulations used for edema experiments on nociceptive behavior induced by formalin are showed. We also tested a purified formulation of vesicles (Fig. 10: BV, sample 1) but the effects were similar to those observed after Hepes administration both in edema and in the formalin experiments. In animals treated with vehicle (Hepes buffer or blank vesicles), the subcutaneous injection of 2.5% zymosan (20 μl/paw) into the dorsal surface of the right hind paw, induces an increase in paw volume that reached the maximal value 3–4 h after the injection, followed by a reduction in the next 24–48 h (Fig. 10, panel A). Local injection of AG (1.12 mg/ml) performed 2 h before zymosan, produced a non-significant reduction of paw edema as observed 3–4 h after zymosan administration (Fig. 10, panel A). When AG was administered as unstructured surfactant formulation at the same concentration (sample S1U) the reducing effects of AG on edema development were potentiated and significant different values from vehicle-treated animals were recorded at 4 and 24 h after zymosan administration (Fig. 10, panel A). When compared to the vehicle-treated animals, maximal edema reduction was observed after the administration of purified formulation of sample 1AG (sample S1). After S1 administration, S1 reduced edema development starting from 1 h until 48 h after zymosan administration (Fig. 10, panel A). This effect was evident and significant at 3, 4 and 24 h after zymosan administration (Fig. 10, panel A). Same effects were observed after sample AG, S1 and S1U administration in the formalin test (Fig. 10 panel B). In the early phase of the formalin test, none of the samples administered was able to change the effects induced by aldehyde. A slight reduction was observed in the early

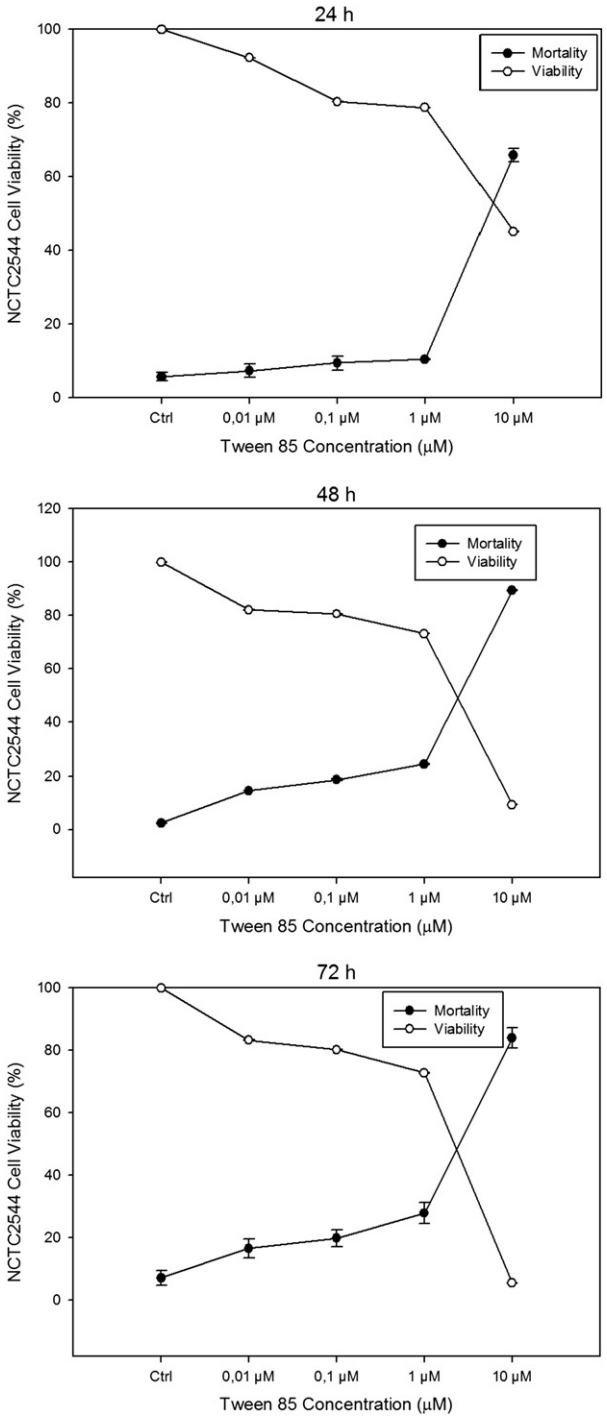


Fig. 8. Dose-dependent cytotoxic effect of niosomes, expressed as Tw85 concentration against NCTC2544 cells as a function of the exposition time. Cytotoxic effect is expressed both as cell viability (filled symbols, MTT test) and cell mortality (hollow symbols, trypan bleu dye exclusion assay). Error bars, if not shown, are within symbols. Results are presented as the mean of six different experiments ± standard deviation.

phase after AG and S1U administration, but this effect was not significant (Fig. 10, panel B). On the contrary, significant reduction of nociceptive behavior was observed after S1 and S1U administration in the late phase of the test (Fig. 10, panel B). AG administration did not change the response to formalin as observed in the late phase of the test (Fig. 10, panel B).

Recently the anti-inflammatory effects of glycyrrhizic acid and 18β-glycyrrhetinic acid were further confirmed in a lipopolysaccharide-stimulated macrophage model [39]. Glycyrrhizic acid or 18β-

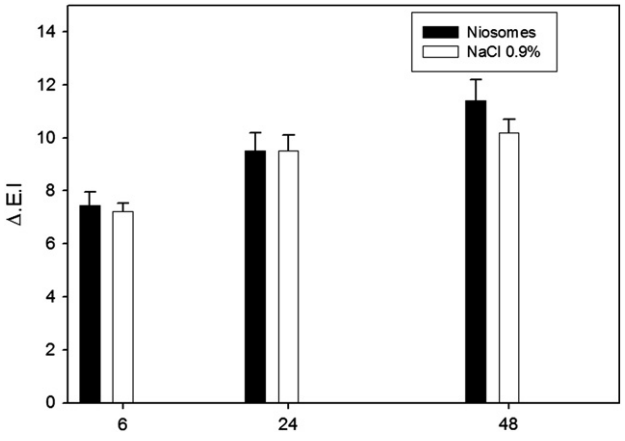


Fig. 9. In vivo human skin tolerability of niosomes after 6, 24 or 48 h of treatment. Results are expressed as a mean value of ΔEI ($n=6$) ± standard deviation.

glycyrrhetinic acid did not affected cell viability but inhibited the production of LPS-induced nitric oxide (NO), prostaglandin E (2) (PGE(2)), and intracellular reactive oxygen species (ROS) reducing the protein and mRNA levels of iNOS and COX-2. Both glycyrrhizic acid and 18β-glycyrrhetinic acid inhibited the activation of NF-κB and the activities of phosphoinositide-3-kinase (PI3K) p110δ and p110γ isoforms and then reduced the production of LPS-induced tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β in a dose-dependent manner. These effects might depend upon different mechanisms: glycyrrhizic acid might act via PI3K/Akt/GSK3beta to reduce cytokine production, while 18β-glycyrrhetinic acid might lead to the dissociation of a glucocorticoid receptor (GR)–HSP90 complex to block inflammation [40]. The antiinflammatory properties of glycyrrhizic acid and its derivatives as AG were also recently confirmed in a model of lipopolysaccharide (LPS)-induced acute lung injury in mice. Pretreatment with AG up-regulated interleukin-10 (IL-10) level and down-regulated the tumor neurosis factor-α (TNF-α) level and inhibited the cyclic adenosine monophosphate-phosphodiesterase (cAMP-PDE) activity in the lung tissue [41]. It is well known that PDE inhibitors are able to reduce edema development when locally administered in the mice paw [42]. This last effects and all above mentioned, might explain the results obtained in the present *in vivo* experiments using AG loaded vesicles.

The potentialities of AG-niosomes as anti-inflammatory agent for topical application were studied also *in vivo* on human volunteers. In this set of experiments skin sites of each volunteer were pre-treated with a solution of methylnicotinate to induce an erythema, the formulations containing ammonium glycyrrhizinate at a concentration of 0.5% (w/v) were then applied. The chemical-induced erythema followed different profiles as a function of the applied anti-inflammatory formulation. As shown in Fig. 11, the ammonium glycyrrhizinate-loaded niosomes were able to reduce the erythema much more rapidly and efficaciously respect to the aqueous solution of ammonium glycyrrhizinate. In fact, in particular, 3 h after the topical application, the chemically induced erythema observed in the sites treated with ammonium glycyrrhizinate-loaded niosomes was reduced by 3.4 fold with respect to the aqueous solution of the drug, which showed a slight reduction with respect to the sites treated with control samples (Hepes buffer or blank vesicles). Following 4 h of treatment with ammonium glycyrrhizinate associated niosomes, the disappearance of the chemically induced erythema was observed with an improvement of the anti-inflammatory activity of the niosome delivered drug. All skin sites treated with ammonium glycyrrhizinate aqueous solution presented an intense erythema up to 5 h.

4. Conclusions

Our findings show that non-ionic surfactant vesicles are a suitable colloidal carrier with flexible physicochemical characteristics to be used as AG delivery device. The stability features of the investigated vesicular carrier were also suitable for a delivery device to be proposed for *in vivo* applications. The AG-loaded non-ionic surfactant

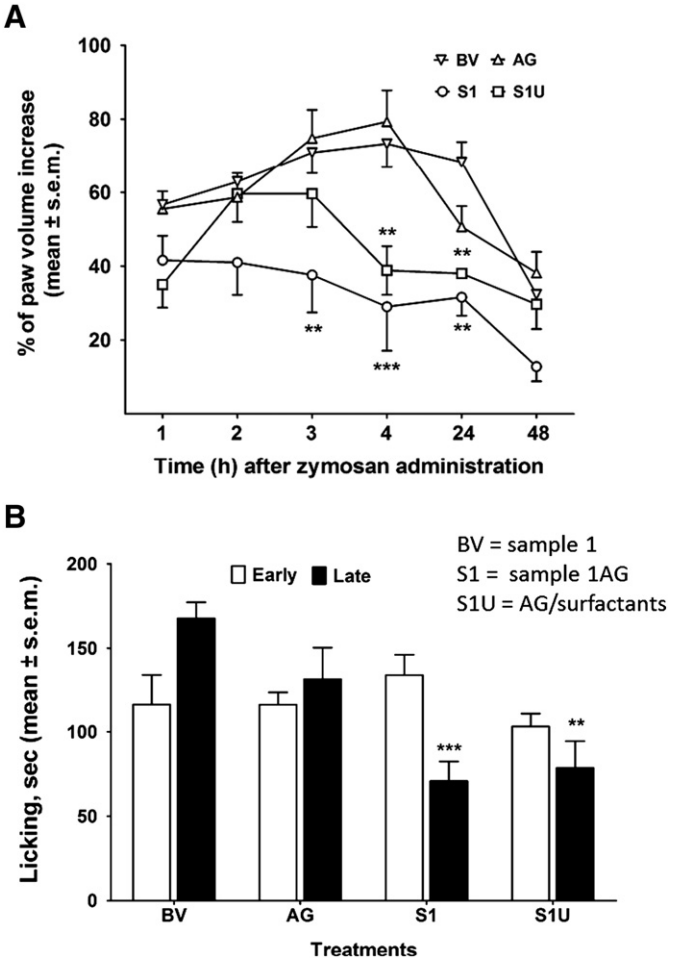


Fig. 10. *In vivo* effects of AG loaded vesicles in edema induced by zymosan (panel A) and on formalin-induced nociception (panel B). Purified formulation of vesicles (S1), unstructured surfactant formulation with the same AG concentration (1.12 mg/ml, S1U) and AG solution at the concentration of 1.12 mg/ml were used. * is for $P<0.05$, ** is for $P<0.01$ and *** is for $P<0.001$ vs. vehicle-treated animals (V). $N=10$–12.

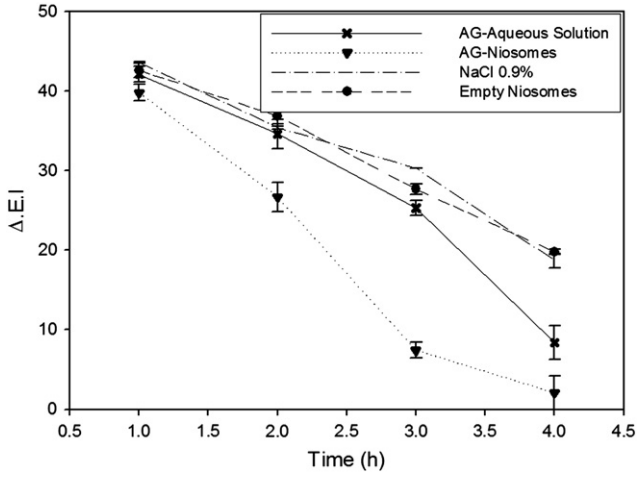


Fig. 11. *In vivo* anti-inflammatory activity in human volunteers of niosomes containing ammonium glycyrrhizinate evaluated as the ability to reduce a skin erythema, chemically induced by pretreatment with an aqueous solution of methyl-nicotinate. Results are expressed as a mean value (six different volunteers) of the erythema index variation ± standard deviation as a function of the time.

vesicles showed no toxicity, good skin tolerability and were able to improve the drug anti-inflammatory activity in mice. At the best of our knowledge, the increased effects of AG entrapped in surfactant vesicles on edema development induced by zymosan and on the reduction of nociceptive behavior, after formalin injection are here reported for the first time.

Furthermore, an improvement of the anti-inflammatory activity of the niosome delivered drug was observed on chemically induced skin erythema in humans.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2012.09.018.

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