# Development of curcumin loaded sodium hyaluronate immobilized vesicles (hyalurosomes) and their potential on skin inflammation and wound restoring

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## ABSTRACT

In the present work new highly biocompatible nanovesicles were developed using polyanion sodium hyaluronate to form polymer immobilized vesicles, so called hyalurosomes. Curcumin, at high concentration was loaded into hyalurosomes and physico-chemical properties and *in vitro/in vivo* performances of the formulations were compared to those of liposomes having the same lipid and drug content. Vesicles were prepared by direct addition of dispersion containing the polysaccharide sodium hyaluronate and the polyphenol curcumin to a commercial mixture of soy

phospholipids, thus avoiding the use of organic solvents. An extensive study was carried out on the physico-chemical features and properties of curcumin-loaded hyalurosomes and liposomes. Cryogenic transmission electron microscopy and small-angle X-ray scattering showed that vesicles were spherical, uni- or oligolamellar and small in size (112-220 nm).

The *in vitro* percutaneous curcumin delivery studies on intact skin showed an improved ability of hyalurosomes to favour a fast drug deposition in the whole skin. Hyalurosomes as well as liposomes were biocompatible, protected *in vitro* human keratinocytes from oxidative stress damages and promoted tissue remodelling through cellular proliferation and migration. Moreover, *in vivo* tests underlined a good effectiveness of curcumin-loaded hyalurosomes to counteract 12-O-tetradecanoilphorbol (TPA)-produced inflammation and injuries, diminishing oedema formation, myeloperoxydase activity and providing an extensive skin reepithelization. Thanks to the one-step and environmentally-friendly preparation method, component biocompatibility and safety, good *in vitro* and *in vivo* performances, the hyalurosomes appear as promising nanocarriers for cosmetic and pharmaceutical applications.

**Keywords:** Hyaluronic acid/Hyaluronan; phospholipid vesicles; polyphenols; skin inflammation; cell oxidative stress; wound healing.

## INTRODUCTION

Many efforts have been recently devoted to improve drug efficacy following topical application. One of the main widely used strategies is to employ nanovesicular carriers, such as liposomes and liposome-like structures, conceptually derived from them, but with different features and/or performances. During last years several reports have shown that different lamellar vesicles, such as niosomes, ethosomes, transfersomes, glycerosomes and PEVs [1–7], are able to increase cutaneous drug accumulation and local bioavailability limiting systemic toxicity. These systems, especially transfersomes, ethosomes and PEVs, seem not to be mere penetration enhancers facilitating drug penetration into the skin, but real delivery systems able to carry drugs to the target tissue controlling

their release and cellular uptake [8]. They represent the latest evolution of liposome-like formulations for topical application but their innovative characteristics such as high drug carrying capacity, long-term stability, and suitable biocompatibility are not usually synergistic, resulting in a partial improvement of overall performances. Moreover, topical use of the vesicular formulations is hampered by their liquid status that allows only a short contact time with the skin and does not provide a prolonged application because of the rapid leakage at the application site. Incorporation of vesicles in a gel can overcome these drawbacks but, unfortunately, may reduce drug release and accumulation in the different skin strata [9,10]. As an alternative, closely-packed semisolid phospholipid vesicles or gel core liposomes have been proposed as innovative tools for topical drug delivery [11–13].

In the present work, new polymer immobilized nanovesicles were produced by an easily reproducible (facilmente riproducibile) and organic solvent-free method, and their suitability as carriers for drug delivery to both intact and damaged skin was evaluated [14]. These vesicles, called hyalurosomes, were obtained by hydrating phospholipids with an aqueous dispersion of the lipophilic curcumin and sodium hyaluronate. Sodium hyaluronate was selected because this polymer is water soluble, and at pH>3 its acid chains are extensively ionized forming strong intermolecular interactions with a consequent development of a structured network having particular visco-elastic properties [15]. Hyaluronan has multifaceted roles in biology and pharmacology; in particular this highly biocompatible polymer has an important role in skin wound healing [16]. Curcumin was chosen as a model drug because it is one of the most potent natural antioxidant, it is safe and exhibits important pharmacological activities such as anti-inflammatory, antioxidant, antiproliferative and antiangiogenic [17–19]. Unfortunately, its therapeutic use is limited by its high lipophilicity (and consequent poor aqueous solubility) and short half-life that lead to a poor bioavailability [20]. Therefore, vesicular carriers are here proposed as tools to improve the local bioavailability and efficacy of this drug. An extensive study on curcumin-loaded hyalurosome physico-chemical features and properties was carried out in comparison with

curcumin-loaded liposomes. Vesicle ability to facilitate drug accumulation in the intact skin was tested *in vitro* as a function of time. *In vitro* biocompatibility of drug-loaded vesicle and their aptitude to protect cells from oxidative stress damage were evaluated using human keratinocytes. Finally, their *in vitro* and *in vivo* activity in wound healing or skin reepithelization was assessed on human keratinocytes and on a mouse model.

## 2. EXPERIMENTAL SECTION

### 2.1 Materials

Enriched soy phosphatidylcholine (Phospholipon® 90G, P90G) was kindly supplied by AVG S.r.l. (Garbagnate Milanese, Milan, Italy). 1,2-dioleolyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rho-PE) was purchased from Lipoid GmbH. Sodium hyaluronate low molecular weight (200-400 kDa) was purchased from DSM Nutritional Products AG Branch Pentapharm (Aesch/Switzerland). Curcumin (CUR), 12-O-tetradecanoylphorbol 13-acetate (TPA) and other reagents of analytical grade were purchased from Sigma-Aldrich (Milan, Italy). Cell mediun, fetal bovine serum, penicillin, and streptomycin were purchased from Life Technologies Europe (Monza, Italy).

### 2.2 Sample preparation

In a typical preparation of hyalurosomes, alternatively 10 or 50 mg of sodium hyaluronate and 100 mg of curcumin were dispersed in distilled water (10 ml) and stirred for 5 h at ~25°C to achieve a final polymer dispersion of 0.1 or 0.5 % w/v, subsequently the polymer dispersions were added to 1.8 g of P90G and left hydrating overnight; finally they were sonicated (5 seconds on and 2 seconds off, 50 cycles; 13 μm of probe amplitude) with a high intensity ultrasonic disintegrator (Soniprep 150, MSE Crowley, London, United Kingdom). To obtain liposomes, curcumin (100 mg) and 1,8 g of P90G were weighed in a glass tube, hydrated with 10 ml of distilled water, left swelling overnight and finally sonicated as above. Samples (1 ml) were freed from the non-incorporated drug by dialysis against water (2.5 litters) using dialysis tubing (Spectra/Por® membranes, 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, The Netherlands) at room

temperature for 4 hours, by replacing the water every hour. The used water (10 litter total) was able to theoretically remove all the drug contained in 1 ml of dispersion (10 mg).

### 2.3 Vesicle characterization

For cryogenic electron transmission microscopy, a thin aqueous film was formed on a glow-discharged holey carbon grid and vitrified by plunging into ethane, using a Vitrobot (FEI Company, Eindhoven, The Netherlands). The vitreous films were transferred to a Tecnai F20 TEM (FEI Company) and the samples were observed in a low dose mode, at 200 kV and at a temperature around ~-172°C.

The average diameter and polydispersity index (PI), as a measure of the size distribution width, were determined by Photon Correlation Spectroscopy (PCS), using a Zetasizer nano-ZS (Malvern Instruments, Worcestershire, United Kingdom). Samples were backscattered by a helium-neon laser (633 nm) at an angle of 173° and a constant temperature of 25°C. Zeta potential was estimated using the Zetasizer nano-ZS by means of the M3-PALS (Mixed Mode Measurement-Phase Analysis Light Scattering) technique, which measures the particle electrophoretic mobility.

Entrapment efficiency (EE%), expressed as the percentage of the drug amount initially used, was determined by high performance liquid chromatography (HPLC) after disruption of vesicles with methanol (1/1000), using a chromatograph Alliance 2690 (Waters, Milano, Italy) equipped with a photodiode array detector and a computer integrating apparatus (Empower$^{TM}$ 3). The column was a SunFire C18 (3.5 μm, 4.6×150 mm). The mobile phase was a mixture of acetonitrile, water and acetic acid (95:4.84:0.16, v/v), delivered at a flow rate of 0.7 ml/min. Curcumin content was quantified at 424 nm, and a standard calibration curve was built up by using working, standard solutions. Calibration graphs were plotted according to the linear regression analysis, which gave a correlation coefficient value ($R^2$) of 0.999. The lipid content of dispersions was determined by the Stewart assay [21]. The concentration of P90G was obtained by measuring the absorbance of the organic solution at 485 nm. Aggregation efficiency (AE%) represents the effective amount of aggregated phospholipids expressed as the percentage of the amount initially used.

### 2.4 Stability studies

The vesicle stability was assessed by monitoring the vesicle average size and zeta potential over 90 days at room temperature (25±1◦C). Drug retention within the vesicles during the storage was evaluated by measuring the vesicular system entrapment efficiency over 90 days.

### 2.5 Rheological studies

Rheological measurements were carried out at 25±1°C, using a Kinexus rotational rheometer (Malvern Instruments, Worcestershire, United Kingdom), equipped with data acquisition and elaboration software rSpace; a cone-plate geometry (CP1/60) was used. Before each analysis, a thin layer of silicon oil (Dimethicone, RFE/Ph. Eur.) was laid on the free surface of the sample to prevent water evaporation. Frequency sweep tests were performed in the range 0.01–10 Hz and a shear stress of 0.1 Pa was employed. Before frequency sweep tests, amplitude sweep experiments were carried out for each sample to determine the linear viscoelastic region where the values of the moduli are independent of the applied deformation. Steady shear experiments were performed in the range 0.01–20 Pa. Viscosity at 1 $s^{-1}$ was used to characterize the flow properties of different dispersions. All measurements were carried out in triplicate.

### 2.6 Small-angle X-ray scattering (SAXS)

SAXS studies were carried out using a S3-MICRO (Hecus X-ray systems, Graz, Austria) coupled to a GENIX-Fox 3D X-ray source (Xenocs, Grenoble, France) and a 50 focused X-ray beam with 0.1542 nm at Cu Kα-line. All scattering curves, recorded at 25°C were reproduced three times, and a representative curve was selected, plotting the scattering intensity (I) as a function of the scattering vector (q). The q range was 0.003-0.6 $Å^{-1}$, where $q = (4\ \pi \sin \theta)/\lambda$ is the modulus of the scattering wave vector, θ the scattering angle and λ the wavelength. SAXS patterns were analyzed in terms of a global model using the program GAP (Global Analysis Program) developed by Pabst [22] that permitted to obtain relevant structural parameters on bilayer-based structures, i.e. vesicles and lamellar phases. From the analysis, the membrane thickness was obtained through the definition $d_B = 2\ (z_H + 2\ \sigma_H)$. $z_H$ and $\sigma_H$ derive from SAXS curve fitting with GAP.

### 2.7 *In vitro* skin delivery studies

Experiments were performed under non-occlusive conditions using Franz cells (diffusion area 0.785 $cm^2$) and newborn pig skin. Oneday-old Goland–Pietrain hybrid pigs (about 1.2 kg) were provided by a local slaughterhouse. The skin (n=6 per formulation), stored at –80°C, was pre-equilibrated in saline at 25°C for 12 hours and then sandwiched between donor and receptor cells. The receptor was filled with 5.5 ml of saline, continuously stirred and thermostated at 37±1°C. Samples (50 μl) were applied onto the skin surface. At regular intervals, up to 24 h, the receiving solution was withdrawn, replaced with pre-thermostated, fresh saline and analyzed by HPLC for drug content. After 1, 2, 4, 8 and 24 h, the skin surface was gently washed (3 times) with 1 ml of distilled water, then dried with filter paper. The stratum corneum was removed by stripping with adhesive tape Tesa® AG (Hamburg, Germany). Epidermis was separated from dermis with a surgical scalpel. Skin strata were cut, placed each in a flask with methanol and sonicated for 2 minutes in an ice bath to extract the drug. The tapes and tissue suspensions were filtered out and assayed for drug content by HPLC.

### 2.8 Antioxidant activity of samples (DPPH assay)

The antioxidant activity of both curcumin ethanolic solution and vesicles was tested by measuring their ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH). Each sample was added (1:50) to DPPH methanolic solution (25 μM), stored at room temperature for 30 min in the dark, and absorbance was measured at 517 nm against blank. All the experiments were performed in triplicate. The percent antioxidant activity was calculated according to the following formula: antioxidant activity (%) = $[(ABS_{DPPH}-ABS_{sample})/ABS_{DPPH}]\times100$ [23].

### 2.9 Cell toxicity and antioxidant activity of samples against induced $H_2O_2$ oxidative stress

Human keratinocytes, at passages 4-5, were grown as monolayer in 75 $cm^2$ flasks, incubated in 100% humidity and 5% $CO_2$ at 37°C, using RPMI1640, supplemented with foetal bovine serum, penicillin/streptomycin and fungizone as culture medium. For toxicity studies cells were seeded into 96-well plates ($7.5\times10^3$ cells/well) and, after 24 h, were treated for 48 h with curcumin dispersion,

empty or curcumin-loaded vesicles at different dilutions (1:200, 1:500, 1:1000, 1:5000, 1:10000 corresponding to 50, 20, 10, 2, 1 μg/ml of drug and 0.9, 0.36, 0.18, 0.036, 0.018 mg/ml of phospholipid). Antioxidant activity was evaluated treating the cells with empty 0.5hyalurosomes (0.9 mg/ml of phospholipid), curcumin dispersion or curcumin-loaded vesicles (50 μg/ml of curcumin) and simultaneously with hydrogen peroxide (1:60000 dilution) for 3-6 h. After incubation, cells were washed 3 times with fresh medium and their viability was determined by the MTT [3(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] colorimetric assay, adding 200 μl of MTT reagent (0.5 mg/ml in PBS) to each well [5]. After 2-3 h, the formed formazan crystals were dissolved in DMSO and their concentration was spectrophotometrically quantified at 570 nm with a microplate reader (Synergy 4, Reader BioTek Instruments, AHSI S.P.A, Bernareggio, Italy). All experiments were repeated at least three times. Results are shown as percent of cell viability in comparison with non-treated control cells (100% viability).

**2.10 *In vitro* scratch assay**

The ability of the different formulations to improve keracinocyte proliferation and migration to remodelling tissue was evaluated measuring the cell expansion on wound surface (scratch assay). Cells were cultured in 6 well plates until complete monolayer confluence. Then a linear wound was generated using a sterile plastic pipette tip. The scattered fragments of cells were removed by washing with fresh medium (RPMI1640). Cells were treated with formulations (50 μg/ml curcumin) labelled with Rho-PE (0.035 mg/ml) and incubated for 24 and 48 h. Cells treated with curcumin dispersion and empty 0.5hyalurosomes were used as control. At the end of experiments cells were fixed with 4% paraformaldehyde and stained with Hoechst 33258 to visualize the cell nucleus. Cells were observed using an inverted confocal laser scanning microscope, FluoView FV1000 (Olympus, Barcelona, Spain) with an UPlanSApo 20x objective and images with a field size of 1024×1024 μm were generated. Curcumin, Rho-PE and Hoechst were excited at 420 nm, 559 nm and 360 nm, and detected at 540 nm, 578 nm and 460, respectively.

**2.11 *In vivo* oedema and myeloperoxidase assays**

Female CD-1 mice (5-6 weeks old, 25-35 g) were obtained from Harlan Laboratories (Barcelona, Spain) and acclimatized for 1 week. All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals.

The back skin of mice was shaved and TPA dissolved in acetone (6 μg/40 μl) was applied to the shaved dorsal area (~2 $cm^2$) to induce cutaneous inflammation and ulceration (day 1). All tested compounds (100 μl), were topically applied 3 and 6 h after TPA application. The procedure was repeated daily for 4 more days. On day 6, mice were sacrificed by cervical dislocation. Each group comprised four mice. The dorsal treated skin area of sacrificed mice was excised, weighted to assess any mass increase indicative of oedema formation. Myeloperoxidase (MPO) activity was evaluated as previously reported [6], homogenizing and centrifuging the skin biopsies, and incubating the supernatant with hydrogen peroxide and tetramethylbenzidine. Finally, MPO was assayed spectrophotometrically at 450 nm and its activity was calculated from the linear portion of a standard curve.

### 2.12 Statistical analysis of data

Results are expressed as the mean±standard deviation and significance was tested at the 0.05 level of probability (p). For size, zeta potential, viscosity, drug accumulation and cytotoxicity, analysis of variance (ANOVA) was used to substantiate statistical differences between groups while Student's t-test was used for comparison between two samples using XLStatistic for Excel. The multiple comparisons of in vivo results was performed by the Scheffe or Dunnet tests using IBM SPSS statistics for Windows.

## 3. RESULTS

### 3.1 Vesicle characterization

In this work, a new formulation for curcumin topical delivery based on swelling and immobilization of phospholipid vesicles in a preformed and structured sodium hyaluronate polymer network was developed. The polymer-drug dispersion was added to the phospholipids and left to swell overnight to obtain the vesicles. Finally, dispersions were sonicated to reduce and uniformate vesicle size. To

achieve a versatile and stable formulation, capable of incorporating high amounts of curcumin, a preformulation study was carried out. Results of this study led us to select a formulation containing 180 mg/ml of phospholipids, which was able to stably incorporate 10 mg/ml of curcumin. Indeed, formulations more or less concentrated in phospholipids were not homogenously dispersed, nor stable with a fast precipitation of the curcumin. Moreover, the hyaluronan was used at low concentrations (0.1 or 0.5%) because an increase of its amount caused a strong enlargement of vesicle size and size distribution converting them into not suitable carrier for skin delivery. The features and performances of the resulting hyalurosomes were tested and compared with those of liposomes containing the same amount of phospholipids and drug. To confirm vesicle formation and evaluate their morphology, liposomes and hyalurosomes were first visualized by cryo-TEM. This technique permits to inspect sample structure in the native form providing valuable structural information. Liposomes exhibited their expected regular spherical shape with a prevalent single membrane surrounding the aqueous core (Figure 1A). Similarly, hyalurosomes were spherical and unilamellar, but oligolamellar vesicles were also observed (Figure 1B). Moreover, Figure 1 shows that both liposomes and hyalurosomes were generally polydispersed with the latter also including some irregular shaped vesicles.

Physico-chemical characteristics of curcumin-loaded liposomes and hyalurosomes were defined and compared with those of the corresponding empty vesicles (Table 1). A global estimate of mean size was determined by Photon Correlation Spectroscopy analyses, which showed that empty liposome and hyalurosome mean diameter was ~115 nm without any statistical differences among samples ($p>0.05$). However, the addition of sodium hyaluronate (0.1, 0.5%) resulted in an improved homogeneity of the samples (PI from 0.31 to 0.25) and a reduction of the zeta potential values (from -17 to -25 mV, $p<0.05$). Curcumin addition strongly affected liposome features increasing the mean diameter up to ~196 nm ($p<0.05$ versus empty liposomes) and the polydispersity index to 0.46. In addition, incorporation of curcumin in liposomes decreased the zeta potential to -30 mV ($p<0.05$ versus empty liposomes), probably because the drug was not only intercalated within the

lipid bilayer but also adsorbed on the vesicle surface, as previously described [6]. Differently, the addition of curcumin in hyalurosomes led to a minor increase in mean diameter (~160 nm, $p<0.05$ versus empty hyalurosomes and curcumin-loaded liposomes) and sample polydispersity (~0.30) and it did not affect the zeta potential value ($p>0.05$) showing that the presence of hyaluronan facilitate curcumin dispersion in the vehicle network. Curcumin actual entrapment efficiency in liposomes was ~66%, whereas in hyalurosomes it reached ~79% ($p<0.05$). Aggregation efficiency, which represents the amount of aggregated phospholipids respect to the initial amount used, was not significantly affected by the presence of polymer, and was ~84% ($p>0.05$) for all formulations.

In a thorough study on the stability of vesicles during 90 days at room temperature (25±1°C), all the analyzed parameters (mean diameter, polydispersity index, zeta potential and entrapment efficiency) of both hyalurosome formulations (i.e. 0.1 and 0.5% w/v) remained constant during the storage period (variation<10%, $p>0.05$) whilst liposomes suffered a change in size and zeta potential after the first month of storage, reaching ~260 nm at 60 days and 380 nm at 90 days ($p<0.05$ versus the initial value), with the zeta potential becoming more negative (Figure 2). Moreover, liposomes also suffered of drug leakage that reduced liposome EE to ~54% ($p<0.05$ versus the initial value), probably, as a consequence of the vesicle aggregation and fusion phenomena pointed out by this study. On the contrary, the hyalurosomes did not show any reduction of EE ($p>0.05$ versus the initial value) on storage, thus, highlighting the actual higher stability of the new vesicular formulations.

To obtain detailed information on vesicle structure, SAXS analyses were performed (Figure 3). Using the method developed by Pabst [22], this technique disclosed the changes imparted by the influence of curcumin and polymer on lamellar organization. SAXS pattern of empty liposomes showed only a first order, broaden and unsharpened peak denoting well defined unilamellar vesicles with a bilayer thickness ($d_B$) ~49 Å, a polar head amplitude ($\sigma_H$) ~3.7 Å, and acyl chain width ($Z_H$) ~17 Å, according to the equation $d_B=2(Z_H+2\sigma_H)$. The addition of curcumin in liposomes did not change the peak shape but altered the electron density and the calculated parameters. In particular,

$d_B$ was lower (~46 Å) due to a contraction of $\sigma_H$ (~2.7 Å) while the $Z_H$ remained constant (~17 Å). The peak of 0.1hyalurosomes was shifted to higher q values than that of liposomes and became slightly more sharpened and intense, hence, indicating a small increase of vesicle lamellarity and stiffness. The lamellarity and stiffness increased to a greater extent in 0.5hyalurosomes, as shown by the broader peak and a plateau in the upper region (Figure 3C). Empty 0.1 and 0.5hyalurosomes showed reduced $d_B$ (~46 and 47 Å) and $\sigma_H$ (~2.4 and 3.1 Å) in comparison with liposomes and the presence of the drug did not cause any further modification of $d_B$ or $\sigma_H$.

An extensive rheological analysis was carried out to investigate possible supramolecular interactions between the vesicles and the polymer. As expected, 0.5hyalurosomes showed the highest viscosity (9.95 Pa s, at shear stress 0.1 Pa, $p<0.05$) in comparison with 0.1hyalurosomes (0.45 Pa s) and liposomes (0.28 Pa s), which showed viscosity values statistically equivalents, ($p>0.05$). To go deeper into the system structure, the different ability to store (G') and dissipate (G") the energy supplied during the rheological experiments was considered. In the mechanical spectra of liposomes both moduli showed the same order of magnitude and a cross-over point of the two moduli was evident in the range 0.1-10 Hz although, unexpectedly, G' was higher than G", (Figure 2D) indicating the existence of strong interactions among the flowing units (close packed vesicles), probably due to the high phospholipid concentration [24]. In the spectrum of hyalurosomes, G' values was much higher than G" (especially for 0.5hyalurosomes) and the cross-over point was shifted toward frequencies lower than 0.1 Hz, indicating a more structured and strong (weak gel) system, with respect to liposomes, probably related to the polymer-vesicle network (Figure 3E) [25,26].

**3.2 *In vitro* skin delivery studies**

To evaluate the vesicle ability to deliver curcumin to the intact skin as a function of time, *in vitro* curcumin deposition in the skin strata and diffusion into the receptor fluid was quantified at different times (1, 2, 4, 8 and 24 h; Figure 4). After 2 h and up to 24 h, liposome and 0.1hyalurosome behaviour was very similar ($p>0.05$ between the two formulations at the same

time): they provided ~30% of drug accumulated in the stratum corneum, ~10% in the epidermis, ~2% in the dermis and only 0.5% in the receptor fluid. Using 0.1hyalurosomes drug deposition was not time-dependent: the amount of drug accumulated in each skin stratum and in the receiver was constant ($p>0.05$) during all the sampling times, reaching the maximum already after 1 h. On the contrary, using liposomes, the mean drug amount retained in the stratum corneum was lower during the first two hours ($p<0.05$ versus higher times) and reached 30% only at 4 h. In comparison with liposomes and 0.1hyalurosomes, the 0.5hyalurosomes showed a superior and time-depending ability to promote drug deposition in the different skin strata. Indeed, at short times, up to 4 h, curcumin was accumulated in high amount but only in the stratum corneum (~57%, $p<0.05$ versus all other values) and epidermis (~18% versus values of stratum corneum), whereas successively (8, 24 h) drug deposition improved highly in the dermis (~16%, $p<0.05$ versus other values found in epidermis) and decreased in the outermost skin strata (i.e. stratum corneum and epidermis), highlighting the ability of this

system to improve the accumulation of the drug in the whole skin.

### 3.3 *In vitro* cytotoxycity and antioxidant activity of curcumin-loaded vesicles

All curcumin samples (dispersion and vesicle-loaded) showed *in vitro* the same scavenging activity of DPPH free radicals (~89%, $p>0.05$), demonstrating their antioxidant capacity.

The cytotoxicity of the different empty and curcumin-containing samples was evaluated on human keratinocytes by the MTT assay. Treating cells with empty formulations, the highest cell viability was provided by 0.5hyalurosomes (~124%, irrespective of the sample dilution, $p<0.05$), followed by 0.1hyalurosomes and empty liposomes (Figure 5A), which showed the lowest viability ($p<0.05$ versus hyalurosomes) at each sample dilution ($p>0.05$ versus dilution) except using 0.018 mg/ml of phospholipid.

At all tested curcumin concentrations (1-50 μg/ml), cell viability was always ≥100% for all vesicles and control (curcumin dispersion). Curcumin dispersion demonstrated a concentration dependent

~~behaviour~~ effect ~~showing~~ providing at the lowest dilutions (20 and 50 μg/ml of drug) a viability ~~up to~~ ~103% that increased up to ~123% at the highest dilutions (1, 2 and 10 μg/ml, $p<0.05$ versus 103%). Viability of cell treated with curcumin-loaded liposomes was like to that of curcumin dispersion (~115%, $p>0.05$ versus 103 and 123%) and independent to the used dilutions ($p>0.05$) while that of cells incubated with 0.1hyalurosomes decreased as the curcumin concentration increased ($p<0.05$ versus dilutions). ~~A similar trend was observed in cells treated with both~~. Furthermore, it is important to underline that in cells treated with curcumin-loaded 0.5hyalurosomes at the lowest concentrations (1, 2, 10 μg /ml) a higher proliferative effect was observed, cell viability reached ~160% ($p<0.05$ versus other values), probably due to the proliferative properties of hyaluronan.

Additionally, liposomes and hyalurosomes showed *in vitro* the capacity to reduce the hydrogen peroxide-induced oxidative stress on keratinocytes and their consequent mortality (Figure 5C). Hydrogen peroxide treatment decreased the viability up to ~60% (3, 6 h), whereas free curcumin and empty 0.5hyalurosomes did not protect significantly the cells from oxidative damage ($p>0.05$ versus 60%) while curcumin-loaded vesicles provided the maximum protective activity increasing cell viability up to ~120% ($p<0.05$ versus 60%).

**3.4 *In vitro* scratch assay**

*In vitro* scratch assay was carried out on a cell monolayer to monitor the effects of the studied formulations on wound closure during 48 h by CLSM. As shown in Figure 6, after 24 h exposure to both hyalurosomes, cells moved toward the opening to close the wound faster than controls (curcumin dispersion and empty 0.5hyalurosomes) and liposomes. At the end of the experiments, the free curcumin dispersion was able to only partially heal the wound while using curcumin-loaded vesicles the effect improved according to the following rank: liposomes< 0.1hyalurosomes< 0.5hyalurosomes. In particular, curcumin-loaded 0.5hyalurosomes provided the highest increase of keratinocytes in the scratched area due to the simultaneous cell proliferation and migration, allowing the complete scratch closure in 48 h (Figure 6) according to biocompatibility study.

### 3.5 *In vivo* skin damage assays

Topical application of TPA was used in shaved mouse skin to induce inflammation, oedema, oxidative stress, infiltration of inflammatory cells, and loss of epidermis or ulceration. Nanoentrapped curcumin had already shown the capability of preventing and reducing TPA damage on a mouse model [6]. Here, curcumin-loaded hyalurosomes were capable of avoiding damage and loss of superficial skin strata after TPA daily application (Figure 7). Animals treated with TPA only, showed a skin lesion area that increased over time: at day 2, an initial loss of skin was evident in the marginal zone, at day 4 the skin damage was more diffuse and at day 6 all the treated area was compromised with several crusts even in the central area (Figure 7). The treatment with TPA and curcumin dispersion reduced the loss of skin that, however, appeared dried with crusts especially after 4 days. On the contrary, at day 6, in the centre of the damaged area, formation of new skin could be observed. Empty 0.5hyalurosomes decreased the skin lesions in the peripheral TPA application zone (2, 4 days) allowing the partial skin reepithelization at day 6. All the animals treated with both TPA and curcumin-loaded formulations presented a significant amelioration of the skin feature. Upon visual inspection, the superior ability of curcumin-loaded hyalurosomes to heal the wound in comparison with controls and liposomes was evident. In particular, using 0.5hyalurosomes, the dangerous effect of TPA was counteracted, thus, moderating the skin lesions (only a slightly loss of superficial skin was observed) in the peripheral application zone (days 2 and 4) and favouring the complete reepithelization of the marginal area at day 6. The treated skin was similar to that of healthy untreated mouse, except for being thinner and for the presence of some minor defects (Figure7).

To confirm the beneficial effect of curcumin formulations, the inhibition of two biomarkers (oedema and MPO) was quantified as well. Oedema and MPO are associated to the skin inflammation and their increase may inhibit the normal reepithelization and the re-establishment of physiological conditions on skin wound. Both hyalurosomes induced a strong inhibition (>80%, $p<0.05$ in comparison with TPA) of the two biomarkers: oedema and MPO (Table 2), whereas the

outcomes provided by free curcumin and empty 0.5hyalurosomes were not effective and not statistically different from those provided by TPA ($p>0.05$). Finally, curcumin-loaded liposomes successfully inhibited oedema (~80%) but they were not able to counteract the MPO activity showing an inhibition statistically similar to that obtained using TPA ($p>0.05$).

## 4. DISCUSSION

In this work, hyaluronic acid sodium salt was used as key component of the vesicular formulations. The pKa value of hyaluronan is ~3.0 and, therefore, at higher pH its carboxylic groups are extensively ionized forming a polymeric network [15], which, used as hydration medium of phospholipids, did not avoid vesicle assembling but, on the contrary, was able to immobilize and stabilize them, forming the hyalurosomes. Considering the great number of curcumin nanocarriers developed for wound healing, the main properties and performances of these innovative systems were compared with those of curcumin in dispersion and loaded in conventional phospholipid vesicles (liposomes), and those of empty 0.5hyalurosomes.

Liposomes are considered as optimal systems for skin delivery of different active compounds as confirmed by the large number of papers focused on these systems. Results obtained during this study demonstrate the higher ability of hyalurosomes to improve the skin bioavailability and therapeutic efficacy of the low-bioavailable curcumin, in comparison with liposomes. Furthermore, the preparation method and the technology used to obtain hyalurosomes enrich the knowledge on phospholipid vesicle assembling because they were produced using an one-step, environmentally-friendly, easily reproducible and organic solvent-free method by mixing the drug and the two natural and highly biocompatible main components (phospholipids and sodium hyaluronate). On the contrary, development of other nanocarriers generally requires 1) a complex preparation method involving several steps, often expensive and difficult to be reproduced on a large-scale; 2) use of organic solvents or not-safe and not-cheap, components; 3) complex chemical synthesis finalized to modify the main components and often involving the use of often toxic reagents or solvents [27–30]. The developed method allowed us to obtain polymer immobilized nanovesicles with peculiar

physico-chemical properties (stability, high viscosity, biocompatibility) and versatile biological behaviour resulting in high delivery performances in both intact and damaged skin. In fact, their efficacy as topical carriers was firstly demonstrated on intact skin and then confirmed by in vitro scratch assay and by in vivo wound healing and inflammation inhibition.

Curcumin-loaded liposomes were unilamellar and polydisperse (PI 0.46) vesicles, with a mean size around 196 nm and hyalurosomes were oligolamellar, smaller (~160 nm, $p<0.05$) and more homogeneous with PI=0.30. Moreover, their analyses were always repeatable as confirmed by the low values of standard deviation obtained from at least six repetitions. All vesicles showed good EE% but hyalurosomes improved significantly the drug loading ($p<0.05$, Table 1), probably as a consequence of the structured vehicle formed by hyaluronan that favoured curcumin thin dispersion in the polymer hydration medium inside vesicles. All vesicles showed highly negative zeta potential values (-24/-30 mV), predictive of a good stability against aggregation and fusion. Zeta potential of all vesicles was negative because P90G is a mixture of phosphatidylcholine, other phospholipids and fatty acids. In aqueous solution at pH~6, the negative phosphate residues of the phospholipids are distributed on the membrane surface leading to a negative potential value [31]. Being phosphatidylcholine a zwitterionic molecule, the orientation and conformation of its headgroup is not constant and can be influenced by binding or adsorption of the negatively charged molecule as well sodium hyaluronate, which can interact with choline groups on the bilayer surface forming a structured vesicle-polymer system [32]. Vesicle interactions with the polymer structure conferred a superior stability to hyalurosomes, which maintained constant their physico-chemical features on storage (90 days at 25±1°C), whereas liposomes lost their properties after 30 days of storage. We assume that these results are due to the structured vehicle produced by the polyanionic hyaluronan, which immobilizes the vesicles, conferring them a higher stability than liposomes. Moreover, the (polymeric) network structure increased system viscosity and resistance (similar to a weak gel) even though these vesicles had a lower captured volume (being smaller and oligolamellar vesicles) than liposomes. The mechanical resistance is important in topical formulations because can facilitate

product application on skin surface avoiding formulation leakage at application site and improving patient compliance. Additionally, hyaluronan structure did not hamper but facilitated vesicle diffusion to the skin, as confirmed by skin delivery study.

According to this model, curcumin did not affect the liposome structure (peak shape of SAXS profile) although reducing the bilayer thickness ($d_B$) as a consequence of polar head amplitude ($\sigma_H$) reduction (Table 2). This is indicative of curcumin interactions with the vesicle surface, accordingly with variation of zeta potential that became less negative. The lamellarity and stiffness of hyalurosomes increased in comparison with liposomes as pointed out by the more sharpened and intense peak. Moreover, as shown by cryo-TEM micrographs, SAXS also proved that in 0.5hyalurosomes oligo- and unilamellar vesicles coexisted, as shown in Figure 3C by the very broad peak with a plateau in the upper region, probably due to the overlapping of different peaks. The presence of the polymer in hyalurosomes altered the electron distribution of the bilayer, resulting in a thinner bilayer ($d_B$) and reduced polar head amplitude ($\sigma_H$), which can be due to the electrostatic interactions between the negative ones of sodium hyaluronate and the positive groups of choline on both internal and external bilayer surface. The polymer in the interlamellar spaces decreased the bilayer repulsion leading to smaller water interlamellar strata and a higher number of lipid lamellae, which formed stiffer and more stable vesicles due to the concentric lipid bilayers alternated with the polymer layers. Therefore, SAXS analysis is in line with bilayer complexation with the polymer, which was distributed inside and outside the vesicle bilayer. On the contrary, the presence of the drug did not cause any further modifications on $d_B$ or $\sigma_H$, suggesting that it was homogenously dispersed in the polymer network as well as within the bilayer and not in the bilayer surface differently from liposomes. Indeed, in hyalurosomes, curcumin did not change the zeta potential value in comparison with the corresponding empty vesicles.

Overall findings of this work allow us to hypothesize that the sodium hyaluronate, did not simply form a superficial coating layer but, interacting with the phospholipids, produced a continuous polymer network in which vesicles and drug were immersed and stabilized. Indeed, the new

vesicles are characterized by high stability, high mechanical resistance, and superior ability to load and retain the curcumin in comparison with conventional liposomes.

Curcumin, thanks to its high antioxidant and antinflammatory activity, may be successfully used as topical therapeutic agent in wound healing but its topical delivery is strongly limited by the poor skin permeability due to its aqueous insolubility [33]. Here, it has been shown that the cutaneous delivery of this molecule can be significantly improved by suitable nanoformulations. Results of the *in vitro* skin penetration and permeation experiments have highlighted an improved and time-dependent deposition of the curcumin in all the skin strata when 0.5hyalurosomes were used. This formulation quick form a curcumin deposit in the outermoster skin layers (SC and epidermis) from which, successively, the drug reaches dermis and receptor fluid in great amount. The higher ability of these vesicles to enhance curcumin delivery into all the intact skin, with respect to liposomes, is probably due to a synergistic penetration enhancement activity of both phospholipids and sodium hyaluronate [34,35].

*In vitro* and *in vivo* assays on cells and damaged tissue confirmed the promising properties of the curcumin-loaded hyalurosomes. Indeed, the *in vitro* keratinocyte viability assay showed their high biocompatibility and their capability of improving the protective effect of curcumin toward oxidative injures. In particular, the curcumin efficacy on inhibit $H_2O_2$ cell damages was strengthened by all the vesicles (liposomes and hyalurosomes) regardless hyaluronan presence. This is likely due to the phospholipid vesicles capability of facilitating cell uptake of curcumin, which may exert its scavenging activity in the intracellular environmental, thus, preventing cell damages and consequent death. However, in the scratch assay, hyalurosomes showed a superior ability than liposomes to potentiate curcumin efficacy in a hyaluronan-concentration depending manner, as a consequence of the reepithelising and tissue remodeling properties of the polymer [36]. The polymer and curcumin synergistically improved not only proliferation but also migration of keratinocytes, resulting in a faster lesion repair provided by the 0.5hyalurosomes with respect to liposomes. Finally, hyalurosomes ability to promote and potentiate curcumin activity on skin repair

was established in vivo. To stimulate the typical anti-inflammatory response, TPA was daily applied on the skin leading to an extended wound associated to an oedematous and inflamed tissue simulating the biochemical events that naturally occur in *in vivo* pathological wounds [6]. The simultaneous treatment with curcumin vesicular samples provided an important reduction of epidermal loss and irritation, thus, proving their efficacy on restoring and/or maintaining healthy skin conditions [6]. The macroscopic evaluation of the damaged skin and the measurement of the biomarkers, oedema and MPO, confirmed a significantly greater anti-inflammatory activity of curcumin-loaded hyalurosomes in comparison with free curcumin dispersion and curcumin-loaded liposomes. Actually, the best performances supplied by 0.5hyalurosomes could be attributed to the opportune association of the three components of the formulation, which allowed the vesicles ~~drug~~ stay at the application site facilitating ~~its~~ curcumin penetration into tissue and cells, ~~mainly~~ due to the mechanical resistance of the formulation (such as a weak gel) and to the delivery ability of phospholipid vesicles, supporting the ~~different~~ therapeutic activity ~~due to the combination~~ of hyaluronan and curcumin.

## 5. CONCLUSION

The *in vitro* and *in vivo* findings highlight that using sodium hyaluronate dispersion as hydrating medium of phospholipids a series of small but substantial changes on vesicle structure are achieved, thus, permitting to reach better physico-chemical properties and biological performances. This is probably due to a synergistic effect of an appropriate amount of hyaluronan, which has already shown restoring properties, curcumin with its antioxidant activity and phospholipid vesicles, which are suitable carriers. Indeed, hyaluronan immobilizes the vesicles greatly improving curcumin entrapment efficiency, vesicle stability, ~~and~~ rheological properties, ~~favouring~~ local drug availability and ~~synergically potentiating~~ formulation therapeutic activity, thus, resulting in *in vivo* fast healing process. Overall results suggest the potential use of curcumin-loaded hyalurosomes as skin delivery systems able to restore the structural and functional conditions on damaged skin resulting appropriate for cosmetic, pharmaceutical and medical devices products.

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## CONFLICT OF INTEREST

Authors declare that no conflict of interest exists for the present work.

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