

**New cationic nanovesicular systems containing lysine-based surfactants for topical administration: toxicity assessment using representative skin cell lines**

Daniele Rubert Nogueira<sup>1</sup>, M. Carmen Morán<sup>1,3</sup>, Montserrat Mitjans<sup>1,3</sup>, Verónica Martínez<sup>1</sup>,  
Lourdes Pérez<sup>2</sup>, M. Pilar Vinardell<sup>1,3,\*</sup>

<sup>1</sup>*Departament de Fisiologia, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII  
s/n, 08028, Barcelona, Spain*

<sup>2</sup>*Departamento de Tecnología Química y de Tensioactivos, IQAC, CSIC, C/Jordi Girona 18-  
26, 08034, Barcelona, Spain*

<sup>3</sup>*Unidad Asociada al CSIC, Spain*

\* Corresponding author. Tel.: +34 934024505; fax: +34 934035901.

*E-mail address:* mpvinardellmh@ub.edu (M. Pilar Vinardell).

## **ABSTRACT**

Cationic nanovesicles have attracted considerable interest as effective carriers to improve the delivery of biologically active molecules into and through the skin. In this study, lipid-based nanovesicles containing three different cationic lysine-based surfactants were designed for topical administration. We used representative skin cell lines and *in vitro* assays to assess whether the cationic compounds modulate the toxic responses of these nanocarriers. The nanovesicles were characterized in both water and cell culture medium. In general, significant agglomeration occurred after 24 h incubation under cell culture conditions. We found different cytotoxic responses among the formulations, which depended on the surfactant, cell line (3T3, HaCaT and THP-1) and endpoint assayed (MTT, NRU and LDH). Moreover, no potential phototoxicity was detected in fibroblast or keratinocyte cells, whereas only a slight inflammatory response was induced, as detected by IL-1 $\alpha$  and IL-8 production in HaCaT and THP-1 cell lines, respectively. A key finding of our research was that the cationic charge position and the alkyl chain length of the surfactants determine the nanovesicles resulting toxicity. The charge on the  $\alpha$ -amino group of lysine increased the depletion of cell metabolic activity, as determined by the MTT assay, while a higher hydrophobicity tends to enhance the toxic responses of the nanovesicles. The insights provided here using different cell lines and assays offer a comprehensive toxicological evaluation of this group of new nanomaterials.

**KEYWORDS:** Cationic nanovesicles, lysine-based surfactants, cell culture, skin drug delivery, cytotoxicity, inflammatory response

## 1. Introduction

Cationic lipid-based nanovesicles have been proposed as biocompatible drug delivery devices, with specific properties and able to overcome the barriers imposed by cell membranes [1]. Among various cationic substances, cationic amphiphile molecules have been successfully used in the composition of such carriers [1,2]. Lipid-based devices have a broad spectrum of applications, one of which is the topical delivery of active ingredients, intensively studied in recent years [3,4]. The inclusion of cationic compounds, such as biocompatible cationic amphiphiles, in the basic membrane of liposomes might be a promising approach to increasing the formulation's stability and its specificity for skin drug delivery. The development of new lipid-like molecules, such as cationic amphiphiles derived from lipoaminoacids [5,6], has become a major feature in the search for natural bioactive molecules that could be used in new drug delivery systems [7]. In this context, cationic lysine-based surfactants are a promising group of amino acid-based amphiphiles that can be regarded as an alternative to conventional synthetic amphiphiles due to their multi-functionality and biodegradability, the renewable source of raw materials used during their synthesis and their low cytotoxic potential [5].

The presence of charges at the vesicle surface may influence topical drug delivery. The skin surface bears a net negative charge [8], which, therefore, favor the positively charged vesicles to increase the permeation rate of different model drugs through the skin [9,10]. In contrast, some authors reported contradictory results, in which vesicles with negative charge have higher skin permeation [11,12]. Although extensive studies have focused on the drug release and penetration properties of cationic vesicles, the potential toxic mechanisms of this kind of carrier have not been explained sufficiently. Some authors have studied the effect of structural properties of cationic vesicles on their cytotoxicity [13], while others assessed the mechanisms of cell death induced by cationic vesicles [14]. The search for reliable

conditions to assess nanomaterials' safety is an emerging field that poses many interesting challenges.

Currently, there are no specific testing requirements for nanotechnology products and, therefore, researchers took liberal approaches to studying toxicity [15,16]. Moreover, it is worth noting that, because of the expense of animal testing in toxicology and pressure from both the general public and government to develop alternatives to *in vivo* testing, *in vitro* cell-based models may be more attractive for preliminary testing of nanomaterials [17]. Here, we developed different formulations of cationic nanovesicles containing biocompatible lysine-based surfactants as surface modification agents, and screened *in vitro* toxicological assays both to understand better the potential health hazards of new nanomaterials designed for topical application and to create predictive toxicology approaches for testing nanotechnology-based products. We specifically studied whether the inclusion of cationic lysine-based surfactants, differing in the cationic charge position and in alkyl chain length, may determine the cytotoxic and phototoxic potential of nanovesicular systems and modulate molecular mechanisms such as inflammatory response in representative skin cell lines. Since there is a knowledge gap between the increasing development and use of nanomaterials and the prediction of possible health risks [18], safety evaluation and greater understanding of nanomaterials' impact on human health are essential before any clinical application is explored.

## **2. Experimental**

### 2.1. Chemicals and reagents

2,5-diphenyl-3-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide (MTT), neutral red (NR) dye, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), cholesterol and dimethylsulphoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Dulbecco's Modified Eagle's Medium (DMEM), RPMI 1640 medium, fetal bovine serum (FBS), phosphate buffered saline (PBS), L-glutamine solution (200 mM), trypsin-EDTA solution (170,000 U/l trypsin and 0.2 g/l EDTA) and penicillin-streptomycin solution (10,000 U/ml penicillin and 10 mg/ml streptomycin) were purchased from Lonza (Verviers, Belgium). The 75 cm<sup>2</sup> flasks and 96-well plates were obtained from TPP (Trasadingen, Switzerland). All other reagents were of analytical grade.

## 2.2. Surfactants included in the nanovesicular systems

Three new biocompatible amino acid-based surfactants derived from N<sup>ε</sup> or N<sup>α</sup>-acyl lysine methyl ester salts with one lysine as the cationic polar head (one cationic charge) and one alkyl chain were used as surface modification agents to prepare the cationic vesicular systems reported in this study: N<sup>ε</sup>-myristoyl lysine methyl ester (MKM) with one alkyl chain of 14 carbon atoms and one positive charge on the α-amino group of the lysine, N<sup>ε</sup>-palmitoyl lysine methyl ester (PKM) with one alkyl chain of 16 carbon atoms and one positive charge on the α-amino group of the lysine and N<sup>α</sup>-myristoyl lysine methyl ester (MLM) with one alkyl chain of 14 carbon atoms and one positive charge on the ε-amino group of the lysine. MKM and PKM have a hydrophobic chain attached to the ε-amino group of the lysine, while MLM has its hydrophobic chain attached to the α-amino group of the lysine. These lysine-based surfactants were synthesized in our laboratory, as described elsewhere [5,6].

## 2.3. Preparation of cationic nanovesicular formulations

The mixed cationic vesicles were prepared by the film hydration method. Briefly, DMPC only or DMPC and cholesterol (CHOL) were mixed with MKM, PKM or MLM in the designed molar ratios and dissolved in the mixed chloroform:methanol (1:1, v/v) solvent in a round-bottom flask. The formulations of DMPC:surfactant were prepared at 80:20 molar ratio, while the molar composition of the vesicular systems composed of DMPC:CHOL:surfactant

were 56:24:20 (30% cholesterol of the total lipid in the formulation). The organic solvent was removed under reduced pressure using a R-210 Rotary Evaporator (Buchi, Switzerland) at 50°C for 60 min to form a homogeneous thin film. To remove residual traces of the organic solvents, the mixed film was freeze-dried (Christ Alpha 2-4 LD freeze-drying system, Martin Christ, Germany) overnight. Ten ml of ultrapurified water was added to hydrate the film, and the resulting suspension was sonicated for 20 min at 60°C in an Ultrasons-H ultrasonic bath (J. P. Selecta, Spain) to promote the formation of uniform vesicles. The total final concentration of each mixed cationic nanovesicle was fixed at 2 mM.

Nanovesicle dispersions were purified by filtration using Vivaspin 2 centrifugal concentrator (PES membrane, 3,000 Da MWCO, Sartorius Stedim Biotech, Goettingen, Germany). The substance filtrated was used to determine the extent of incorporation of the cationic surfactants into the vesicles. The amount of unincorporated surfactant was assessed by high-performance liquid chromatography (HPLC), following the analytical method previously described [5].

#### 2.4. Nanovesicle characterization

The mean hydrodynamic size and the polydispersity index (PDI) of the cationic nanovesicles were determined at 25°C by dynamic light scattering (DLS) using a Malvern Zetasizer ZS (Malvern Instruments, Malvern, UK). The measurements were performed in ultrapurified water immediately after preparation ( $t = 0$  h) and in cell culture medium with 5% FBS at  $t = 0$  h and after a 24 h incubation at 37°C ( $t = 24$  h). Each measurement was performed using at least three sets of at least ten runs.

The zeta potential (ZP) values of the nanovesicle formulations were assessed by determining the electrophoretic mobility of a particle with the Malvern Zetasizer ZS equipment. The measurements were performed in ultrapurified water and cell culture medium at 25°C using at least three sets of at least 20 runs.

The morphology and size analysis of the vesicular systems were analysed by transmission electron microscopy (TEM) and the images were obtained with a Jeol JEM-1010 electron microscope (Jeol Ltd., Tokyo, Japan) operating at an acceleration voltage of 80 kV. A droplet (5  $\mu$ l) of the vesicles dispersed in ultrapure water was placed on a carbon-coated copper grid, forming a thin liquid film. The negative staining of samples was obtained with a 2% (w/v) solution of phosphotungstate acid (pH 6.5, with KOH). The excess solution was removed by a filter paper and followed by thorough air-drying.

## 2.5. Cell cultures

The murine Swiss albino fibroblasts, 3T3, and the spontaneously immortalized human keratinocyte HaCaT cell lines were grown in DMEM medium (4.5 g/l glucose) supplemented by 10% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin at 37°C, 5% CO<sub>2</sub>. The 3T3 and HaCaT cells were routinely cultured in 75 cm<sup>2</sup> culture flasks and were trypsinized using trypsin-EDTA when the cells reached approximately 80% confluence. The human monocytic leukemia cell line THP-1 was grown in RPMI 1640 medium supplemented by 10% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 50  $\mu$ M 2-mercaptoethanol at 37°C, 5% CO<sub>2</sub>. The three cell lines were obtained from Eucellbank (Universitat de Barcelona, Spain).

## 2.6. Cytocompatibility assays

3T3 ( $1 \times 10^5$  cell/ml) and HaCaT ( $7.5 \times 10^4$  cell/ml) cells were seeded into 96-well cell culture plates in 100  $\mu$ l of complete culture medium. Cells were incubated for 24 h under 5% CO<sub>2</sub> at 37°C and the medium was then replaced with 100  $\mu$ l of fresh medium supplemented by 5% FBS containing the vesicular system dispersions in the 0.5 – 100  $\mu$ M concentration range. THP-1 cells were seeded into 24-well plates at a density of  $1 \times 10^6$  cell/ml and then treated with vesicle dispersions in the 1 – 100  $\mu$ M concentration range. The final volume in each well

was 500  $\mu$ l and the medium used contained 5% FBS. Each concentration was tested in triplicate and control cells were exposed to medium with 5% FBS only. The cell lines were exposed for 24 h to each nanovesicle treatment.

To determine whether the NVs interact with the viability assays, UV-visible absorbance measurements were carried out [19,20]. NVs at 100  $\mu$ M were suspended in DMEM medium (without FBS and phenol red) containing MTT (0.5 mg/ml) or NR (50  $\mu$ g/ml) dyes. After 3 h incubation under cell culture conditions, the NVs were pelleted by ultracentrifugation, rinsed and extracted with DMSO or a solution containing 50% ethanol absolute and 1% acetic acid in distilled water for MTT and NR dyes, respectively. The extracted solution was transferred to a quartz cuvette and the absorbance read at 550 nm and at intervals from 300 to 700 nm on a Shimadzu UV-160A spectrophotometer (Shimadzu, Kyoto, Japan).

#### 2.6.1. MTT assay

The MTT assay is based on the protocol first described by Mossmann [21]. In this assay, living cells reduce the yellow tetrazolium salt MTT to insoluble purple formazan crystals. After 24 h exposure of 3T3 and HaCaT cells to the various vesicular systems, the treatment-containing medium was removed, and 100  $\mu$ l of MTT in PBS (5 mg/ml) diluted 1:10 in FBS-free medium without phenol red was then added. Plates were further incubated for 3 h, after which time the medium was removed. The purple formazan product was then dissolved by adding 100  $\mu$ l of DMSO to each well. Plates were then placed in a microtitre-plate shaker for 10 min at room temperature and the absorbance of the resulting solutions was measured at 550 nm using a Bio-Rad 550 microplate reader.

After 24 h incubation of THP-1 cells with the treatments, the plates were centrifuged and supernatants were collected and kept at  $-80^{\circ}\text{C}$  till subsequent analysis (see section 1.9). Then, 300  $\mu$ l of a MTT solution of 0.75 mg/ml was added to each well. Cells were incubated

for 3 h at 37°C, plates were then centrifuged, medium discarded and cells lysed in 250µl/well of a mixture of HCl/isopropanol. 100 µl of the resulting solutions were transferred to a 96-well plate and the absorbance was read as described above. Cell viability was calculated as the percentage of tetrazolium salt reduction by viable cells on each sample against the untreated cell control (cells with medium only).

#### 2.6.2. NRU assay

Based on the protocol described by Borenfreund and Puerner [22], the NRU assay was performed following exposure to the nanovesicles. 3T3 and HaCaT cells were incubated for 3 h with NR dye solution (50 µg/ml) dissolved in medium without FBS and phenol red. Cells were then washed with PBS, followed by the addition of 100 µl of a solution containing 50% ethanol absolute and 1% acetic acid in distilled water to extract the dye. Plates were gently shaken for 10 min to ensure complete dissolution. We then measured the absorbance of the extracted solution at 550 nm using a Bio-Rad 550 microplate reader. The effect of each treatment was calculated as the percentage of uptake of NR dye by lysosomes against the untreated cell control (cells with medium only).

#### 2.6.3. LDH assay

LDH leakage was determined in the conditioned medium 24 h after cationic vesicles' treatment in 3T3 and HaCaT cell lines, using a commercially available kit (Takara Bio Inc, Otsu, Japan), in line with the instructions provided by the manufacturer. This assay quantifies cytotoxicity based on the measurement of LDH activity released from dead or plasma membrane-damaged cells into the supernatant. Results are expressed as percentage of control, with 1% Triton-X used as positive control.

#### 2.7. Cell morphology analysis

3T3 cells at a density of  $1 \times 10^5$  cells/ml were grown on sterile cover glass in 24-well plates and then treated without (control) or in the presence of the  $IC_{50}$  concentrations (determined by the MTT assay) of the various formulations of cationic vesicles for 24 h. After incubation, cell morphology was analyzed by a phase contrast microscope (Olympus BX41, Olympus, Japan). Images were digitised by an Olympus XC50 camera connected to Olympus cell^B computer software.

## 2.8. Phototoxicity assay

Cell lines 3T3 and HaCaT were used as in vitro models to predict cutaneous phototoxicity. Two plates were seeded with cells; one for irradiation (+UVA) and the other wrapped in foil and therefore non-irradiated (-UVA). The cells were treated with the various vesicle formulations, as described in section 2.6. After treatment application, the plates were pre-incubated for 1 h at  $37^\circ\text{C}$  in a humidified 5%  $\text{CO}_2$  and then irradiated with a dose of  $2.5 \text{ J/cm}^2$  UVA light. Following irradiation, the plates were incubated again under the same conditions to complete 24 h exposure. MTT and NRU endpoint assays were used to assess the phototoxic potential of the formulations. The phototoxic effect was evaluated by the OECD phototoxic validation test [23], with some modifications. A photoirritation-factor (PIF) was calculated, using the following formula:

$$\text{PIF} = \text{IC}_{50} (- \text{UVA}) / \text{IC}_{50} (+ \text{UVA}) \quad (1)$$

Based on the results, a test substance with a  $\text{PIF} < 2$  predicts “no phototoxicity”; a  $\text{PIF} > 2$  and  $< 5$  predicts “probable phototoxicity” and a  $\text{PIF} > 5$  predicts “phototoxicity”.

## 2.9. Cytokine production

IL-1 $\alpha$  and IL-8 concentrations, as markers of induced inflammatory stimuli, were measured by commercially available sandwich enzyme-linked immunosorbent assay (ELISA)

kits (Diacclone Research, France and ImmunoTools, Friesoythe, Germany, respectively). The results are expressed in pg/ml.

To assess whether there was an inflammatory reaction measured by the presence of IL-1 $\alpha$ , HaCaT cells at a density of  $1 \times 10^5$  cells/ml were grown in 24-well plates and then exposed to a range of concentrations from 1 to 100  $\mu$ M of the various vesicle formulations diluted in medium with 1% FBS. After 24 h exposure, conditioned medium was recovered, centrifuged and used for the determination of extracellular IL-1 $\alpha$  (IL-1 $\alpha$  release). Monolayers were washed with PBS, then lysed in 300  $\mu$ l of PBS containing 0.5% of Triton X-100 and used for the determination of intracellular IL-1 $\alpha$  (cell-associated IL-1 $\alpha$ ). The protein content of the cell lysate determined by a commercial kit (Bio-Rad, Hercules, CA, USA) based on the dye-binding procedure of Bradford [24]. Cell viability was determined by the LDH leakage assay as described previously.

IL-8 release was assessed in cell-free supernatants after incubation of the various cationic vesicle formulations with THP-1 cells. The supernatants were collected directly from the cytotoxicity assay plates, centrifuged and stored at -80°C until analysis.

## 2.10. Statistical analysis

All *in vitro* experiments were performed at least three times, using three replicate samples for each formulation concentration tested. Results are expressed as mean  $\pm$  standard error of the mean (SEM). Statistical analyses used the Student's *t*-test or one-way analysis of variance (ANOVA) to determine the differences between the datasets, followed by Dunnett's *post-hoc* test for multiple comparisons using SPSS<sup>®</sup> software (SPSS Inc., Chicago, IL, USA).  $p < 0.05$  and  $p < 0.005$  were considered significant.

## 3. Results and discussion

Cationic lipid-based vesicles have attracted considerable interest because of their use as effective drug delivery systems [25,26]. More specifically, these kinds of carriers have been studied to improve the delivery of biologically active molecules into and through the skin [10,12]. Among a range of applications, cationic nanovesicles could be used in cosmetic formulations and epicutaneous drug release, because the addition of polar amphiphiles increases the likelihood of skin penetration [27]. In the present study, we provided new cationic nanocarriers as possible vehicles for dermal and transdermal applications. The cationic amphiphiles used as surface modification agents have biocompatible properties and can be considered much more suitable for practical applications than current commercial surfactant systems [28]. Furthermore, we showed previously that amphiphiles with the positive charge on the  $\alpha$ -amino group of lysine (MKM and PKM) have pH-dependent membrane lytic activity [29,30]. Some researchers have reported that the use of pH-sensitive lipid vesicles for skin delivery of biologically active molecules resulted in enhanced effects [31].

Here experiments were performed to investigate the physicochemical parameters of the nanovesicles and, more particularly, to see how their composition affects their interaction with cells that are representative of the skin and play a key role in irritant, inflammatory and immunological reactions. The *in vitro* methods proposed here to assess the safety of new nanomaterials are adapted to nanoscale colloids. They are a direct extension of methods known for other macroscopic biomaterials or soluble drug toxicology assessments. However, since there are no defined protocols for assessing nanotoxicity [15,16], the use of the current assays to evaluate the toxic potential and the mechanisms involved is of great importance in determining structure/function relationships between nanomaterials and toxicity.

### 3.1. Characterization of cationic nanovesicles

Nanomaterial size and zeta potential are very important parameters in drug delivery applications. As keeping nanomaterials in solution for longer periods results in aggregation [32], we prepared fresh formulations for each subsequent study to guarantee nanosized vesicles. Table 1 shows the results for each formulation. Average nanovesicle hydrodynamic diameter is between 90 and 255 nm, as determined by DLS analysis. The addition of cholesterol decreased the mean nanovesicle diameter, with the exception of the formulations containing MKM. When the nanovesicles containing MKM and PKM were dispersed in cell culture medium (DMEM with 5% FBS), the size increase was slight by 0 h, but significant agglomeration to micron-sized structures occurred after 24 h incubation under cell culture conditions. The easy aggregation in cell culture medium is probably attributed to the high ionic nature of the solution, resulting in the formation of the secondary particles [33]. In contrast, the nanovesicles containing MLM did not suffer agglomeration in cell culture medium, which can be attributed to their higher charge density (pKa MLM = 8.1) [29], corroborated by the ZP values determined in cell culture medium. The ZP values of all formulations are highly positive ( $> 40$  mV) and did not differ significantly from each other. In contrast, in cell culture medium almost neutral values were obtained. The high positive ZP values in water indicate the stability of the prepared formulations and reflect the net charge on the surface of the vesicles. This is also of great importance in preventing fusion or aggregation of nanovesicles [13]. It has been reported that a physically stable formulation would have a minimum  $\pm 30$  mV ZP as a borderline value of colloidal stability [34]. The PDI values reported in Table 1 range from 0.23 to 0.42, indicating a relatively homogenous vesicular population. A PDI value lower than 0.3 indicates a homogenous and monodisperse population [34].

The TEM morphological analysis showed that all the cationic vesicle formulations displayed clear negative staining images with a roughly spherical shape (Fig. 1). The

nanovesicles containing the surfactants with the positive charge on the  $\alpha$ -amino group of lysine (PKM and MKM) were in general much smaller ( $\sim 20$ – $50$  nm) than those obtained by DLS. These differences were especially significant for the nanovesicles with MKM (Figs. 1a, b), while the formulations with PKM showed more heterogeneous size distribution (Fig. 1e, f). The mean hydrodynamic particle size measured by DLS did not capture the real population distribution of the nanovesicles observed by TEM. The latter's aggregate population was undetected even as a peak by DLS analysis. This disparity between DLS and TEM analysis, previously reported [32,35,36], might be a result of the resolution limitations of DLS. DLS provides a scattered intensity-based size of a colloidal particle, and thus the mean diameter is biased towards larger vesicles, even though they may occupy a much smaller fraction of the vesicle population [35]. Moreover, these observed differences might be attributed to aggregation [32,37] or to the swelling of the nanovesicles in the presence of water [36]. In contrast, the nanovesicles containing the surfactant MLM, which have the positive charge on the  $\epsilon$ -amino group of lysine, did not show the same significant disparity between DLS and TEM analysis (Figs. 1c, d). In general, the TEM images corroborated the mean size obtained by DLS. Moreover, TEM images also revealed the formation of a multilayered membrane in the vesicles containing the surfactant MLM (Figs. 1c, d), while those containing the surfactants MKM and PKM showed unilamellar membranes in both presence and absence of cholesterol in the basic membrane (Figs. 1a, b, e, f).

The HPLC analysis of the filtrated samples (obtained from the purification process performed to remove the unincorporated amount of surfactant) revealed that the cationic surfactants were highly incorporated into the formulations (from 75 to 99% incorporation). (Table 1). It is noteworthy that the vesicles containing cholesterol have a lower degree of surfactant incorporation than those with DMPC only. Moreover, the formulations with the surfactant PKM have almost total incorporation of this compound into the nanovesicle. This

observation was fully expected since, the longer the alkyl chain of a surfactant is, the greater is its tendency to incorporate into lipid-based vesicles due to its hydrophobicity [38].

### 3.2. Cytocompatibility studies

The cytotoxic potential of new nanovesicles designed for biomedical application was evaluated on representative skin cells using established *in vitro* methods. HaCaT keratinocyte and 3T3 fibroblast cultures gave an appropriate *in vitro* model for skin irritation [39], while the human monocytic leukemia cell line THP-1 is considered surrogate of cutaneous dendritic cells in *in vitro* skin sensitization studies [40]. The combination of different cell lines and cytotoxicity assays gives information concerning general and specific toxicological mechanisms [41] that could help in understanding the toxic response of nanomaterials.

All the formulations containing the surfactants showed, as expected, higher cytotoxicity than the vesicles formulated without surfactant (data not shown). The cationic charge of the vesicles is probably responsible for the initial binding to the surface membrane by ionic interaction with the negatively charged cell membrane [42], which might enhanced the toxic effects of these formulations. Indeed, the cytotoxic effects observed showed many disparities between formulations that, in fact, depend on the surfactant, cell line and endpoint assay. The vesicles containing MKM and PKM (positive charge on the  $\alpha$ -amino group of lysine) have a clear and dose-dependent decrease in MTT activity in the three cell lines studied after 24 h, while the NRU and LDH assays showed a significant decline in cell viability only at doses  $> 50 \mu\text{M}$  (Fig. 2). In contrast, the vesicular systems containing MLM (positive charge on the  $\epsilon$ -amino group of lysine) showed similar cytotoxic responses by the three endpoint assays. These differences are reflected by comparing the  $\text{IC}_{50}$  values of the vesicles, as shown in Table 2.

In studies of MTT and NR dye interactions with nanovesicles, we observed minimal interference with each dye. These data were proved by the UV-vis measurements (data not

shown). Only the formulations containing PKM induced a slight increase in the MTT absorbance values at 550 nm. This might be due to a false positive reaction in which MTT was converted to formazan in the absence of cells. However, these small interferences did not result in further increases in cell viability with increasing concentration (false viability), which is in contrast to previous reported data for other types of nanomaterials [19,20]. These data prove that these viability endpoints are suitable for the intended purpose.

A key finding of our research was that the structural characteristic of the surfactants included in the nanovesicular systems directly affect the toxicological effects of such nanomaterials. Firstly, the position of the cationic charge in the amphiphile molecule was critical in determining the sensitivity of the endpoint used to assess the formulation's cytotoxicity. On the one hand, the nanovesicles containing the compounds with the positive charge on the  $\alpha$ -amino group of lysine (MKM and PKM) have greater cytotoxicity detected by MTT than by NRU and LDH endpoints. On the other hand, the nanovesicles containing MLM (with the positive charge on the  $\epsilon$ -amino group of lysine) displayed in general the same level of cytotoxicity with the three endpoints. These effects might be due to a different interaction mechanism of the vesicular systems within cell as a function of the cationic charge position on the amphiphile included in them. The MTT assay is a measurement of cell metabolic activity within the mitochondrial compartment, while the NRU and LDH assays measure membrane integrity. NR dye diffuses through intact cell membranes to accumulate within lysosomes, while the LDH endpoint detects cells in the last stages of cell death [43]. Based on the mechanisms of cell damage detected by each cytotoxicity assay, our overall results suggest that the vesicles containing MKM and PKM interacted early with the mitochondrial compartment, first affecting cell metabolic activity, while the plasma membrane is affected in an lesser extent. The early interaction of these nanovesicles with the mitochondria might be due to their cell internalization before any damage to the cell membrane. The ability of these

nanomaterials to be cell internalized was corroborated using the fluorophores Nile Red and Calcein as drug models (unpublished results). Moreover, the pH-sensitive activity of MKM and PKM [29,30] could favor this increased mitochondrial damage: the nanovesicles containing these compounds might have the ability to lyse the endosome membrane after cell internalization, enhancing, thus, their potential toxicity in the cell cytoplasm (especially to the mitochondria). Finally, it is also worth noting that the length of the surfactant alkyl chain also affected the nanovesicles' cytotoxicity. Regardless of the cell line and endpoint used, the vesicular systems containing the amphiphile PKM were the most cytotoxic. Therefore, we can conclude that, the longer the alkyl chain of the surfactant is, the greater the cytotoxicity of the resulting vesicular system. In line with these findings, our previous studies revealed that the amphiphiles also displayed significant differences in their phospholipid bilayer-perturbing properties [29,30], corroborating that the interaction processes with cell membrane are directly dependent on surfactant structure.

Together with the disparities observed with different cytotoxic assays, we also found that each cell line used showed different sensitivity to the cationic nanovesicles. Unfortunately, no clear conclusion was achieved about which cell was the most sensitive. Although we have no explanation for these differences at present, these data showed cell-specific differences in cationic vesicle processing and toxicity. The results given are in line with previous studies [42,44], in which was reported that cytotoxic effects of particulate carrier systems differ, depending on the cell lines used, due to the innate nature, metabolic abilities (e.g. enzymes present) and capabilities of these cells. All in all, our results showed that the surfactants have a strong effect on the interaction of nanovesicles with the cells and, thus, on their cytotoxicity. These findings corroborated previous studies that found that the type and concentration of surfactant strongly affected the toxic responses of solid lipid

nanoparticles [44] and also have a slightly effect on the cytotoxicity of lipoplex formulations [1,2].

### 3.3. Morphological analysis

Light microscopy analysis was used to view the effects of nanovesicles on the morphology of 3T3 cells after 24 h of incubation. Untreated control cells showed a well-spread and flattened morphology (Table 2). In contrast, 3T3 cells treated with the IC<sub>50</sub> concentrations of the nanovesicles displayed prominent morphological changes, including rounding, reduced spreading and shrunken cells. These morphological changes corroborate and are directly attributed to the cytotoxic effects of each vesicular system.

### 3.4. Phototoxicity assessment

Keratinocytes and fibroblasts are considered biologically relevant targets for skin irritants and photoirritants [45]. We therefore chose HaCaT and 3T3 cells as model cell systems to study the cutaneous phototoxic potential of nanovesicles. No potential phototoxic effects were observed, with PIF values lower than 2 (non-phototoxic) in all cases (Fig. 3). In general, the IC<sub>50</sub> values in irradiated (+UVA) and non-irradiated (-UVA) plates were similar to the HaCaT cells, and only the formulations containing the amphiphile MKM showed a significant increase ( $p < 0.005$ ) in the toxic response after irradiation, as determined by the NRU assay. In contrast, more significant differences between (-UVA) and (+UVA) plates were observed with 3T3 cells, especially with the NRU assay. Although the MTT assay gave lower IC<sub>50</sub> values (for the formulations containing MKM and PKM) for the (+UVA) plates than the NRU assay did (as also found in the cytotoxicity assays, see section 3.2), the latter assay was more sensitive in detecting the UVA irradiation effects in both cell lines studied. These results suggest that the nanovesicle treatment followed by UVA irradiation has a greater effect on the plasma membrane of the cells than on their metabolic activity. Furthermore, the

results of nanovesicle phototoxicity revealed the different sensitivity of the two cell lines regardless of the endpoint used, with HaCaT cells being, in general, less sensitive to the phototoxic effects. This variability in the effects of photoirritants on human keratinocytes and 3T3 cells was previously demonstrated in the original phototoxicity validation study [46] and in the studies of other amino acid-based surfactants [47]. All these data indicate the importance of assessing specific effects with different endpoints in a variety of different cell types.

As also discussed above for the general cytotoxicity assays, it is worth mentioning that the structural features of the amphiphiles also affected the phototoxicity of the nanovesicles. Vesicles containing MKM (positive charge on the  $\alpha$ -amino group of lysine) had a significant phototoxic effect with both cell lines and endpoint assays, while those containing MLM (positive charge on the  $\epsilon$ -amino group of lysine) had practically no phototoxicity in either cell line. Moreover, the length of the amphiphile alkyl chain also affected the phototoxicity of the formulations. Although the vesicles containing PKM (with 16 carbon atoms) were the most cytotoxic formulations, they showed lower phototoxic effects than those containing MKM (with 14 carbon atoms). Altogether, the results obtained showed that even though the formulations with MKM were the least cytotoxic in almost all conditions tested (see section 2.2), they displayed the highest potential phototoxicity of the nanovesicular systems.

### 3.5. Inflammatory response

To gain an insight into possible inflammatory reactions attributable to the nanovesicle formulations, secretion of cytokines (IL-1 $\alpha$  and IL-8) by two different cell lines (HaCaT and THP-1, respectively) were examined by ELISA. THP-1 cells were stimulated with 5 ng/ml of lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 (Sigma, St Louis, MO), in order to examine the capacity of this cell model to up-regulate cytokine (IL-8) production [48]. We found significantly higher IL-8 concentrations ( $652.14 \pm 13.86$  pg/ml,  $p < 0.005$ ), while cell

viability was 87.5% as detected by the MTT assay. THP-1 cells were chosen because they were proposed as an *in vitro* model able to produce and release the potent pro-inflammatory cytokine IL-8 [48,49]. Therefore, this *in vitro* model is a promising tool for the screening of new nanomaterials with possible inflammatory response. IL-8 (Fig. 4) release was induced in THP-1 cells in a dose-dependent manner by the formulations containing the amphiphiles MKM and PKM. The cationic vesicles containing MKM showed significant cytokine release at concentrations higher than 2.5  $\mu\text{M}$  ( $p < 0.005$ ), while those containing PKM induced significant IL-8 release at concentrations higher than 1  $\mu\text{M}$  and 5  $\mu\text{M}$ , for the formulations with DMPC only or DMPC and cholesterol as lipid matrix, respectively. Interestingly, the surfactant MLM, which differs in the position of the cationic charge, did not show a dose-response release of IL-8, but only induced a significant increase in the level of this cytokine at the highest concentration. Indeed, since the significant release at the highest concentrations might be related to cell death, it is reasonable to consider cytokine release responses given by concentrations that displayed viability higher than 75% as significant [40,49]. Therefore, we can reasonably consider as significant IL-8 releases those induced at concentrations equal to or lower than 2.5  $\mu\text{M}$  and 5  $\mu\text{M}$  for the formulations containing PKM and MKM, respectively, while no significant response can be attributed to the formulations containing MLM, as even a 10-fold higher concentration (50  $\mu\text{M}$ , viability  $> 75\%$ ) did not induce IL-8 release. The IL-8 release induced by nanomaterials has also been studied in different cell lines and, in line with our findings, only slight or negligible inflammatory response due to increased levels of IL-8 was found [16,50].

Keratinocytes participate actively in inflammatory and immunological skin reactions [51]. The human keratinocyte HaCaT cell line was stimulated with SDS 20  $\mu\text{g/ml}$ , as positive control, to confirm IL-1 $\alpha$  up-regulation [52]. Under these conditions, we found significantly higher cell-associated IL-1 $\alpha$  concentrations ( $239.12 \pm 9.87$  pg/mg protein,  $p < 0.005$ ) with cell

viability of 85.7% as detected by the LDH assay. Therefore, the combination of keratinocyte and cytokine production (IL-1 $\alpha$  as a pro-inflammatory cytokine) offers a simplified *in vitro* model to evaluate the potential toxicity of new nanomaterial-based formulations with cutaneous applications. In this study, IL-1 $\alpha$  production (both cell-associated and that released into extracellular media) was investigated in human keratinocytes following their exposure to nanovesicle formulations. The intracellular amount of IL-1 $\alpha$  was standardized with the total cell protein content and the LDH assay was used as an indicator of cell viability. Fig. 5 shows our results for cell-associated IL-1 $\alpha$  (pg/mg protein) and IL-1 $\alpha$  release (pg/ml) after cationic vesicle treatment. Neosynthesis or release of IL-1 $\alpha$  only achieved significant levels above the basal values (negative control) at the highest exposure conditions (25 to 100  $\mu$ M) in almost all cases, but the levels obtained were below those of the positive control SDS. For some formulations there was a down-regulatory effect in cell-associated IL-1 $\alpha$  and an increase in IL-1 $\alpha$  release at the highest concentrations, which can be directly attributable to the loss of cell membrane integrity (as seen in the LDH assay results, see Figs. 2e,f). The formulations containing cholesterol in the lipid matrix induced, in general, lower IL-1 $\alpha$  production. Moreover, the nanovesicles containing MLM were those that induced lower production of IL-1 $\alpha$ , as also observed for IL-8 release. This means that the inflammatory response of the nanovesicles might also be directly related, as reported for the cytotoxic and phototoxic studies, to the position of the cationic charge of the amphiphile: the formulations containing the surfactant with the positive charge on the  $\epsilon$ -amino group of the lysine have the least inflammatory potential. In the same way as described for the IL-8 and since the significant release at the highest concentrations might be related to cell death, the neosynthesis or release of IL-1 $\alpha$  were not significant. All in all, and despite some significant responses found for IL-8 and IL-1 $\alpha$  release in comparison with control cells, our results suggest that only a very slight inflammatory and allergenic effect was induced by nanovesicle formulations.

#### **4. Conclusions**

We demonstrated that the cytotoxicity of cationic nanovesicles differed between the three representative skin cell lines as well as when the *in vitro* endpoint varied, showing that the selected cell type and assay can affect the final outcome. Moreover, no potential phototoxic effect was induced by all nanovesicular systems, while only a slight inflammatory response occurred due to cytokine release. The formulations with MLM showed the lowest tendency to induce phototoxicity and inflammation. Overall, our findings showed that nanovesicle composition plays a primary role in the underlying toxicity. The cytotoxic responses of the nanovesicles varied especially as a function of the cationic charge position on the amphiphile included in them. Furthermore, the surfactant with the highest hydrophobicity tends to enhance the toxic potential of the formulations. All these findings suggest that differential toxicity according to vesicle composition could be an important concept when developing new nanomaterials for biomedical applications. In conclusion, the combination of all assays used in the present study offers an in-depth and comprehensive evaluation of the potentially toxic effects of nanomaterials. Due to their generally low toxicity, the nanovesicles containing MKM and MLM are especially recommended for topical administration.

#### **Conflict of interest statement**

The authors state that they have no conflict of interest.

#### **Acknowledgments**

This research was supported by Projects CTQ2009-14151-C02-02 and CTQ2009-14151-C02-01 of the *Ministerio de Ciencia e Innovación* (Spain). We also thank Dr. Núria Cortadellas for her expert technical assistance with the TEM experiments. Daniele Rubert Nogueira holds a PhD grant from MAEC-AECID (Spain).

## References

- [1] A.M.S. Cardoso, H. Faneca, J.A.S. Almeida, A.A.C. C. Pais, E.F. Marques, M.C.P. Lima, A.S. Jurado, Gemini surfactant dimethylene-1,2-bis(tetradecyldimethylammonium bromide)-based gene vectors: A biophysical approach to transfection efficiency, *Biochim. Biophys. Acta* 1808 (2011) 341-351.
- [2] D. Lundberg, H. Faneca, M.C. Morán, M.C.P. Lima, M.G. Miguel, B. Lindman, Inclusion of a single-tail amino acid-based amphiphile in a lipoplex formulation: Effects on transfection efficiency and physicochemical properties, *Mol. Membr. Biol.* 28 (2011) 42-53.
- [3] G. Betz, A. Aeppli, N. Menshutina, H. Leuenberger, In vivo comparison of various liposome formulations for cosmetic application, *Int. J. Pharm.* 296 (2005) 44-54.
- [4] P. Mura, F. Maestrelli, M.L. Gonzalez-Rodriguez, I. Michelacci, C. Ghelardini, A.M. Tabasco, Development and characterization and in vivo evaluation of benzocaine-loaded liposomes, *Eur. J. Pharm. Biopharm.* 67 (2007) 86-95.
- [5] L. Pérez, A. Pinazo, M.T. García, M. Lozano, A. Manresa, M. Angelet, M.P. Vinardell, M. Mitjans, R. Pons, M.R. Infante, Cationic surfactants from lysine: Synthesis, micellization and biological evaluation, *Eur. J. Med. Chem.* 44 (2009) 1884-1892.
- [6] A. Colomer, A. Pinazo, M.A. Manresa, M.P. Vinardell, M. Mitjans, M.R. Infante, L. Pérez, Cationic surfactants derived from lysine: effects of their structure and charge type on antimicrobial and hemolytic activities, *J. Med. Chem.* 54 (2011) 989-1002.
- [7] M.C. Morán, M.R. Infante, M.G. Miguel, B. Lindman, R. Pons, Novel Biocompatible DNA Gel Particles, *Langmuir* 26 (2010) 10606-10613.
- [8] A. Manosroi, L. Kongkaneromit, J. Manosroi, Stability and transdermal absorption of topical amphotericin B liposome formulations, *Int. J. Pharm.* 270 (2004) 279-286.
- [9] N. Katahira, T. Murakami, S. Kugai, N. Yata, M. Takano, Enhancement of topical delivery of a lipophilic drug from charged multilamellar liposomes, *J. Drug Target.* 6 (1999) 405-414.
- [10] N. Dragicevic-Curic, S. Grafe, B. Gitter, S. Winter, A. Fahr, Surface charged temoporfin-loaded flexible vesicles: in vitro skin penetration studies and stability, *Int. J. Pharm.* 384 (2010) 100-108.
- [11] T. Ogiso, T. Yamaguchi, M. Iwaki, T. Tanino, Y. Miyake, Effect of positively and negatively charged liposomes on skin permeation of drugs, *J. Drug Target.* 9 (2001) 49-59.

- [12] A. Gillet, P. Compère, F. Lecomte, P. Hubert, E. Ducat, B. Evrard, G. Piel, Liposome surface charge influence on skin penetration behaviour, *Int. J. Pharm.* 411 (2011) 223-231.
- [13] C.-H. Liang, T.-H. Chou, Effect of chain length on physicochemical properties and cytotoxicity of cationic vesicles composed of phosphatidylcholines and dialkyldimethylammonium bromides, *Chem. Phys. Lip.* 158 (2009) 81-90.
- [14] J.-H.S. Kuo, M.-S. Jan, C.-H. Chang, H.-W. Chiu, C.-T. Li, Cytotoxicity characterization of cationic vesicles in RAW 264.7 murine macrophage-like cells, *Coll. Surf. B Bioint.* 41 (2005) 189-196.
- [15] D.R. Boverhof, R.M. David, Nanomaterial characterization: considerations and needs for hazard assessment and safety evaluation, *Anal. Bioanal. Chem.* 396 (2010) 953-961.
- [16] J. Robbens, C. Vanparys, I. Nobels, R. Blust, K.V. Hoecke, C. Janssen, K.D. Schampelaere, K. Roland, G. Blanchard, F. Silvestre, V. Gillardin, P. Kestemont, R. Anthonissen, O. Toussaint, S. Vankoningsloo, C. Saout, E. Alfaro-Moreno, P. Hoet, L. Gonzalez, P. Dubruel, P. Troisfontaines, Eco-, geno-, and human toxicology of bio-active nanoparticles for biomedical applications, *Toxicology* 269 (2010) 170-181.
- [17] J.M. Hillegass, A. Shukla, S.A. Lathrop, M.B. MacPherson, N.K. Fukagawa, B.T. Mossman, Assessing nanotoxicity in cells *in vitro*, *WIREs Nanomed. Nanobiotechnol.* 2 (2009) 219-231.
- [18] M. Ema, J. Tanaka, N. Kobayashi, M. Naya, S. Endoh, J. Maru, M. Hosoi, M. Nagai, M. Nakajima, M. Hayashi, J. Nakanishi, Genotoxicity evaluation of fullerene C<sub>60</sub> nanoparticles in a comet assay using lung cells of intratracheally instilled rats, *Regul. Toxicol. Pharmacol.* 62 (2012) 419-424.
- [19] N.A. Monteiro-Riviere, A.O. Inman, L.W. Zhang, Limitations and relative utility of screening assays to assess engineered nanoparticle toxicity in a human cell line, *Toxicol. Appl. Pharmacol.* 234 (2009) 222-235.
- [20] N.A. Monteiro-Riviere, S.J. Oldenburg, A.O. Inman, Interactions of aluminum nanoparticles with human epidermal keratinocytes, *J. Appl. Toxicol.* 30 (2010) 276-285.
- [21] T. Mosmann, Rapid colorimetric assay to cellular growth and survival: application to proliferation and cytotoxicity assays, *J. Immunol. Methods* 65 (1983) 55-63.
- [22] E. Borenfreund, J.A. Puerner, Toxicity determined *in vitro* by morphological alterations and neutral red absorption, *Toxicol. Lett.* 24 (1985) 119-124.

- [23] OECD, Organisation for Economic Co-operation and Development. Guideline for Testing of Chemicals: *In Vitro* 3T3 NRU phototoxicity test, No. 432, 2004.
- [24] M.M. Bradford, A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* 72 (1976) 248-254.
- [25] M. Ramezani, M. Khoshhamdam, A. Dehshahri, B. Malaekheh-Nikouei, The influence of size, lipid composition and bilayer fluidity of cationic liposomes on the transfection efficiency of nanolipoplexes, *Coll. Surf. B: Biointerf.* 72 (2009) 1-5.
- [26] J. Ding, C. Xiao, C. He, M. Li, D. Li, X. Zhuang, X. Chen, Facile preparation of a cationic poly(amino acid) vesicle for potential drug and gene co-delivery, *Nanotechnology* 22 (2011) 1-9.
- [27] G. Cevc, Lipid vesicles and other colloids as drug carriers on the skin, *Adv. Drug Deliv. Rev.* 56 (2004) 675-711.
- [28] A. Colomer, A. Pinazo, T. Garcia, M. Mitjans, P. Vinardell, M.R. Infante, V. Martínez, L. Pérez, pH sensitive surfactants from lysine: assessment of their cytotoxicity and environmental behavior, *Langmuir* 28 (2012) 5900-5912.
- [29] D.R. Nogueira, M. Mitjans, M.C. Morán, L. Pérez, M.P. Vinardell, Membrane-destabilizing activity of pH-responsive cationic lysine-based surfactants: role of charge position and alkyl chain length, *Amino Acids* 43 (2012) 1203-1215.
- [30] D.R. Nogueira, M. Mitjans, M.A. Busquets, L. Pérez, M.P. Vinardell, Phospholipid bilayer-perturbing properties underlying lysis induced by pH-sensitive lysine-based surfactants in biomembranes, *Langmuir* 28 (2012) 11687-11698.
- [31] J.Y. Park, H. Choi, J.S. Hwang, J. Kim, I.S. Chang, Enhanced depigmenting effects of N-glycosylation inhibitors delivered by pH-sensitive liposomes into HM3KO melanoma cells, *J. Cosmet. Sci.* 59 (2008) 139-150.
- [32] P. Venkatesan, N. Puvvada, R. Dash, B.N.P. Kumar, D. Sarkar, B. Azab, A. Pathak, S.C. Kundu, P.B. Fisher, M. Mandal, The potential of celecoxib-loaded hydroxyapatite-chitosan nanocomposite for the treatment of colon cancer, *Biomaterials* 32 (2011) 3794-3806.
- [33] M.Horie, H. Kato, K.Fujita, S. Endoh, H. Iwahashi, *In vitro* evaluation of cellular response induced by manufactured nanoparticles, *Chem. Res. Toxicol.* 25 (2012) 605-619.

- [34] L. Di Marzio, C. Marianecchi, M. Petrone, F. Rinaldi, M. Carafa, Novel pH-sensitive non-ionic surfactant vesicles: comparison between Tween 21 and Tween 20, *Coll. Surf. B Biointerf.* 82 (2011) 18-24.
- [35] V.A. Ojogun, H.-J. Lehmler, B.L. Knutson, Cationic-anionic vesicle templating from fluorocarbon/fluorocarbon and hydrocarbon/fluorocarbon surfactants, *J. Coll. Interf. Sci.* 338 (2009) 82-91.
- [36] A. Mehrotra, R.C. Nagarwal, J.K. Pandit, Lomustine loaded chitosan nanoparticles: characterization and in-vitro cytotoxicity on human luna cancer cell line L132, *Chem. Pharm. Bull.* 59 (2011) 315-320.
- [37] W. Bai, Z. Zhang, W. Tian, X. He, Y. Ma, Y. Zhao, Z. Chai, Toxicity of zinc oxide nanoparticles to zebrafish embryo: a physicochemical study of toxicity mechanism, *J. Nanopart. Res.* 12 (2009) 1645-1654.
- [38] A. Maza, L. Coderch, P. Gonzalez, J.L. Parra, Subsolubilizing alterations caused by alkyl glucosides in phosphatidylcholine liposomes, *J. Control. Release* 52 (1998) 159-168.
- [39] L. Sanchez, M. Mitjans, M.R. Infante, M.P. Vinardell, Assessment of the potential skin irritation of lysine-derivative anionic surfactants using mouse fibroblasts and human keratinocytes as an alternative to animal testing, *Pharm. Res.* 21 (2004) 1637-1641.
- [40] H. Sakaguchi, T. Ashikaga, M. Miyazawa, Y. Yoshida, Y. Ito, K. Yoneyama, M. Hirota, H. Itagaki, H. Toyoda, H. Suzuki, Development of an in vitro skin sensitization test using human cell lines; human Cell Line Activation Test (h-CLAT) II. An inter-laboratory study of the h-CLAT, *Toxicol. in Vitro* 20 (2006) 774-784.
- [41] D.R. Nogueira, M. Mitjans, M.R. Infante, M.P. Vinardell, Comparative sensitivity of tumor and non-tumor cell lines as a reliable approach for *in vitro* cytotoxicity screening of lysine-based surfactants with potential pharmaceutical applications, *Int. J. Pharm.* 420 (2011) 51-58.
- [42] T. Xia, M. Kovichich, M. Liong, J.I. Zink, A.E. Nel, Cationic polystyrene nanosphere toxicity depends on cell-specific endocytic and mitochondrial injury pathways, *ACS Nano* 2 (2008) 85-96.
- [43] B.J. Marquis, S.A. Love, K.L. Braun, C.L. Haynes, Analytical methods to assess nanoparticle toxicity. *Analyst* 134 (2009) 425-439.

- [44] N. Schöler, C. Olbrich, K. Tabatt, R.H. Müller, H. Hahn, O. Liesenfeld, Surfactant, but not the size of solid lipid nanoparticles (SLN) influences viability and cytokine production of macrophages, *Int. J. Pharm.* 221 (2001) 57-67.
- [45] T. Benavides, V. Martínez, M. Mitjans, M.R. Infante, M.C. Morán, P. Clapés, R. Clothier, M.P. Vinardell, Assessment of the potential irritation and photoirritation of novel amino acid-based surfactants by *in vitro* methods as alternative to the animal tests, *Toxicology* 201 (2004) 87-93.
- [46] R. Clothier, A. Willshaw, H. Cox, M. Garle, H. Bowler, R. Combes, The use of human keratinocytes in the EU/COLIPA international *in vitro* phototoxicity test validation study and the ECVAM/COLIPA study on UV filterchemicals, *ATLA* 27 (1999) 247-259.
- [47] M.P. Vinardell, T. Benavides, M. Mitjans, M.R. Infante, P. Clapés, R. Clothier, Comparative evaluation of cytotoxicity and phototoxicity of mono and diacylglycerol amino acid-based surfactants, *Food Chem. Toxicol.* 46 (2008) 3837-3841.
- [48] L. Allermann, O.M. Poulsen, Interleukin-8 secretion from monocytic cell lines for evaluation of the inflammatory potential of organic dust, *Environm. Res.* 88 (2002) 188-198.
- [49] M. Mitjans, B. Viviani, L. Lucchi, C.L. Galli, M. Marinovich, E. Corsini, Role of p38 MAPK in the selective release of IL-8 induced by chemical allergen in naïve THP-1 cells. *Toxicol. In Vitro* 22 (2008) 386-395.
- [50] E. Rytting, M. Bur, R. Cartier, T. Bouyssou, X. Wang, M. Krüger, C.-M. Lehr, T. Kissel, *In vitro* and *in vivo* performance of biocompatible negatively-charged salbutamol-loaded nanoparticles, *J. Control. Release* 141 (2010) 101-107.
- [51] J.N. Barker, R.S. Mitra, C.E. Griffiths, V.M. Dixit, B.J. Nickoloff, Keratinocytes as initiators of inflammation, *Lancet* 337 (1991) 211-214.
- [52] V. Martínez, E. Corsini, M. Mitjans, A. Pinazo, M.P. Vinardell, Evaluation of eye and skin irritation of arginine-derivative surfactants using different *in vitro* endpoints as alternatives to the *in vivo* assays, *Toxicol. Lett.* 164 (2006) 259-267.

**Figure captions:**

**Fig. 1.** TEM images of cationic nanovesicles (a) DMPC:MKM (80:20, molar ratio), (b) DMPC:CHOL:MKM (56:24:20, molar ratio), (c) DMPC:MLM (80:20, molar ratio), (d) DMPC:CHOL:MLM (56:24:20, molar ratio), (e) DMPC:PKM (80:20, molar ratio), (f) DMPC:CHOL:PKM (56:24:20, molar ratio). Scale bars correspond to 100 nm.

**Fig. 2.** Cell viability measured by the (a,b) MTT, (c,d) NRU and (e,f) LDH assays on (a,c,e) 3T3 and (b,d,f) HaCaT cell lines. Each cell line was exposed to increasing concentrations of the nanovesicle formulations, ranging from 0.5 to 100  $\mu$ M. Results are given as a percentage of untreated control cells. The nanovesicles did not induce loss of cell viability when concentrations were lower than 10  $\mu$ M, as determined by the LDH assay (data not shown). Results are expressed as mean  $\pm$  SEM of three independent experiments, performed in triplicate.

**Fig. 3.** Cytotoxicity and phototoxicity induced by the nanovesicle formulations expressed as IC<sub>50</sub> values on 3T3 and HaCaT cell lines. Values were obtained from MTT and NRU assays. Black bars = non-irradiated cells and white bars = UVA-irradiated cells (2.5 J/cm<sup>2</sup>). The photoirritation-factor (PIF) was calculated as described in Section 1.9. Results are expressed as mean  $\pm$  SEM of three independent experiments, performed in triplicate. Statistical analyses were performed using the Student's *t*-test. \*  $p < 0.05$ , \*\*  $p < 0.005$  denote significant differences.

**Fig. 4.** IL-8 release (bars) induced by THP-1 cells with increasing concentrations of each nanovesicle formulation. Cell viability (line) was determined by MTT assay and is expressed as percentage of control. Results are expressed as mean  $\pm$  SEM of three independent experiments, performed in triplicate. Statistical analyses were performed using ANOVA followed by Dunnett's multiple comparison test. \*  $p < 0.05$ , \*\*  $p < 0.005$  denote significant differences. Different scale bars were used to express the results better.

**Fig. 5.** (a) Cell-associated IL-1 $\alpha$  (pg/mg protein) and (b) IL-1 $\alpha$  release (pg/ml) to the culture medium by HaCaT cells with increasing concentrations of each nanovesicle formulation. The concentrations tested were 1  $\mu$ M (white bars), 2.5, 5, 10, 25, 50 and 100  $\mu$ M (black bars). Results are expressed as mean  $\pm$  SEM of three independent experiments, performed in triplicate. Statistical analyses were performed using ANOVA followed by Dunnett's multiple comparison test. \*  $p < 0.05$ , \*\*  $p < 0.005$  denote significant differences.

**Table 1.** Characterization parameters of the different cationic nanovesicles.

	DMPC:MKM (80:20)	DMPC:CHOL:MKM (56:24:20)	DMPC:PKM (80:20)	DMPC:CHOL:PKM (56:24:20)	DMPC:MLM (80:20)	DMPC:CHOL:MLM (56:24:20)
	<i>Size (nm) ± SEM<sup>a</sup></i>					
t = 0 h water	94.16 ± 2.05	107.33 ± 0.94	253.07 ± 26.05	184.77 ± 6.64	174.40 ± 7.16	127.50 ± 1.96
t = 0 h DMEM 5% FBS	94.42 ± 6.50	159.77 ± 5.71	229.37 ± 12.64	197.73 ± 6.57	229.63 ± 16.33	170.27 ± 9.49
t = 24 h DMEM 5% FBS <sup>b</sup>	1781.67 ± 45.72/ 110.3 ± 5.17	2028.67 ± 21.23/ 154.33 ± 11.95	1059.50 ± 10.61/ 118.93 ± 2.09	1488 ± 19.59/ 125.67 ± 11.94	193.47 ± 7.75	151.77 ± 4.44
	<i>PDI ± SEM<sup>a</sup></i>					
t = 0 h water	0.231 ± 0.004	0.278 ± 0.019	0.427 ± 0.017	0.331 ± 0.020	0.394 ± 0.003	0.256 ± 0.006
t = 0 h DMEM 5% FBS	0.385 ± 0.015	0.236 ± 0.001	0.522 ± 0.001	0.288 ± 0.001	0.445 ± 0.007	0.319 ± 0.029
t = 24 h DMEM 5% FBS <sup>b</sup>	0.903 ± 0.075	1.00 ± 0.000	0.605 ± 0.002	0.973 ± 0.027	0.294 ± 0.001	0.352 ± 0.007
	<i>Zeta potential (mV) ± SEM<sup>a</sup></i>					
t = 0 h water	42.7 ± 0.90	41.2 ± 1.66	52.8 ± 1.15	55.17 ± 0.67	78.7 ± 2.56	44.9 ± 0.40
t = 0 h DMEM 5% FBS	1.23 ± 1.64	-3.13 ± 0.81	6.78 ± 0.94	0.86 ± 0.11	13.00 ± 0.49	8.61 ± 0.16
	<i>% incorporation of surfactant into NVs ± SEM<sup>a</sup></i>					
	88.56 ± 0.009	75.96 ± 0.054	99.10 ± 0.007	98.96 ± 0.006	90.85 ± 0.053	79.05 ± 0.038

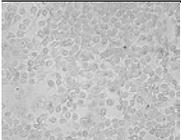
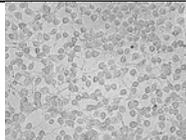
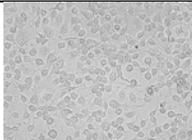
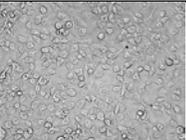
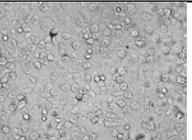
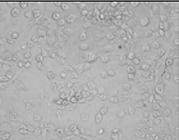
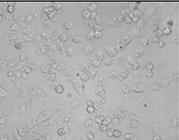
<sup>a</sup> Mean of three experiments ± standard error of the mean (SEM).

<sup>b</sup> Incubated under cell culture conditions: 37°C, 5% CO<sub>2</sub>.

**Table 2.** Cytotoxicity of the nanovesicle formulations expressed as IC<sub>50</sub> values (μM) on 3T3, HaCaT and THP-1 cell lines.

Nanovesicle formulation	IC <sub>50</sub> (μM) <sup>a</sup>						
	3T3			HaCaT			THP-1
	MTT	NRU	LDH	MTT	NRU	LDH	MTT
DMPC:MKM	28.16	71.14	60.01	8.03	162.22	129.43	13.92
DMPC:CHOL:MKM	31.05	72.59	79.82	19.51	191.32	141.35	21.18
DMPC:PKM	3.76	41.62	33.24	2.12	47.68	46.94	12.31
DMPC:CHOL:PKM	3.86	43.88	35.56	2.26	70.31	46.34	11.73
DMPC:MLM	36.41	39.23	32.23	65.66	64.32	84.42	89.41
DMPC:CHOL:MLM	39.11	42.96	36.14	73.25	71.41	77.53	102.48

Cell morphology – 3T3 <sup>b</sup>						
Control	DMPC:MKM	DMPC:CHOL: MKM	DMPC:PKM	DMPC:CHOL: PKM	DMPC:MLM	DMPC:CHOL: MLM
						

<sup>a</sup> Mean of three independent experiments.

<sup>b</sup> Induced by treatment with the IC<sub>50</sub> determined by MTT assay. Morphological changes was analysed under a phase contrast microscope.

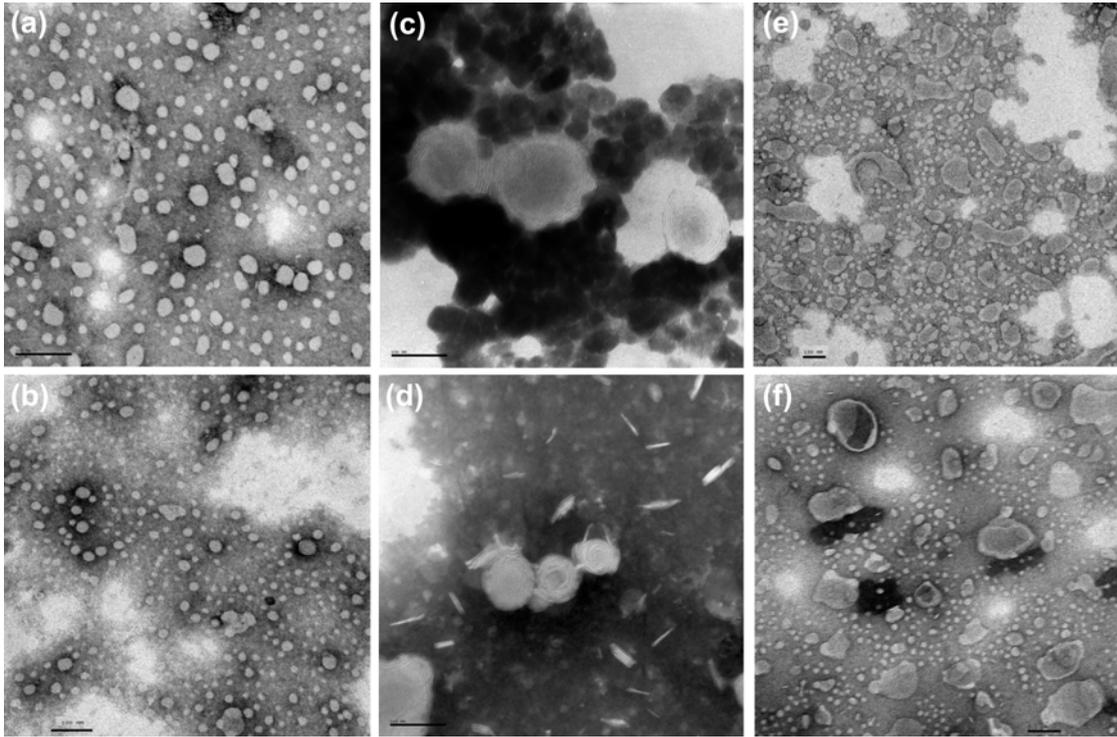


Fig. 1

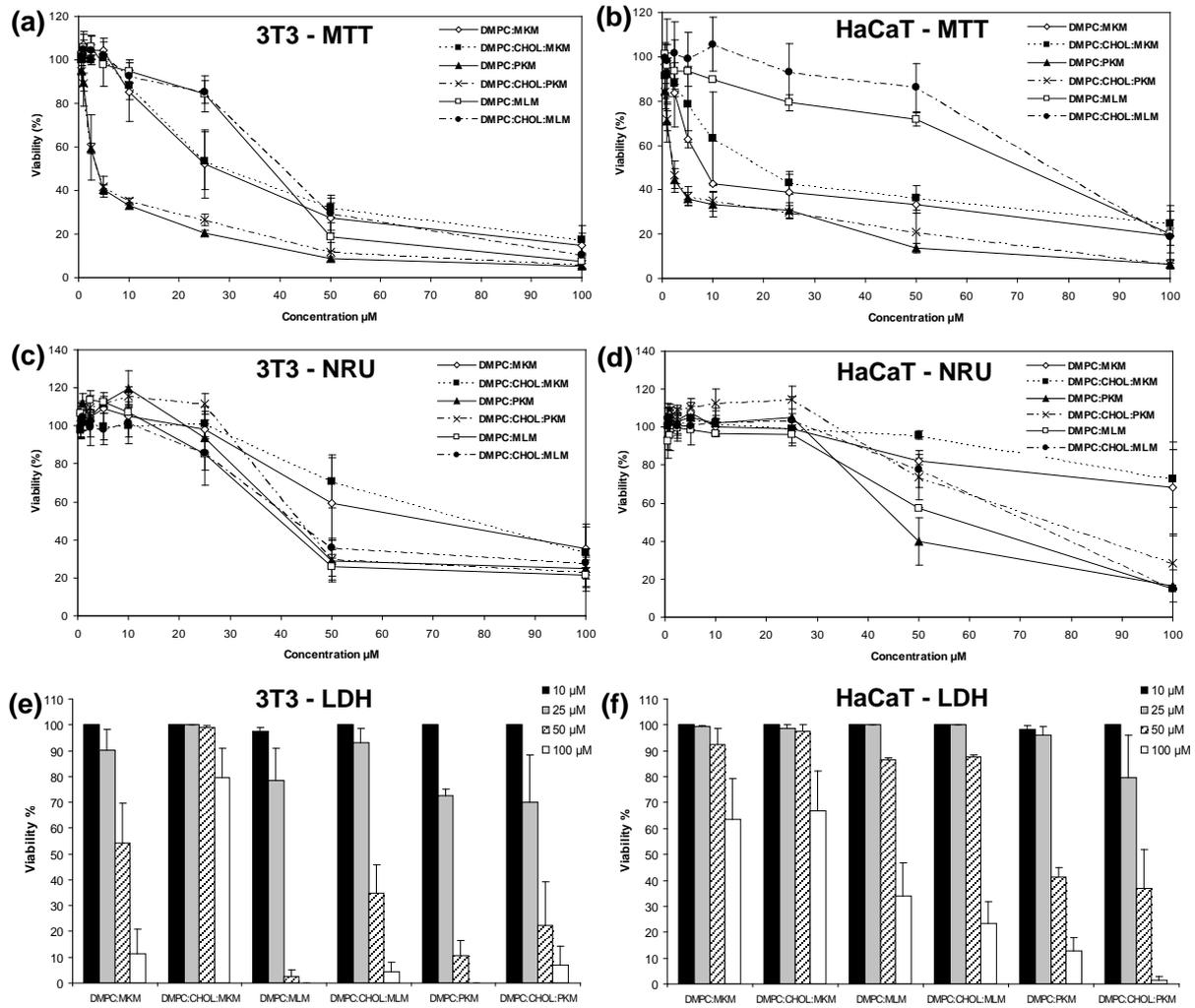


Fig. 2

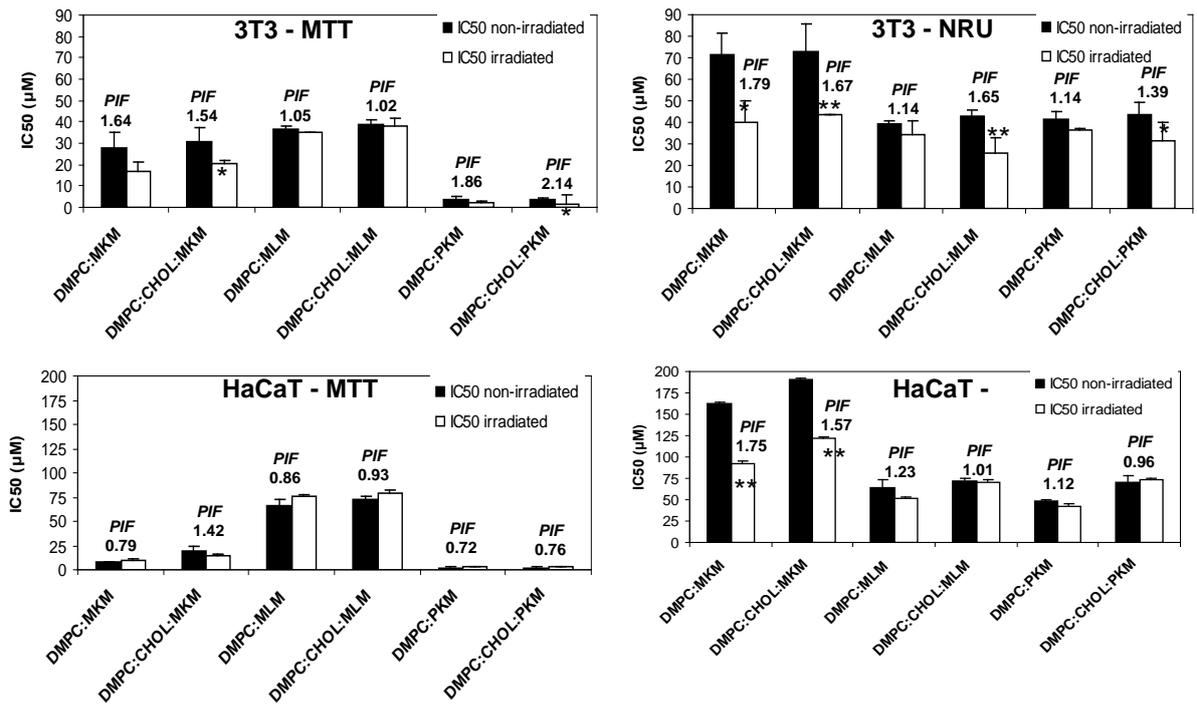


Fig. 3

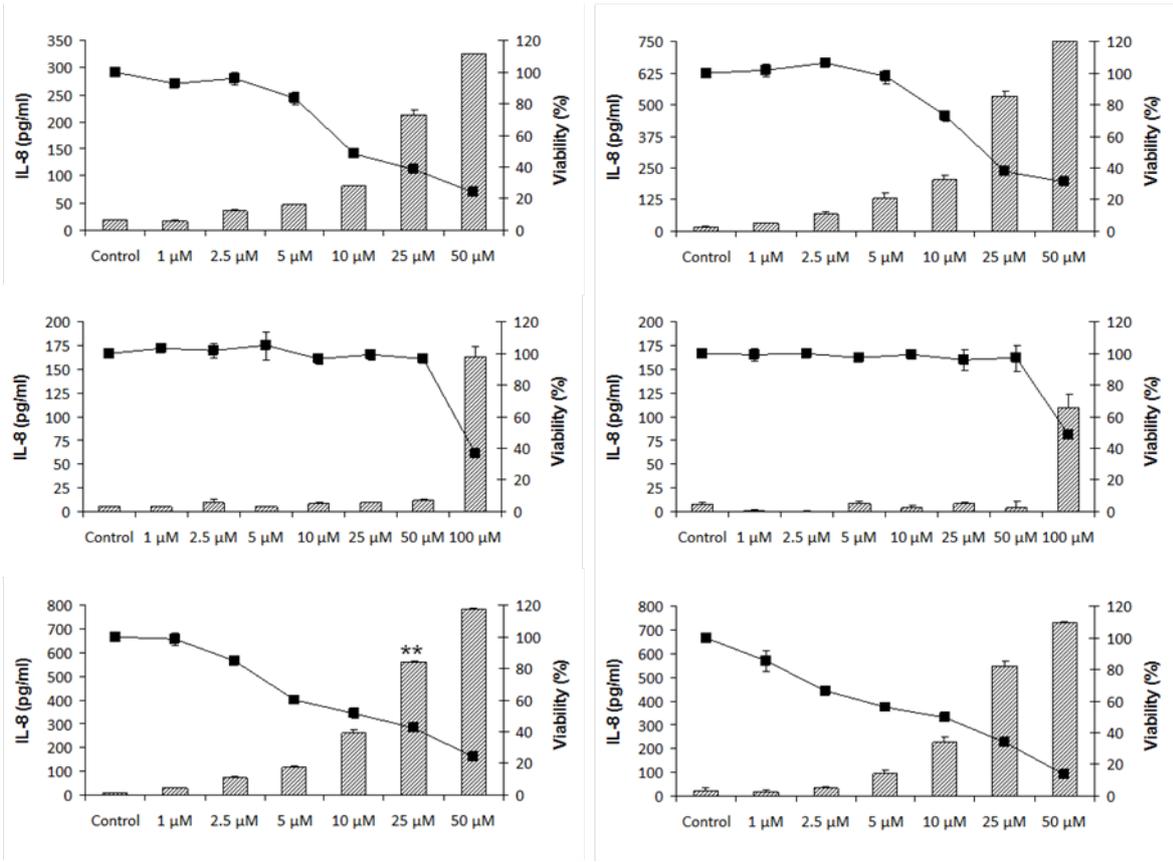


Fig. 4

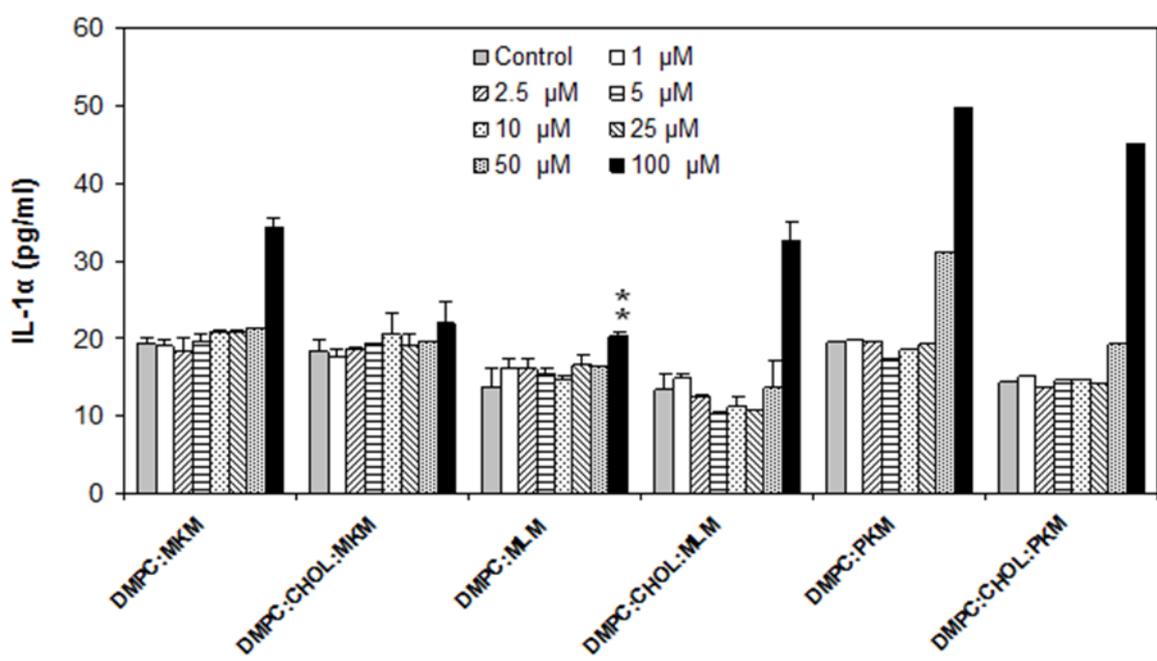
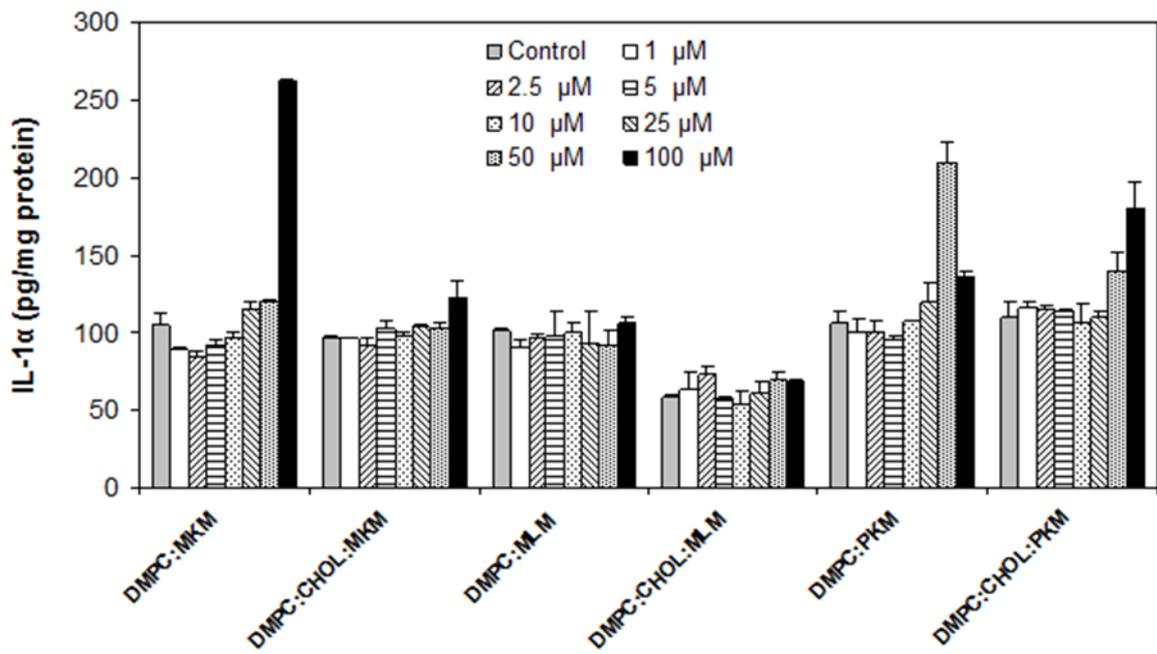


Fig. 5