Experimental Dermatology ADF

Effect of new Curcumin-containing nanostructured lipid dispersions on human keratinocytes proliferative responses

Journal:	*Experimental Dermatology*
Manuscript ID:	EXD-14-0473
Manuscript Type:	Regular Article
Date Submitted by the Author:	20-Oct-2014

Complete List of Authors:	Esposito, Elisabetta; University of Ferrara, Life Sciences and Biotechnology Sticozzi, Claudia; University of Ferrara, Life Sciences and Biotechnology Ravani, Laura; University of Ferrara, Life Sciences and Biotechnology Drechsler, Markus Muresan, Ximena; University of Ferrara, Life Sciences and Biotechnology Cervellati, Franco; University of Ferrara, Life Sciences and Biotechnology Cortesi, Rita; University of Ferrara, Life Sciences and Biotechnology Valacchi, Giuseppe; University of Ferrara,

Keywords:	Curcumin, Nanoparticles, NFKB, Cyclin D1, Keratinocytes

Effect of new Curcumin-containing nanostructured lipid dispersions on human keratinocytes proliferative responses.

Elisabetta Esposito[a#], Claudia Sticozzi[a#], Laura Ravani[a], Markus Drechsler[b], Ximena M Muresan[a], Franco Cervellati[a], Rita Cortesi[a], Giuseppe Valacchi[a*]

[a]*Department of Life Sciences and Biotechnology, Section of Medicinal and Health Products, University of Ferrara, I-44121 Ferrara, Italy*

[b]*Macromolecular Chemistry II, University of Bayreuth, Germany*

# Equally contributed to the study

*Correspondence to:

Giuseppe Valacchi, PhD
Department of Life Sciences and Biotechnology,
University of Ferrara,
44121 Ferrara, Italy
(39) 0532 455 482
giuseppe.valacchi@unife.it

Abstract

The present study describes the production and characterization of nanostructured lipid dispersions (NLD) containing curcumin (CUR) as new tools for curcumin topical delivery.

Four types of NLD based on monoolein in association with different emulsifiers were produced: Na cholate and poloxamer 407 (NLD1), poloxamer alone (NLD2), the mixture of Na cholate and Na caseinate (NLD3) and Na cholate alone (NLD4). Morphology, dimensional distribution of lipid dispersions were investigated by cryo-TEM and Photon Correlation Spectroscopy (PCS). In vitro studies based on Franz cell and membrane nylon, and stratum corneum-epidermis (SCE) were carried out in order to compare CUR diffusion from selected lipid dispersions. In addition the NLDs cytotoxicity was evaluated in human keratinocytes.

PCS studies showed differences in particles diameter among the different NLDs. Cytotoxicity results in HaCaT cells evidenced that NLD1 and NLD2 were toxic at doses over 1 μM. Therefore Cryo-TEM was determined only for NLD3 and NLD4 showing the unlammelar vescicles without any effect on the structure by CUR. Diffusion measurement in SCE and nylon membrane evidenced that CUR had a time delayed release for NLD4. The "wound healing" effect of NLD3 and NLD4 with and without CUR was analysed keratinocytes in vitro. Results showed a clear inhibition of cell proliferation/migration by CUR. This effect was mediated by the inhibition of cyclin D1 expression as a consequence of the impaired NFkB activation.

This study confirms the antiproliferative properties of CUR and evidenced a new possible model of CUR topical delivery for hyperproliferative cutaneous diseases such as psoriasis.

Keywords: cubosomes, curcumin, HaCaT, cyclinD1, NFkB

Abbreviations: Nanostructured lipid dispersions (NLD); curcumin (CUR); Cryogenic Transmission Electron Microscopy (cryo-TEM), Photon Correlation Spectroscopy (PCS) stratum corneum-epidermis (SCE).

48

1. Introduction

Curcumin (CUR) is a polyphenol derived from the spice turmeric, with an age-old tradition as a drug [1, 2]. Among its numerous activities, CUR is able to affect skin proliferative properties by modulating the expression of growth related proteins [1, 2]. Despite its efficacy, CUR is scarcely soluble in water and in particular it is not readily absorbed through the skin, hence arduous to be administered.

At this regard many delivery strategies for CUR have been proposed, mainly based on the use of lipid matrixes such as liposomes, solid lipid nanoparticles, and cyclodextrins [3, 4]. In particular our group has proposed the solubilization of CUR in nanostructured lipid dispersions (NLD) [5, 6]. Lipid dispersions enable to gain dissolution and controlled delivery of active molecules, at the same time improving drug bioavailability and reducing side-effects [7, 8]. The emulsification of unsaturated long-chain monoglycerides in water in the presence of an emulsifier results in the formation of aqueous nanostructured dispersions with a complex disperse phase based on lyotropic liquid crystalline phases (lamellar, hexagonal, and cubic) [9, 10]. The emulsifier exerts an important role since it influences both structure and stability of the disperse phase [6, 11].

In the present investigation four NLD based on monoolein in association with different emulsifiers and loaded with CUR have been produced and characterized with the aim to obtain new tools to possibly modulate keratinocytes proliferation.

Morphology and dimensional distribution of NLDs have been investigated by Cryogenic Transmission Electron Microscopy (cryo-TEM) and Photon Correlation Spectroscopy (PCS).

The permeability of CUR from NLDs has been investigated by Franz cell alternatively associated to stratum corneum-epidermis (SCE) or nylon membranes. The latest have been chosen in order to simulate cutaneous tissue. In our studies we were able to evidence that CUR did not affect the diameter and the structure of the particles (NLDs). “In vitro” study showed that a slight cytotoxicity of NLD1 and NLD2; while, CUR loaded in NLD3 and NLD4 was able to affect cell proliferation by

inhibiting the activationof NFkB/cyclin D1 pathway, suggesting a possible new toll for CUR delivery.

2. Methods

2.1 Materials

Glyceryl monooleate RYLO MG 19 (monoolein) was a gift from Danisco Cultor (Grindsted, Denmark). Pluronic F127 (Poloxamer 407, poloxamer) (PEO_{98}-POP_{67}-PEO_{98}) was obtained from BASF (Ludwigshafen, Germany). Curcumin (CUR), (1E,6E)-1,7-bis(4-Hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, sodium cholate (Na cholate) (3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid sodium salt), and sodium caseinate (Na caseinate) (αS1, αS2, β, κ) were purchased from Sigma Chemical Company (St Louis, MO, USA). Solvents were of HPLC grade and all other chemicals were of analytical grade.

2.2. NLD preparation

Production of dispersions was based on the emulsification of monoolein and emulsifier in water, as previously described [5, 6]. NLD compositions are reported in Table I.

After emulsification, the dispersions were subjected to homogenization (15,000 rev min¯1, Ultra Turrax, Janke & Kunkel, Ika-Werk, Sardo, Italy) at 60°C for 1 min, then cooled and maintained at room temperature in glass vials.

To produce CUR containing NLD (CUR-NLD), 7.5 mg of CUR (0.33% w/w with respect to the monoolein, 0.015% w/w with respect to the dispersion) were added to the molten monoolein/emulsifier mixture and dissolved before addition to the aqueous solution. During production the vial was protected from light with an aluminium foil to prevent photo-degradation of CUR.

2.2 Characterization of NLD

2.2.1 Cryo-Transmission Electron Microscopy (Cryo-TEM)

Samples were vitrified as described in a previous study [6, 12]. The vitrified specimen was transferred to a Zeiss EM922Omega (Carl Zeiss Microscopy, Oberkochen, Germany) transmission

electron microscope using a cryoholder (CT3500, Gatan, Munich, Germany). Sample temperature was kept below 100K throughout the examination. Specimens were examined with reduced doses of about 1000-2000 e/nm^2 at 200 kV. Images were recorded by a CCD digital camera (Ultrascan 1000, Gatan, Munich, Germany) and analysed using a GMS 1.8 software (Gatan, Munich, Germany).

2.2.2 Photon Correlation Spectroscopy (PCS)

Submicron particle size analysis was performed using a Zetasizer 3000 PCS (Malvern Instr., Malvern, England) equipped with a 5 mW helium neon laser with a wavelength output of 633 nm. Glassware was cleaned of dust by washing with detergent and rinsing twice with sterile water. Measurements were made at 25 °C at an angle of 90° with a run time of at least 180 sec. Samples were diluted with bidistilled water in a 1:10 v:v ratio. Data were analysed using the "CONTIN" method [13]. Measurements were performed on NLD after production.

2.3. In vitro diffusion studies

In vitro diffusion studies were performed by Franz type diffusion cells supplied by LGA (Berkeley, CA) associated to SCE or nylon membranes (Millipore, 0.45 □m pore size).

Samples of adult human skin (mean age 36±8 years) were obtained from breast reduction operations and treated as previously reported [6, 8, 14].

Samples of dried SCE or nylon membranes were rehydrated by immersion in distilled water at room temperature for 1 h before being mounted in Franz cell. The exposed skin surface area was 0.78 cm^2 (the diameter of the orifice was 1 cm). The receptor compartment contained 5 ml of a mixture of phosphate buffer 60 mM pH 7.4 and methanol (50:50, v/v) as above reported. This solution was stirred with the help of a magnetic bar at 500 rpm and thermostated at 32 ± 1°C during all the experiments [15].

Approximately 500 µl of each formulation was placed on the skin surface in the donor compartment and the latter was sealed to avoid evaporation. At predetermined time intervals comprised between

1 and 24 hours, samples (0.15 ml) of receptor phase were withdrawn and CR concentration in the receptor phase was measured using HPLC. Each removed sample was replaced with an equal volume of simple receptor phase. The CUR concentrations were determined six times in independent experiments and the mean values ± standard deviations were calculated. The mean values were then plotted as a function of time. The diffusion coefficients, computed from the linear portion of the accumulation curve, represent the experimentally observed fluxes (F_o).

Normalized fluxes F_n were then calculated by the following equation:

$$F_n = F_o/C \quad (1)$$

where C is the CUR concentration (in mg/ml) in the analysed form.

2.4 HPLC Procedure

HPLC determinations were performed using a two-plungers alternative pump (Jasco, Japan), an UV-detector operating at 425 nm, and a 7125 Rheodyne injection valve with a 50 µl loop. Samples were loaded on a stainless steel C-18 reverse-phase column (15×0.46 cm) packed with 5 µm particles (Grace® - Alltima, Alltech, USA).

Elution was performed with a mobile phase containing methanol, 2% acetic acid and acetonitrile 5:30:65 v/v at a flow rate of 0.5 ml/min. Retention time of CUR was 7.0 min.

2.5 Cell culture and treatments

HaCaT cells, (a cell line gift from Dr. F. Virgili), were grown in Dulbecco's modified Eagle's medium High Glucose (Lonza, Milan, Italy), supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM l-glutamine as previously described [16]. Cell suspension containing 10 or 1×10^5 viable cells/ml were used. Cells were incubated at 37 °C for 24 h in 95% air/5% CO_2 until 80% confluency.

HaCaT cells were treated with different doses of these preparations (1 and 10 nM) at different time points (1 and 24 h).

After treatment, cells or medium were collected for the several assays described below.

CUR were dissolved in ETOH at a concentration of 10 mM as a stock solution. The stock was diluted to the required concentrations directly in the medium. The final concentration of ETOH in culture medium during compounds treatment did not exceed 0.1% (v/v).

2.6 Cellular viability

Viability studies were performed 24 h after treatments by cytofluorimetric (Muse Cell Analyzer) and LDH release assay. The cytofluorimetric assay was perfomed by using Muse Count & Viability Kit (Millipore, Corporation, Billerica, MA, USA). Briefly, 380 µL of Muse Count&Viability working solution were added to cells ($1x10^6$ cell/mL), and 20 µL of this cell suspension were incubated for 5 minutes at room temperature in the dark.

In addition, the LDH release was measured by enzymatic assay: in the first step NAD+ is reduced to NADH/H+ by the LDH-catalyzed conversion of lactate to pyruvate; in the second step the catalyst (diaphorase) transfers H/H+ from NADH/H+ to tetrazolium salt which is reduced to formazan. Prior to each assay, the cells were lysed with 2% (V/V) Triton X-100 in culture media for 30 min at 37 °C to obtain a representative maximal LDH release as the positive control with 100% toxicity The amounts of LDH in the supernatant were determined and calculated according to kit instructions (EuroClone Milan, Italy). All tests were performed in triplicate repeated at least three times.

2.8 Wound healing "in vitro" assay

HaCaT cells (2×10^6 per well) were seeded in a 60 × 15 mm Petri dishes and grown until confluent. Cells were then treated with CUR, CUR-NLD3 and CUR-NLD4 (1 and 10nM) for 24 h. Each monolayer was scratched with the use of a 200 µl pipette tip to generate a cell-free zone (0.8–1 mm in width). After washing with DMEM, cells were incubated for 24 h at 37°C with DMEM 10% FBS. Cells were photographed 1hrs after wounding, the wounded area was marked on the base of

the Petri dish, and the same field was photographed again after incubation for 24 h. The migration of cells into the wound area was evaluated. Images were acquired on a Leica imaging microscope.

2.9 Western blot analysis

After protein extraction in RIPA buffer, 60 µg boiled protein were loaded onto 10% sodium dodecyl sulphate–polyacrylamide electrophoresis gels and separated by molecular size. Gels were electro-blotted onto nitrocellulose membranes and then blocked for 1 h in Tris-buffered saline, pH 7.5, containing 0.5% Tween20 and 5% milk. Membranes were incubated overnight at 4 °C with the rabbit anti- cyclin D1 (Millipore Corporation, Billerica, MA, USA [clone EPR 224 (IHC)-32]) or β-actin (Millipore Corporation, Billerica, MA, USA). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (anti-rabbit) for 1 h, and the bound antibodies were detected by chemiluminescence (BioRad, Milan, Italy). β-Actin was used as loading control. Images of the bands were digitized and the densitometry analysis was performed using Image-J software.

2.10 Immunocytochemistry

Briefly, HaCaT cells were grown on coverslips in medium with FBS 10%, at a density of 1×10^5 cell/ml, and after treatments were fixed in 4% paraformaldehyde in PBS for 30 min at 4 °C. Cells were permeabilized and then blocked in PBS containing 5% BSA at RT for 1 h. Coverslips were then incubated for 1 h with primary antibody (anti p65 subunit, SantaCruz, CA), followed by 1 h with secondary antibodies. Nuclei were stained with 1 µg/ml DAPI (Molecular Probes) for 1 min after removal of secondary antibodies. Coverslips were mounted onto glass slides using with anti-fade mounting medium 1,4-diazabicyclo[2.2.2]octane in glycerine (DABCO) and examined by the Leica light microscope equipped with epifluorescence at 63X magnification. Negative controls were performed by omitting primary antibodies.

Statistical Analysis

For each of the variables tested, two-way analysis of variance (ANOVA) was used. A significant effect was indicated by a P-value < 0.05. Data are expressed as mean ± S.D. of triplicate determinations obtained in 5 indipendent experiments.

3. Results

3.1 Preparation and characterization of NLD

In the present study we have studied the cytotoxicity of four different NLD obtained in the presence of (a) Na cholate and poloxamer 407 (NLD 1), (b) poloxamer alone (NLD 2), (c) the mixture of Na cholate and Na caseinate (NLD3) and (d) Na cholate alone (NLD4), (see Table I).

PCS studies were conducted to determine the dimensional distribution of NLD, in the absence and in the presence of CUR. Table II summarizes the obtained results expressed as intensity mean diameter and polydispersity index (P.I.). It can be observed that the mean diameter of the NLD disperse phase after production is comprised between 150 and 230 nm. The lowest diameter was obtained by the use of Na cholate and poloxamer 407 while the highest in the case of Na cholate and Na caseinate. P.I. was not affected by the employed surfactant.

3.2 Cytotoxicity studies

Cytotoxicity of CUR-NLDs ranging from 1 to 100 nM was evaluated in HaCaT cells by means of cytofluorimetric assay and LDH release. Treatment for 24hrs with NLD1 and NLD2, induced cytotoxic effect (data not shown), while, NLD3 and NLD4 treatment did not affect cell viability as it is shown in Fig. 1A. These results were in line with the LDH release. In fact, as it is depicted in Fig. 1B, treatment with NLD3 and NLD4 did not induce the release of LDH in the media.

3.3 CUR containing NLD

The cytotoxicity study enabled to select NLD3 and NLD4 as the less toxic nano-dispersions to be employed for keratinocytes studies. To this aim NLD were produced in the presence of CUR (CUR-NLDs) and characterized in term of size and morphology.

Concerning dimensions, from data reported in Table III, it should be noted that the mean diameter of NLD disperse phase is not affected by the presence of CUR.

Cryo-TEM analyses were conducted in order to investigate the internal structures of NLDs and to compare the influence of the emulsifier on the nanostructure of the dispersed phase. Figure 2 reports cryo-TEM images of NLD3 (panels A and B) and CUR- NLD3 (panels C and D). All panels show mixtures of vesicles, cubosomes and hexasomes. In particular, in Figure 2A mainly unilamellar vesicles can be observed together with invaginated ones. Figure 2B shows a particle with the typical inner cubic structure. In both Figures 2C and 2D some hexasomes in different formation state are reported, besides plain vesicles and vesicles with invaginations. The amphiphilic protein casein, exhibits self-association into micellar aggregates in water and it is able to form cubosomes in the presence of monoolein, as previously demonstrated by other authors [17, 18]. CUR does not influence NLD disperse phase.

The morphology of NLD4 and CUR- NLD4, whose cryo-TEM images are respectively reported in Fig. 3A and 3B, was characterized by unilamellar vesicles. The black dots are ascribed to ice crystals contamination due to sample preparation. Also in this case the presence of CUR does not seem to affect NLD aspect.

3.4. CUR permeability study

Franz cell was employed to compare the diffusion kinetics of CUR from CUR-NLD3 and CUR-NLD4. SCE and nylon membranes were employed and their performances were compared.

A non-physiological receptor phase with 50% v/v of ethanol was used, in order to allow the establishment of the sink conditions and to sustain permeant solubilization [8, 19]. Figure 4 summarizes the results obtained by SCE (panel A) and nylon (panel B) membranes.

CUR-NLD4 resulted in the fastest diffusion of the drug both in the case of SCE and nylon membranes. The differences between the CUR diffusion data were extremely significant ($p<0.0001$).

The slope, which represents the release rate, steady-state flux, was calculated by linear regression of the linear portion of the curve. In the case of SCE membrane the CUR calculated fluxes from NLD3

and NLD4 were 0.093 and 0.22 x 10^{-3} cm/h respectively, while by the use of nylon membrane the fluxes were 9.24 and 42.93 x 10^{-3} cm/h for NLD3 and NLD4 respectively. Hence CUR diffuses more slowly from NLD3 than from NLD4.

The CUR fluxes obtained by nylon were about 200 fold higher than those obtained by SCE, this result was expectable since SCE porosity is about 80 nm while nylon membrane displays a porosity of 450 nm.

3.5. Wound healing study

Since it is well known that CUR is a natural compound with anti-proliferative effect on several cell type, we have examined the impact of NLDs plus CUR in an "in vitro" scratch wound model. As it is shown in Fig. 5, CUR-NLDs hampered the ability of HaCaT cells to regenerate the monolayer in the scratched area, resulting in a delayed closure of the wound. In the specific, NLD4+CUR (1 and 10nM) was able to significantly delay the wound closure already after 20hr from the "scratch" (33%). This effect was also noticed for NLD3+CUR (10nM) although it was less evident and reached the significance only at 24h. No differences were noticed respect to NLDs when the cells were treated with the only CUR (data not shown).

3.6 Cyclin D1expression

As shown in Fig. 5, CUR treatment was able to reduced the expression of Cyclin D1 in a dose dependent manner after 24 hr of treatment (20% for 1nM and 70% for 10 nM). Similar results were observed when the cells were treated with NLD3+CUR (1 and 10nM). On the contrary, only 10nM NLD4+CUR were able to significantly affect Cyclin D1 protein levels (57%).

3.7 NFkB nuclear translocation study

Being Cyclin D1 under the control of the transcription factor NFkB, we have evaluated the activation (nuclear translocation) of this transcription factor. As shown in Fig. 7, 1h after the

treatment of the cells with 1 and 10 nM of CUR, there was an evident decrease in NFkB activation (green color) as measured by its nuclear translocation. Similar effect was observed when the cells were treated with sodium NLD4 (1 and 10nM) and NLD3 (10 nM) and these data parallel with the Cyclin D1 results.

Discussion

In the current paper we demonstrate that NLDs-CUR are a strong inhibitors of keratinocytes proliferation. Because keratinocytes abnormal proliferation in psoriatic skin mimics an overshot wound-healing process, we tested the potential effect of NLDs-CUR in an "in vitro" scratch wound model. We found that CUR hampered the monolayer reconstitution in the wound site, markedly delaying spontaneous wound closure. Further work is needed to assess whether NLDs-CUR impaire wound healing by simply blocking cell proliferation or, additionally, by decreasing cell motility.

CUR is a component of turmeric and it has been used, for first time, as a medical remedy in Southeast Asia for centuries. This compound, in fact, has different properties including antioxidant, anti-carcinogenic, anti-viral, and anti-infectious as well as anti-inflammatory and anti- proliferative activity [20–22]. Many of CUR functions have been attributed to its ability to modulate transcription factors and lots of work has been done on the effect of CUR on NFkB [23, 24]. Several papers have now evidenced the ability of CUR to inhibit the activation of NFkB and therefore to regulate several cellular processes among which cellular proliferation is a key component in cutaneous tissues. Indeed, cellular proliferation and differentiation are tightly regulated in skin and their alteration can lead to several pathologies. For instance, psosirasis is characterized by hyper-proliferative and altered differentiation of keratinocytes [25]. Several natural products have been investigated to ameliorate or cure psoriasis and CUR has been extensively studied in cutaneous tissues where can be applied topically.

Although the numerous beneficial properties, CUR molecule is not easily absorbed through the skin. Therefore the aim of this study was to produce and characterize NLDs containing CUR as new tools for its topical delivery.

It is well known that the emulsification of monoolein in water in the presence of a surfactant such as poloxamer 407 leads to the production of cubosome dispersions [10, 26, 27]. Some authors have found a moderate toxicity toward blood components exhibited by cubosomes stabilized by poloxamer 407 [28] due to the synergistic action of poloxamer 407 and monoolein. The produced

nanostructures would be able to exert a very low but detectable hemolytic activity. To possibly overcome this drawback we have produced and characterized heterogeneous monoolein dispersions by the use of alternative emulsifiers such as Na cholate and Na caseinate [5, 6]. We found that the mean diameter of the NLD disperse phase after production is comprised between 150 and 230 nm. The lowest diameter was obtained by the use of Na cholate and poloxamer 407 while the highest in the case of Na cholate and Na caseinate. P.I. was not affected by the employed surfactant. In addition loading the NLDs with CUR did not affect the size and the properties of the nanostructured lipids.

Concerning the membranes for Franz diffusion studies, excised human or animal skin can be preferably used in order to obtain reliable permeability results [29]. Nonetheless, synthetic membranes can also be employed when biological skin is not readily available [30]. Synthetic membranes should have minimum diffusion resistance to drugs and only act as a support to separate the formulation from the receptor medium, in this respect in the present study SCE was chosen to reproduce the human skin, while nylon was employed to simulate the cutaneous wounds, where the skin is not continuous and integer but is damaged instead. In any case CUR diffusion appears fastest by the use of NLD4. In this regards the different inner morphology NLD should be considered: the disperse phase of NLD3 is constituted of a mixture of well organized structures such as cubosomes, hexasomes and different types of vesicles, while NLD4 disperse phase is mainly based on vesicles. Hence it is conceivable that CUR transition through NLD4 is more easy than through NLD3, where the different supramolecular structures perplex the CUR passage, at last leading to a slower diffusion.

In addition our results demonstrated that NLD3 and NLD4, did not have any toxic effect in human keratinocyets and especially for NLD4 there was a significant delay in cell proliferation by the use of an in vitro scratch wound model. This effect could be the consequence of the inhibition of NFkB activation that subsequently affect Cyclin D1 expression.

Skin injury activated a number of signaling pathways that induce the increased cell proliferation, resulting in the formation of scar tissue. These pathways are mediated by the transcription regulator NFkB. Prior to injury, NFkB exists as a pair of dimers (p50/p65) within the cytoplasm. When activated by a number of inflammatory stimuli, including surgical injury, NFkB dimers translocate into nucleus, where they bind to DNA, and induce the transcription of over 200 genes responsible for cell proliferation, cell migration, cell cycling, and the inhibition of apoptosis [31-33].

The activation of NF-kB requires phosphorylation at multiple serine/threonine-specific sites as well as tyrosinespecific sites [33, 36]. The process of activating NFkB requires the removal of its inhibitory protein, IkB, by phosphorylation of its kinase, IkB kinase. IkB kinase is a serine/threonine kinase which is activated by PhK. It has been demonstrated that PhK is inhibited by CUR [37].

Therefore it is possible to hypothesize that CUR-NLDs are capable of penetrating injured skin, such as in psoriasis, sufficiently to decrease adenosine triphosphate (ATP)-phosphorylase b phosphotransferase PhK levels within the epidermis. These data are in agreement with the study of Heng et al. [38] that demonstrated that CUR gel is also capable of penetrating at least as far as the superficial dermis in post-surgical scars, with significant resolution of post-surgical scarring. Our data support the fact that NLDs could be a good alternative of delivering “beneficial” molecules to the skin ameliorating the ability to be absorbed thank also to the slow time release. In this specific case, NLD4 seems to release CUR faster than NLD3 showing even a lower cellular toxicity. These molecules could be also used not only for hyper-proliferative diseases but, as it has shown in the work by Heng et al., also to optimize the time of wound-closure to avoid the formations of scars [38]. In conclusion NLDs are a new and promising tool to ameliorate the pathogenesis of several skin diseases thanks to the possibility to apply them topically.

References

[1] Aggarwal B B, Harikumar K B. Potential therapeutic effects of curcumin, the anti-inflammatory agent, against neurodegenerative, cardiovascular, pulmonary, metabolic, autoimmune and neoplastic diseases. Int J Biochem Cell Biol 2009: 41: 40-59.

[2] Thangapazham R L, Sharma A, Maheshwari R K. Beneficial role of curcumin in skin diseases. Adv Exp Med Biol 2007: 595: 343-357.

[3] Bhawana B, Basniwal R K, Buttar H S, *et al.* Curcumin nanoparticles: preparation, characterization, and antimicrobial study. J Agric Food Chem 2011: 59: 2056-2061.

[4] Tonnesen H H, Masson M, Loftsson T. Studies of curcumin and curcuminoids. XXVII. Cyclodextrin complexation: solubility, chemical and photochemical stability. Int J Pharm 2002: 244: 127-135.

[5] Puglia C, Cardile V, Panico A M, *et al.* Evaluation of monooleine aqueous dispersions as tools for topical administration of curcumin: characterization, in vitro and ex-vivo studies. J Pharm Sci 2013: 102: 2349-2361.

[6] Esposito E, Ravani L, Mariani P *et al.* Curcumin containing monoolein aqueous dispersions: A preformulative study. Materials Science and Engineering C 2013: 33: 4923–4934.

[7] Esposito E, Eblovi N, Rasi S, *et al.* Lipid based supramolecular systems for topical application: a preformulatory study. AAPS PharmSci 2003: 5: E30.

[8] Esposito E, Cortesi R, Drechsler M, *et al.* Cubosome Dispersions as Delivery Systems for Percutaneous Administration of Indomethacin. Pharm Res 2005: 22: 2163-73.

[9] Siekmann B, Bunjes H,. Koch MHJ *et al.* Preparation and structural investigations of colloidal dispersions prepared from cubic monoglyceride/water phases. Int J Pharm 2002: 244: 33-43.

[10] Gustafsson J, Ljusberg-Wharen H, Almgrem M, *et al.* Cubic lipid/water phase dispersed into submicron particles. Langmuir 1996: 12: 4611-4613.

[11] Worle G, Drechsler M, Koch MHJ, *et al.* Influence of composition and preparation parameters on the properties of aqueous monoolein dispersions. Int J Pharm 2007: 329: 150–157.

[12] Esposito E, Mariani P, Ravani L, *et al.* Nanoparticulate lipid dispersions for bromocriptine delivery: characterization and in vivo study. Eur J Pharm Biopharm 2012: 80: 306-314.

[13] Pecora R. Dynamic Light Scattering Measurement of Nanometer Particles in Liquids. J. Nanoparticle Res 2000: 2: 123-131.

[14] Kligman A M, Christophers E. Preparation of isolated sheets of human stratum corneum. Arch Dermatol 1963: 88: 702-705.

[15] Siewert M, Dressman J, Brown C K, *et al.* FIP/AAPS guidelines to dissolution/in vitro release testing of novel/special dosage forms. AAPS PharmSciTech 2003: 4: E7.

[16] Sticozzi C, Belmonte G, Pecorelli A, *et al.* Cigarette smoke affects keratinocytes SRB1 expression and localization via H2O2 production and HNE protein adducts formation. PLoS One. 2012: 7(3): e33592.

[17] Golding M, Sein A. Surface rheology of aqueous casein-monoglyceride dispersions. Food Hydrocoll 2004: 18: 451-461.

[18] Zhai J, Waddington L, Wooster T J. *et al.* Revisiting β-casein as a stabilizer for lipid liquid crystalline nanostructured particles. Langmuir 2011: 27: 14757-14766.

[19] Toitou E, Fabin B. Altered skin permeation of highly lipophilic molecule: Tetrahydrocannabinol. Int J Pharm 1988: 43: 17-22.

[20] Shishodia S, Sethi G, Aggarwal B B. Curcumin: getting back to the roots. Ann N Y Acad Sci 2005: 1056: 206–17.

[21] Duvoix A, Blasius R, Delhalle S, *et al.* Chemopreventive and therapeutic effects of curcumin. Cancer Lett 2005: 223: 181–90.

[22] Campbell FC, Collett GP. Chemopreventive properties of curcumin. Future Oncol 2005: 1: 405–14.

[23] Singh S, Aggarwal B B. Activation of transcription factor NF-kappa B is suppressed by curcumin (diferuloylmethane) [corrected]. J Biol Chem 1995: 270: 24995–5000.

[24] Chen A, Zheng S. Curcumin inhibits connective tissue growth factor gene expression in activated hepatic stellate cells in vitro by blocking NF-kappa B and ERK signaling. Br J Pharmacol 2008: 153: 557–567.

[25] Schon M P, Boehncke W H Psoriasis. N Engl J Med 2005: 352: 1899–1912.

[26] Lindstrom M, Ljusberg-Wahren H, Larsson K *et al.* Aqueous lipid phases of relevance to intestinal fat digestion and absorption. Lipids 1981:16: 749-754.

[27] Buccheim W, Larsson K. Cubic lipid-protein-water phases. J Colloid Interface Sci 1987: 117: 582-583.

[28] Bodea J C, Kuntsche J, Funari S S, *et al.* Interaction of dispersed cubic phases with blood components. Int J Pharm 2013: 448: 87-95.

[29] Bronaugh R L, Stewart R F, Simon M, Methods for in vitro percutaneous absorption studies. VII: Use of excised human skin. J Pharm Sci 1986: 75: 1049-1097.

[30] Ng S F, Rouse J, Sanderson D, *et al.* A comparative study of transmembrane diffusion and permeation of ibuprofen across synthetic membranes using Franz diffusion cells. Pharmaceutics 2010: 2: 209–223.

[31] Aggarwal B B. Nuclear factor-kappa B: the enemy within. Cancer Cell 2004: 6: 203–208.

[32] Verma IM, Stevenson JK, Schwarz EM, et al. Rel/NF-jB family: intimate tales of association and disassociation. Genes Dev 1995: 270: 2723–2735.

[33] Takada Y, Singh S, Aggarwal B B. Identification of p65 peptide that selectively inhibits NF-kappa B activation induced by various inflammatory stimuli and its role in downregulation of NF-kappaB-mediated gene expression and upregulation of apoptosis. J Biol Chem 2004: 279: 15096–15104.

[34] Lallena M J, Diaz-Meco M T, Bren G, *et al.* Activation of IjBa by protein kinase C isoforms. Moll Cell Biol 1999: 19: 2180–2188.

[35] cHuang W C, Chen J J, Chen C C. c-Src dependent tyrosine phosphorylation of IKKbeta is involved in tumor necrosis factor-alpha-induced intercellular adhesion molecule-1 expression. J Biol Chem 2003: 278: 9944– 9952.

[36] Yang F, Yamashita J, Tang E, *et al.* The zinc finger C417R of I-kappa B-kinase gamma impairs lipopolysaccharide and TNF-mediated NF-kappa B activation through inhibiting phosphorylation of the I-kappa B kinase beta activation loop. J Immunol 2004 : 172: 2446–2452.

[37] Reddy S, Aggarwal B B. Curcumin is a non-competitive and selective inhibitor of phosphorylase kinase. FEBS Lett 1994: 341: 19–22.

[38] Heng M C. Wound healing in adult skin: aiming for perfect regeneration. Int J Dermatol 2011: 50(9): 1058-66.

Figure legends

Figure 1. Cell Viability measured by using cytofluorimetric assay (A) and LDH release (B) in HaCaT cells after CUR, NLDs or NLDs+CUR. Triton X represent 100% of LDH release. Data are expressed as percentage of control (averages of five indipendent experiments).

Figure 2. Cryo-transmission electron microscopy images (cryo-TEM) of NLD stabilized by Na cholate and Na caseinate (NLD 3) in the absence (A, B) and in the presence (C, D) of CUR.

Figure 3. Cryo-transmission electron microscopy images (cryo-TEM) of NLD stabilized by Na cholate (NLD 4) in the absence (A) and in the presence (B) of CUR.

Figure 4. CUR release profiles from CUR-NLD 3 and CUR-NLD 4 obtained by Franz cell associated to SCE (A) or nylon membranes (B). Data represent the mean of 4 independent experiments ± S.D.

Figure 5. Confluent HaCaT cells were scratch wounded, washed and allowed to regenerate from scratch wounding in normal conditions (control) or after NLDs+CUR treatment for 24 h and photographed. Multiple photographs of the wound were obtained and the distance between the cells borders (after wound) represent 0% recovery. Using image analysis Image J software the distance calculated in percentage of cellular recovery areas was determined. Data are expressed as percentage of 1h (averages of five indipendent experiments).

Figure 6. Representative Western blot for Cyclin D1 protein expression after NLDs+CUR treatment is showed in the upper panel. Quantification of the Cyclin D1 bands is shown in the bottom panel. Data are expressed as arbitrary units (averages of five indipendent experiments, $*p < 0.05$). β-actin was used as loading control.

Figure 7. Immunofluorescence of HaCaT cells after NLDs+CUR treatment for 1h showed the localization of NF-κB (p65 subunit, green). Images are merged and representative of at least 100 cells viewed in each experiments (n = 5). Nuclei (blue) were stained with DAPI. Original Magnification X 630.

.

Table I. Composition of NLD

Component (% w/w)	NLD 1	NLD 2	NLD 3	NLD 4
Monooleine	4.5	4.5	4.5	4.5
Poloxamer 407	0.5	0.5	-	-
Na Cholate	0.15	-	0.15	0.15
Na caseinate	-	-	0.07	-
H_2O	94.85	95	95.28	95.35

[a]*In the case of drug containing NLD, curcumin content was 0.015 %*

Table II. Size distribution parameters of NLD, as determined by PCS.

Dimensional parameter		
Formulation	**Intensity mean diameter (nm)**	**Polydispersity Index**
NLD 1	154.5	0.29
NLD 2	198.9	0.26
NLD 3	227.5	0.28
NLD 4	189.8	0.27

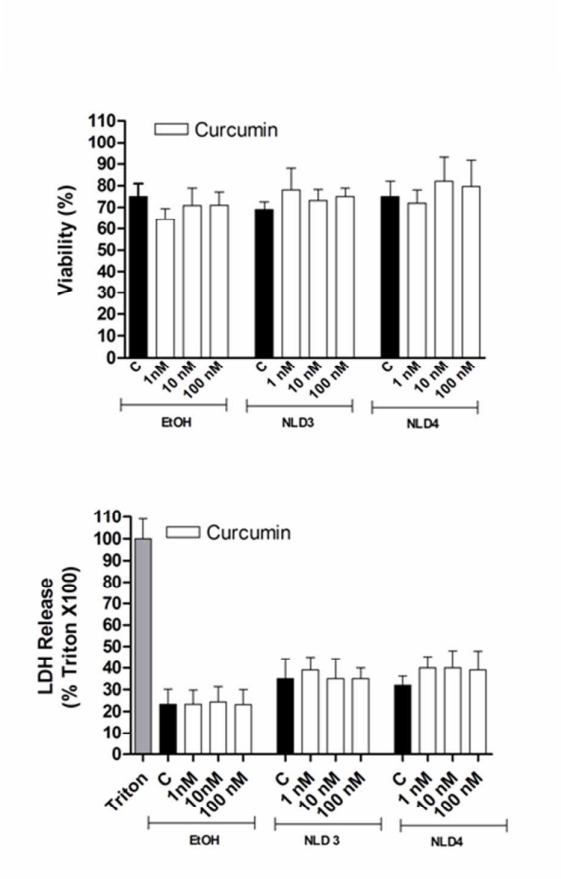
PCS data are means of 5 determinations on different batches of the same type of dispersion, SD was always comprised between ± 5%

Table III. PCS parameters of NLD 3 and 4 in the presence of CUR.

Parameter	**NLD 3**	**NLD 3 *CUR***	**NLD 4**	**NLD 4 *CUR***
Z-Average (nm)	227.20	218.38	210.70	206.80
P.I.	0.23	0.29	0.23	0.27

CUR denotes the formulation produced in the presence of curcumin; PI: Polidispersity Index.
PCS data are means of 5 determinations on different batches of the same type of dispersion, SD was always comprised between ± 5%

Fig. 1



555
556
557
558
559

Fig. 2

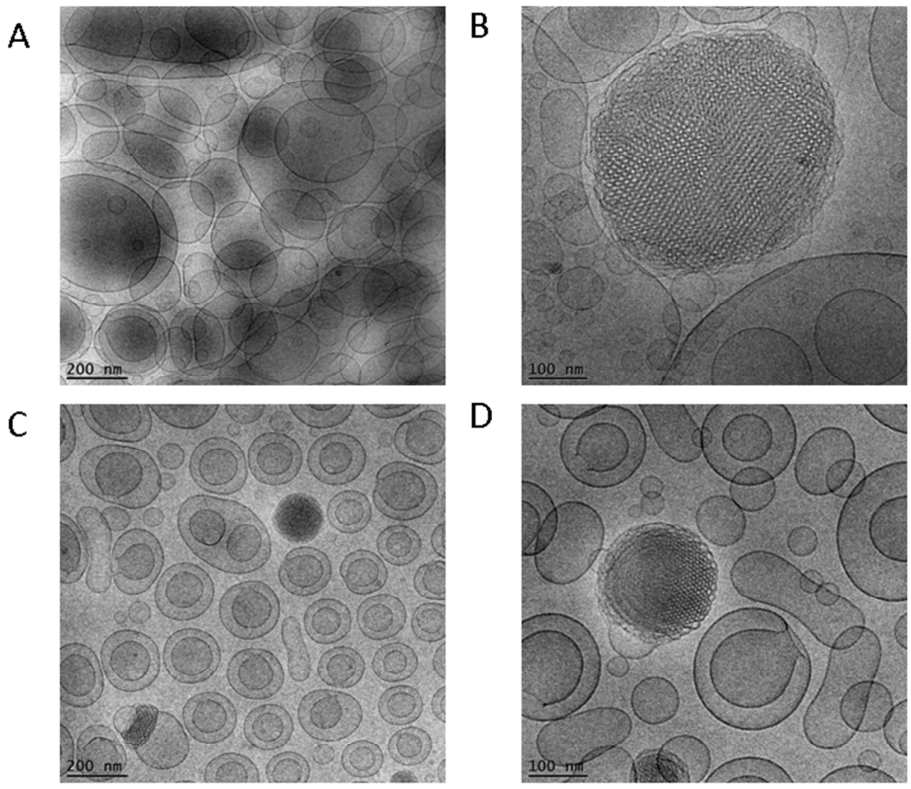


Fig. 3

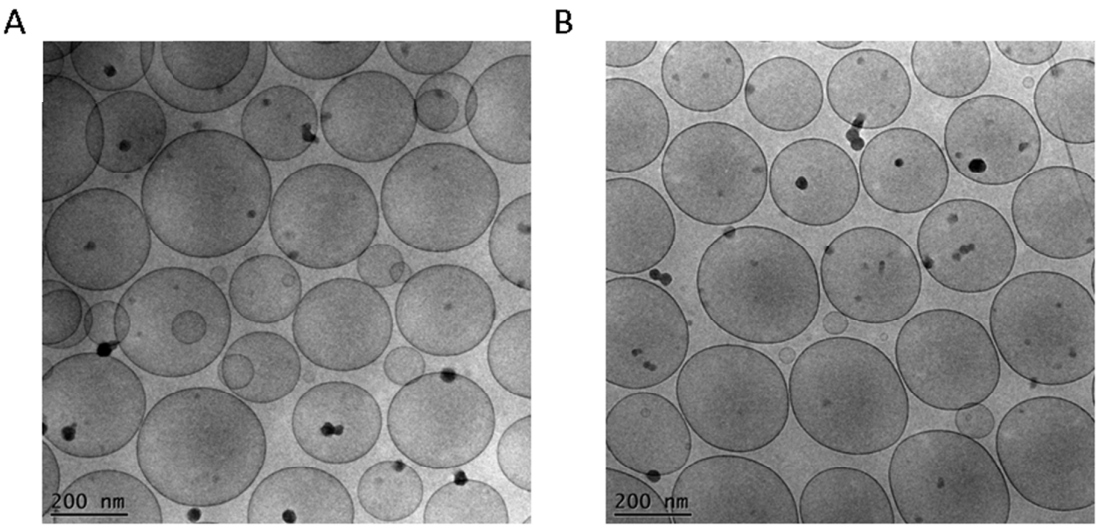


Fig. 4

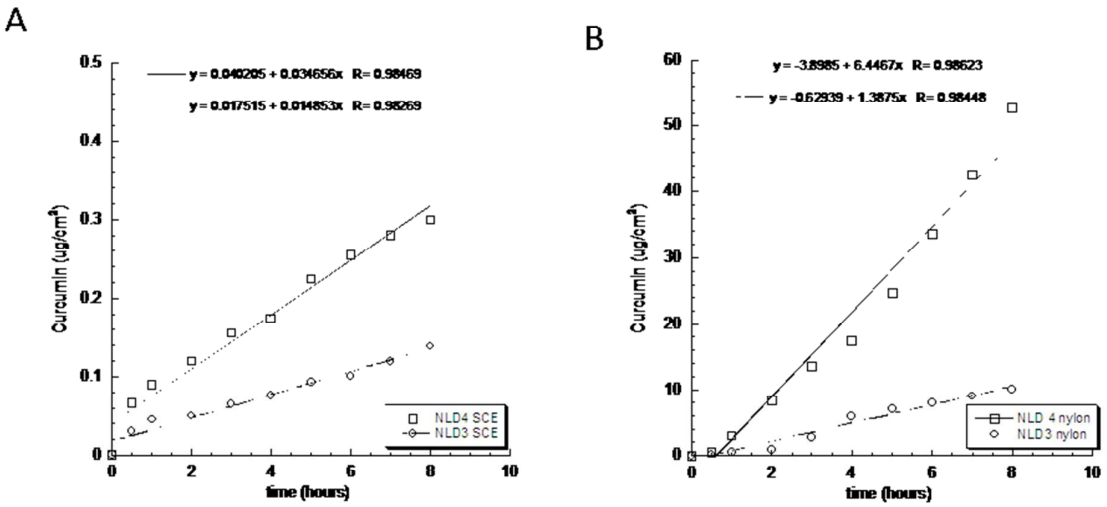


Fig. 5

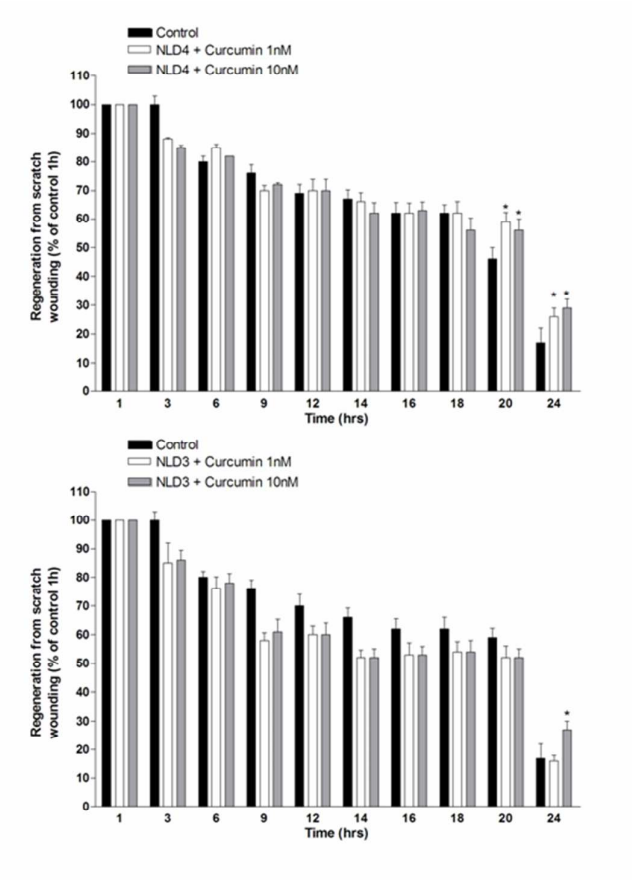
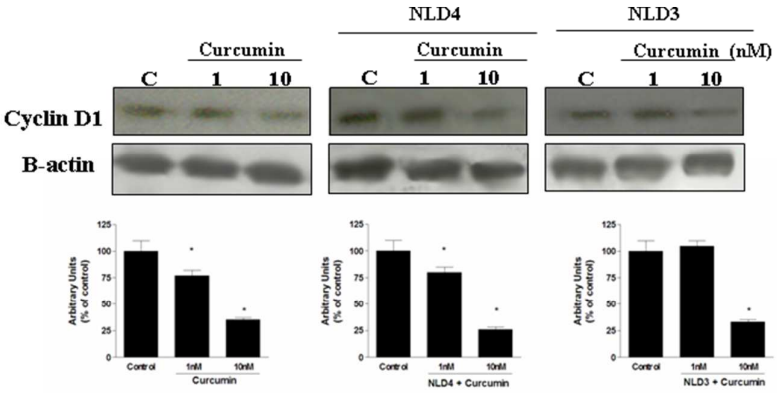
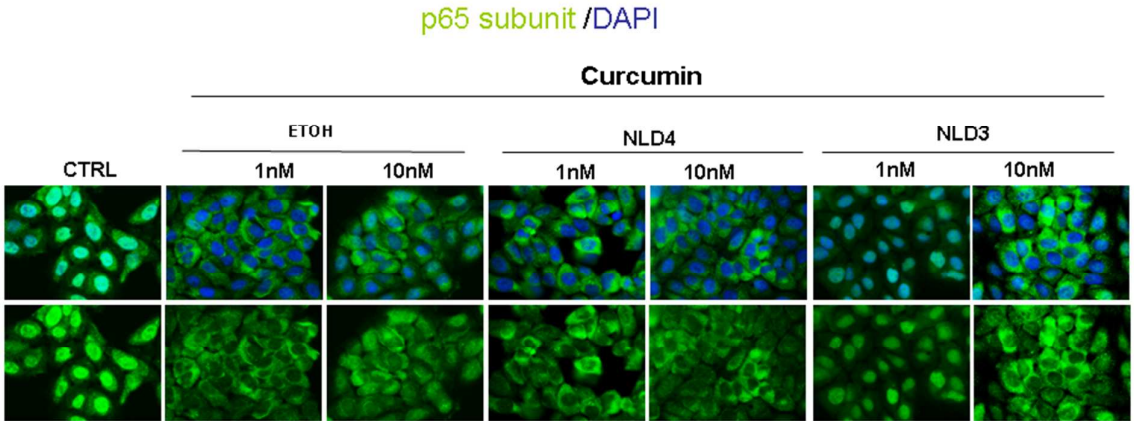

Control
NLD4 + Curcumin 1nM
NLD4 + Curcumin 10nM
Regeneration from scratch wounding (% of control 1h)
Time (hrs)
Control
NLD3 + Curcumin 1nM
NLD3 + Curcumin 10nM
Regeneration from scratch wounding (% of control 1h)
Time (hrs)

Fig. 6


NLD4
NLD3
Curcumin
Curcumin
Curcumin (nM)
C
1
10
C
1
10
C
1
10
Cyclin D1
B-actin
Arbitrary Units (% of control)
Control
1nM
10nM
Curcumin
NLD4 + Curcumin
NLD3 + Curcumin

689

Fig. 7



690