Applied Clay Science 168 (2019) 382–395

Contents lists available at ScienceDirect

Applied Clay Science

journal homepage: www.elsevier.com/locate/clay

Research Paper

Folic acid-layered double hydroxides hybrids in skin formulations: Technological, photochemical and in vitro cytotoxicity on human keratinocytes and fibroblasts

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ARTICLE INFO

Keywords:
Folic acid
Layered double hydroxides
Photochemistry
Cytotoxicity
Keratinocytes
Fibroblasts

ABSTRACT

Folic acid (FA) is a key factor in the physiological processes of cell metabolism; as it is involved in DNA synthesis and repair, it can be used in the treatment of aged and photo-damaged skin. The main drawbacks associated to FA use, particularly for topical applications, are the limited solubility and the sensitivity to UV rays.

Hybrids of FA with two kinds of layered double hydroxides (ZnAl-LDH and MgAl-LDH) were prepared and characterized showing suitable particle size, flow properties and UV photoprotection. The introduction of FA-LDHs in the external water phase of O/W emulgels produced an enhancement of their flow properties; moreover, the spectrophotometric analyses showed that very good photostability is maintained even after their introduction into the formulations. In-vitro release studies showed that the FA-LDH containing emulgels promoted a sustained FA release as expected. Finally, the safety of FA-LDH was evaluated by in-vitro studies performed on human keratinocytes (HaCaT) and human primary dermal fibroblasts (as a skin representative). The obtained results showed a high cytotoxic effect of ZnAl-LDH-FA in both cell lines.

1. Introduction

Due to its involvement in DNA synthesis and repair, folic acid (FA) topical applications have shown remarkable effects for the treatment of aged and photo-damaged skin (Crider et al., 2012; Ammar et al., 2016; Watson et al., 2018). Debowska et al. observed a fast cellular repair after incubating skin cells carrying damaged DNA with FA. Moreover, a second study demonstrated that UV induced apoptosis was prevented in presence of FA (Debowska et al., 2005; Debowska et al., 2006; Knott et al., 2008).

Many topical formulations containing FA are available on the market for the treatment of photo-damaged or aged skin; however, the aspect of FA UV susceptibility often is not taken into account. Ultraviolet exposure of FA generates two reactive photoproducts: 2-amino-4-hydroxy-6-formyl pteridine (pterine-6-carboxaldehyde) and p-aminobenzoyl-L-glutamic acid (Off et al., 2005; Juzeniene et al., 2016). After prolonged exposure time, the pterine-6-carboxaldehyde is converted into the corresponding 6-carboxylic acid (pterine-6-carboxylic acid, PCA) and then into the decarboxylated 2-amino-4-hydroxy pteridine (Juzeniene et al., 2016). Pterine-6-carboxaldehyde and PCA are able to induce the production of reactive oxygen species, which is responsible for cytotoxicity and DNA damages (Off et al., 2005; Butzbach and Bernd, 2013).

The very low FA solubility (1.6 mg/L) (Hofsäss et al., 2017) makes difficult its homogeneous dispersion in hydrophilic solvents; thus, surfactants, co-surfactants or co-solvents must be employed to obtain a homogeneous formulation. Ethanol is commonly used as co-solvent; however, because of its volatility at room temperature, it may quickly evaporate causing crystallization of the active ingredient. For this reason, this approach, although widely used in the industry, is unsuitable for our purposes. As a result, UV susceptibility, combined with low solubility, limits the FA effectiveness when formulated in topical products.

It is therefore necessary to find a technological approach to protect FA from UV rays enhancing the workability, the efficacy and the safety of its topical use.

The aim of this work is to enhance FA dispersibility/workability and stability to UV rays, and to overcome the aforementioned safety issues.

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https://doi.org/10.1016/j.clay.2018.12.009
Received 15 June 2018; Received in revised form 14 November 2018; Accepted 9 December 2018
Available online 13 December 2018

The final purpose is to offer a suitable alternative to the conventional formulations based on FA actually available on the market.

In this work, we report how inorganic-organic hybrids MgAl-LDH-FA and ZnAl-LDH-FA were used as FA sources to prepare innovative topical O/W emulgels.

Lamellar double hydroxides (LDH) are anionic layered clays containing exchangeable anions; their lamellae are constituted by bivalent (Mg^{2+} or Zn^{2+} in the LDH used) and trivalent (Al^{3+}) metal cations occupying the centers of octahedra whose vertices contain hydroxide ions. Each OH group is shared by three octahedral cations and the hydrogen points towards the interlayer region similar to brucite, $Mg(OH)_2$ (Trifirò and Vaccari, 1996). Considering FA drawbacks (UV susceptibility, poor solubility), the use of LDH as carrier was considered a suitable strategy. In fact, LDH are very interesting materials known for their ability to: i) photoprotect molecules susceptible to UV rays, ii) improve the dissolution rate of poor soluble molecules, iii) improve the rheological properties of semisolid formulations (Conterosito et al., 2013; Perioli et al., 2015; Arrigo et al., 2018).

Thus, the UV stability and rheological properties of the FA-LDH emulgels were studied using free crystalline FA emulgel as control. Moreover, the aspect of safety was evaluated by in-vitro studies on human keratinocytes and dermal fibroblasts as representative of skin.

2. Materials and methods

2.1. Materials

FA and glycerol were purchased from A.C.E.F. (Fiorenzuola D'Arda, Italy). Lactic acid (Carlo Erba, Cornaredo, Milano, Italy) was supplied by DueM (Perugia, Italy). Liquid paraffin and vaseline (Caelo, Hilden, North Rhine Westphalia, Germany) were purchased from Comifar (Perugia, Italy). Hydroxyethylcellulose (HEC), Natrosol® 250 HX, was supplied by Hercules incorporated - Aqualon division (Wilmington, DE, USA). Hydroxypropyl methylcellulose (HPMC, viscosity 2600–5600 mPa·s) was supplied by Sigma Aldrich (Milano, Italy). Gelucire®50/13 and Transcutol®P were a gift from Gattefossé SAS (Saint Priest, France). Cetostearyl alcohol was purchased from Galeno (Carmignano, Italy). Ultrapure water was obtained by reverse osmosis process in a MilliQ system Millipore (Rome, Italy). Other reagents and solvents were of analytical grade and were used without further purification.

The fluids used in the release studies were:

(a) synthetic sweat phosphate buffer solution at pH 5.5 (adjusted by adding ammonia solution 30% dilution 1:100) composed by sodium chloride 0.5%, urea 0.1% and lactic acid 0.1% (Colin et al., 1999); the percentages are expressed in mass and.
(b) K_2CO_3 solution 0.025 N (Nakayama et al., 2004).

For cell-based assays, Dulbecco's Modified Eagle's Essential Medium (DMEM), L-glutamine, trypsin, ethylenediaminetetraacetic acid disodium and tetrasodium salt (EDTA) were obtained from Microtech Srl (Pozzuoli, NA, Italy). Fetal Bovine Serum (FBS), dimethyl sulfoxide (DMSO) and penicillin-streptomycin were instead obtained from Thermo Fisher Scientific (Waltham, MA, USA). 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich Srl (St. Louis, MO, USA).

2.2. Hybrids preparation and characterization

The hybrids MgAl-LDH-FA and ZnAl-LDH-FA were prepared as follows: FA water solutions (alkalinized by NaOH 1 M until pH 8.0), free from carbonate ions, were prepared. FA and LDHs were used in the molar ratio LDH:FA 1:2. Pristine LDHs, MgAl-LDH-NO_3 and ZnAl-LDH-NO_3, obtained by coprecipitation method and urea respectively (Pagano et al., 2016), were dispersed in the solutions. The obtained suspensions were stirred vigorously at 600 rpm, 60 °C for 3 and 4 days (MgAl-LDH-FA and ZnAl-LDH-FA respectively). After this time the solids were recovered by centrifugation, washed and dried in a heater at 37 °C for 24 h.

Metal analyses were performed with a Varian 700-ES series Inductively Coupled Plasma-Optical Emission Spectrometers (ICP-OES).

Coupled thermogravimetric and differential thermal analyses were performed with a Netzsch STA 449C apparatus, in air flow and heating rate of 10 °C/min to determine the weight loss (water and drug) as a function of increasing temperature.

Carbon, hydrogen and nitrogen elemental microanalyses were performed by Elemental UNICUBE analyzer using sulfanilamide as a reference standard with an accuracy of ca. 2 mmol g^{-1}.

The content of FA in the hybrids was measured starting from a solution obtained with 10 mg of each sample solubilized in 2 drops of HNO_3 conc. then diluted with distilled water until 10 ml. The concentration of FA was measured by:

- UV–vis spectrophotometry (UV–Visible Agilent model 8453) using a standard curve in water (λ_{max} = 290.0 nm, r = 0.9998),
- HPLC using the method reported in the monograph of Folic Acid in the European Pharmacopoeia 9.0th edition (Ph. Eur. 9.0th Ed. 2017a): stationary phase: column Zorbax SB C18 4.6 × 250 mm 5 µm (Agilent, Santa Clara, CA, United States), mobile phase: methanol/water solution of potassium dihydrogen phosphate (11.16 g/l) and dipotassium phosphate (5.50 g/l) 12/88 *v*/v, isocratic flow, flow rate: 1 ml/min; detection: UVspectrophotometer at 280.0 nm, retention time: 8.50 min.

Based on these analyses the formulas of the compounds are:

$[Mg_{0.61}Al_{0.39}(OH)_2](FA)_{0.123}(NO_3)_{0.144}$ * 1.4 H_2O MW = 147.63 g/mol

Anal calc.: C 19.00, N 9.53, H 4.66, Mg 9.92, Al 7.13%

Found.: C 22.00, N 9.97, H 4.47, Mg 10.31, Al 7.50%.

FA Loading: 36.6% g/g.

$[Zn_{0.66}Al_{0.34}(OH)_2](FA)_{0.104}(NO_3)_{0.132}$ * 1.2 H_2O MW = 161.63 g/mol

Anal calc.: C 14.67, N 7.45, H 3.82, Zn 26.70, Al 5.68%

Found.: C 16.58, N 8.16, H 3.65, Zn 26.41, Al 5.50%.

FA Loading: 28.3% g/g.

SEM micrographs were registered by FE-SEM LEO 1525 Zeiss - LEO Electron Microscopy Inc., One Zeiss Drive (Thornwood, NY). Powder samples were prepared by deposition on the stab under nitrogen flow. The raw powder was not sifted. Before observation, samples were sputtered and coated with chromium for 5 min under an Argon atmosphere.

The sample size distribution was measured by an Accusizer C770 (PSS Inc., Santa Barbara, CA, USA) using the technique “Single Particle Optical Sensing”. The size was expressed as cumulative area distribution percentage. D10, D50, and D90 are defined as the size values corresponding to cumulative distributions at 10%, 50%, and 90%, respectively.

The flow properties of the samples were determined by C.I. (Eq. (1)) and H.R. (Eq. (2)) calculation following the procedure reported in the Ph. Eur. 9.0th Ed. (par. 2.9.34) by the determination of the apparent volume before and after powder settling (ERWEKA SVM 101/201).

$$C.\,I. = (V_0 - V_f)/V_0 * 100 \quad (1)$$

$$H.\,R. = V_0/V_f \quad (2)$$

where V_0 = initial volume and V_f = final volume.

Table 1
Composition of the prepared emulgels.

		Emulgel*				
Ingredients (g)		Control	1	2	3	4
Water phase (W)	FA	0.50	0.50	0.50	–	–
	MgAl-LDH-Cl	–	5.00	–	3.63	–
	ZnAl-LDH-Cl	–	–	5.00	–	3.23
	MgAl-LDH-FA	–	–	–	1.37	–
	ZnAl-LDH-FA	–	–	–	–	1.77
	HPMC	2.00	2.00	2.00	2.00	2.00
	Glycerol	5.00	5.00	5.00	5.00	5.00
	Ultrapure water	64.30	59.30	59.30	59.80	59.80
Oil phase (O)	Gelucire®50/13	15.00	15.00	15.00	15.00	15.00
	cetostearyl alcohol	7.20	7.20	7.20	7.20	7.20
	Transcutol®P	6.00	6.00	6.00	6.00	6.00

Emulgel 1: Physical mixture FA/MgAl-LDH-Cl.
Emulgel 2: Physical mixture FA/ZnAl-LDH-Cl.
Emulgel 3: MgAl-LDH-FA.
Emulgel 4: ZnAl-LDH-FA.
* Emulgel control: crystalline FA.

2.3. Photostability studies

The photochemical behavior and solidity of the specimens were spectrophometrically investigated. Absorption spectra of the powder and emulgel samples were recorded by a Varian (Cary 4000) spectrophotometer, equipped with a 150-mm integration sphere; a barium sulfate tablet was used as reference. Solutions spectra were registered by a spectrophotometer PERKIN ELMER UV/VIS lambda 10. The spectra were analyzed by the Kubelka-Munk equation.

Steady-state irradiation experiments were performed using a Xenon lamp (150 W) as light source, the irradiation wavelength (340 nm for the solutions and 440 nm for powders and emulgels) was selected by a monochromator and a band-pass slit of 35 nm was used. The spectra analysis was carried out at different irradiation times.

2.4. Emulgel manufacturing

The hybrids were dispersed in the external water phase of an O/W emulgel having the following composition: Oil phase (O): Gelucire® 50/13 (15%), Transcutol®P (6%), cetostearyl alcohol (7.2%). Water phase (W): HPMC (2%) hydrogel (for the exact composition see Table 1). Emulgels were loaded with FA (in crystalline form or as hybrid) in order to get a final content of 0.5%.

For emulgels 3 and 4 containing the hybrids (Table 1), the amount was calculated according to FA loading values. For all the formulations, in order to maintain the same total amount of LDH in the water phase (Table 1), MgAl-LDH-Cl or ZnAl-LDH-Cl were added to reach the final content of 5%. Their use was preferred to MgAl-LDH-NO_3/ZnAl-LDH-NO_3 (used as precursor in the intercalation procedure) because of safety concerns involving nitrates (Santamaria, 2006).

Emulgels were prepared as follows:

Gelucire®50/13, cetostearyl alcohol and Transcutol®P, representing the oil internal phase (O), were melt in a steam bath. The water external phase (W) was prepared in another flask by adding the polymer (HPMC) to a glycerol-water solution free from carbonate ions, which was held in a steam bath and kept under mechanical stirring (500 rpm). Upon complete polymer hydration and water gelation, the resulting hydrogel was used as the water phase (W), to which the oil phase (O) was added under mechanical stirring (500 rpm); the mixture was maintained in these conditions until complete cooling (room temperature). Folic Acid (in one of the three forms explained in Table 1) was added to HPMC, well mixed by using mortar and pestle, finely dispersed into the water phase (hydrogel), then emulsified with the oil phase to obtain the hydrophilic O/W emulgel.

2.5. In vivo evaluation of the formulation skin-feel

The studies were performed (in triplicate) by applying emulgels containing LDH (free from FA) on the face skin of three healthy volunteers. Each of them was asked to make a judgment with a score about (i) pleasant or unpleasant sensation during and after application, (ii) physical appearance, and (iii) greasiness degree.

2.6. X-ray analysis

Powder X-ray diffraction patterns (PXRD) of samples were collected with a Philips X'Pert PRO MPD diffractometer operating at 40 kV and 40 mA, with a step size 0.03° 2theta, and step scan 40 s, using Cu Kα radiation and an X'Celerator detector.

2.7. Rheological measurements

Rheological measurements were performed by means of a controlled stress rheometer Stresstech HR (Reologica Instruments, AB Milan, Italy) equipped with a cone-plate geometry (diameter 40 mm, angle 1°). Samples were applied to the lower plate using a spatula to ensure that formulation shearing did not occur. The shear stress range investigated was 1–100 Pa.

The viscosity was measured at 32 °C in order to evaluate the formulation flow properties at skin surface temperature. The extrudability of each emulgel was estimated by yield stress measurements performed at 25 °C (room temperature).

The experiments were performed in triplicate, each reported result represents the average of the three measurements.

2.8. Cytotoxicity assay

2.8.1. Cell culture

The human immortalized keratinocyte cell line, HaCaT, were purchased from I.Z.S.L.E.R. (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna) and used as a model to study the epidermal homeostasis and its pathophysiology. In-house established human primary dermal fibroblasts were kindly supplied by Dr. A. Dardis (Regional Coordinator Centre for Rare Diseases, AMC Hospital of Udine, Italy).

Both cell lines were cultured according to standard procedures in Dulbecco's modified Eagle's medium (DMEM), and supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 2 mM of L-glutamine and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin). The cells were maintained in a cell incubator at 37 °C in a humidified atmosphere containing 5% CO_2. When the cells reached 80–90% of confluence, the routine culture medium was aspirated, and the cells washed two times with PBS 1×. The cells were then harvested by 0.05% trypsin in 0.02% Na_4EDTA and suspended 1:6 for HaCaT and 1:3 for fibroblasts in supplemented growth medium to be maintained in the exponential growth phase. Both cell lines were tested for mycoplasma contamination before use. For the experiments, the cells were counted by using trypan blue dye exclusion assay, seeded and cultured in absence or presence of samples at different concentrations and for different times. All studies on human primary fibroblasts were carried out using cells from passages 4–12. In all experiments, untreated cells were used as negative controls.

2.8.2. Samples preparation

The powders, crystalline FA, MgAl-LDH-FA or ZnAl-LDH-FA (amount corresponding to 8.6 mg of FA), were dispersed in 5 ml of DMEM, vortexed and incubated at 37 °C for 24 h.

To obtain the solutions, after incubation, the samples were centrifuged for 10 min at 4000 rpm and the supernatant used for cells treatment.

To obtain the suspension, after incubation, the samples were

vortexed and then used for cells treatment.

2.8.3. Cell viability

Cellular viability was assessed by the reduction of MTT to formazan (Ceccarini et al., 2016a).

Human immortalized keratinocytes and human primary fibroblasts were seeded onto 96-well plate at a density of 3×10^3 cells/well and 1×10^4 cells/well, respectively, with DMEM complete medium. After 24 h, the medium was completely replaced with fresh one for the treatment (for 24 h) with different volumes of the supernatants obtained from each sample diluted with DMEM to get to the final solution volume of 180 µl. After 24 h the MTT reagent was dissolved in PBS 1 ×, and added to the culture at 0.5 mg/ml final concentration (20 µl). After 3 h of incubation at 37 °C, the supernatant was carefully removed and formazan salt crystals were dissolved in 200 µl of DMSO. After 30 min the absorbance (OD) values were measured spectrophotometrically at 540 nm using an automatic microplate reader (Eliza MAT 2000, DRG Instruments, GmbH). Each experiment was performed twice in triplicate. Cell viability was expressed as a percentage relative to that of the control cells as described previously (Ceccarini et al., 2016b).

2.9. In vitro release studies

For this investigation, a vertical Franz diffusion cell was used (PermeGear, Inc., Bethlehem, PA, USA, diameter 20 mm); it is formed by a water jacketed receptor chamber (15 ml), containing the synthetic sweat at pH 5.5. The receptor fluid was maintained at 32 °C and magnetically stirred at 600 rpm. The two chambers were separated by a cellulose membrane (Whatman 41, Whatman GmbH, Dassel, Germany) and the formulation (200 mg) was placed on top of the membrane in the upper donor chamber. 2 ml of a 0.025-N K_2CO_3 solution (simulating air CO_2) were placed into the donor chamber and then sealed with parafilm®. At regular time intervals (15, 30, 60, 120, 240, 360, 480 and 1440 min) samples of the receiving phase were withdrawn and FA content was determined by a UV–vis spectrophotometer (UV–Visible Agilent model 8453). The quantification of FA in each sample was accomplished using a standard curve constructed in synthetic sweat pH 5.5 (λ_{max} = 280.0 nm, r = 0.9998). All experiments were performed in triplicate, each result represents an average of three measurements and the error was expressed as standard deviation (SD).

3. Results and discussions

3.1. Hybrids preparation and characterization

At first, the solids MgAl-LDH-FA and ZnAl-LDH-FA were dimensionally and morphologically characterized. By comparing each starting material (MgAl-LDH-NO_3 and ZnAl-LDH-NO_3) to the corresponding hybrids, it can be asserted that the intercalation procedure does not induce substantial modifications of the particle size (Fig. SI1, Table 2).

The cumulative distributions reported in Fig. SI1 (Supplementary material) show that the ZnAl-LDH-FA's population is more homogeneous than the MgAl-LDH-FA's. However, the dimensional ranges D10-D90 measured for ZnAl-LDH-FA (2.90–11.30 µm) and MgAl-LDH-FA (2.90–17.29 µm) did not show any noteworthy difference (Table 2).

SEM micrographs showed a flat structure of MgAl-LDH-NO_3 crystals (Fig. SI2A and SI2B, Supplementary material) when modified by FA intercalation to produce MgAl-LDH-FA, to afford smaller crystals with irregular and rounded edges (Fig. SI2C, Supplementary material). For ZnAl-LDH-NO_3 crystals, a typical “desert like” structure can be observed (Fig. SI2D and SI2E, Supplementary material). Also in this case, the intercalation procedure is responsible for the modification of the crystals morphology characterized by rounded edges (Fig. SI2E, Supplementary material). Furthermore, the single crystals are still visible and separated in ZnAl-LDH-NO_3. Crystals of small dimensions can be detected in both cases; the two hybrids are suitable for formulation without any preventive grinding treatment. Due to the particles' very small size range (< 60 µm), the powder can be classified as very fine according to the Ph. Eur. 9.0th Ed. classification of powders by fineness (par. 2.9.35). Thus, it is reasonable to hypothesize that the hybrid powders could be considered non-abrasive and thus not tactilely perceived by the user during the application and skin massage.

Table 2
Dimensional data of pristine LDH and the corresponding hybrids.

Sample	Mode (µm)	D10 (µm)	D50 (median) (µm)	D90 (µm)
MgAl-LDH-NO_3	11.30	3.98	10.80	23.36
MgAl-LDH-FA	9.43	2.90	7.55	17.29
ZnAl-LDH-NO_3	5.47	3.00	5.23	8.23
ZnAl-LDH-FA	5.99	2.90	5.26	11.30

The flow properties of a powder influence its workability characteristics. In order to evaluate this aspect, the compressibility index (C.I.) and Hausner ratio (H.R.) of crystalline FA and the corresponding hybrids, ZnAl-LDH-FA and MgAl-LDH-FA, were calculated according to the procedure described in Ph. Eur. 9.0th Ed. For the raw material (crystalline FA), the measured parameters were: C.I. 50 and H.R. 2 identifying a powder with “very poor” flow character as classified in the Ph. Eur. 9.0th Ed. (Ph. Eur. 9.0th Ed. 2017b, par. 2.9.34) scale of flowability. The two hybrids showed a decrease of the parameter values resulting in the improvement of the flow character. With C.I. of 23 and H.R. of 1.33, ZnAl-LDH-FA was classified as “passable” Instead, with C.I. of 17.5 and H.R. of 1.21, MgAl-LDH-FA was classified as “fair”. These results suggested that, once intercalated into LDH, FA flow character improves influencing positively the workability of the powder allowing an easy and homogenous dispersion of the active ingredient in the formulation.

3.2. Photochemical characterization of the hybrids

FA is a photosensitive molecule; its exposure to light, especially in the presence of oxygen, induces the formation of two photoproducts, 6-formyl pteridine and p-aminobenzoyl-L-glutamic acid, that are responsible for cytotoxic damages (Hirakawa et al., 2003; Song and Hwang, 2007; Dántola et al., 2010). Folic Acid's photochemical

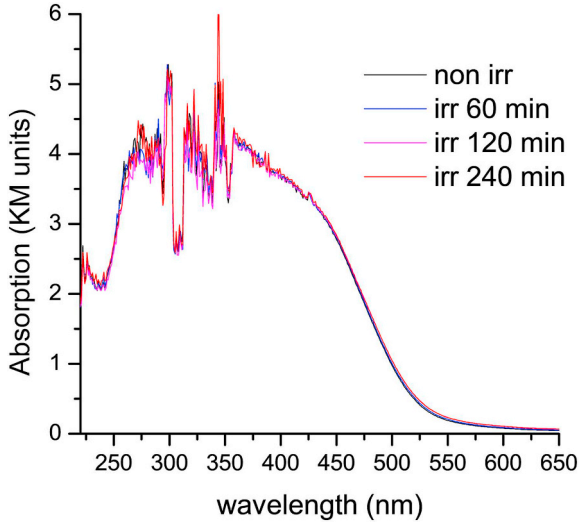

Fig. 1. Absorption spectra in Kubelka-Munk units of FA powder recorded after 0 (black line), 60 (blue line), 120 (pink line), 240 (red line) min of irradiation at 440 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

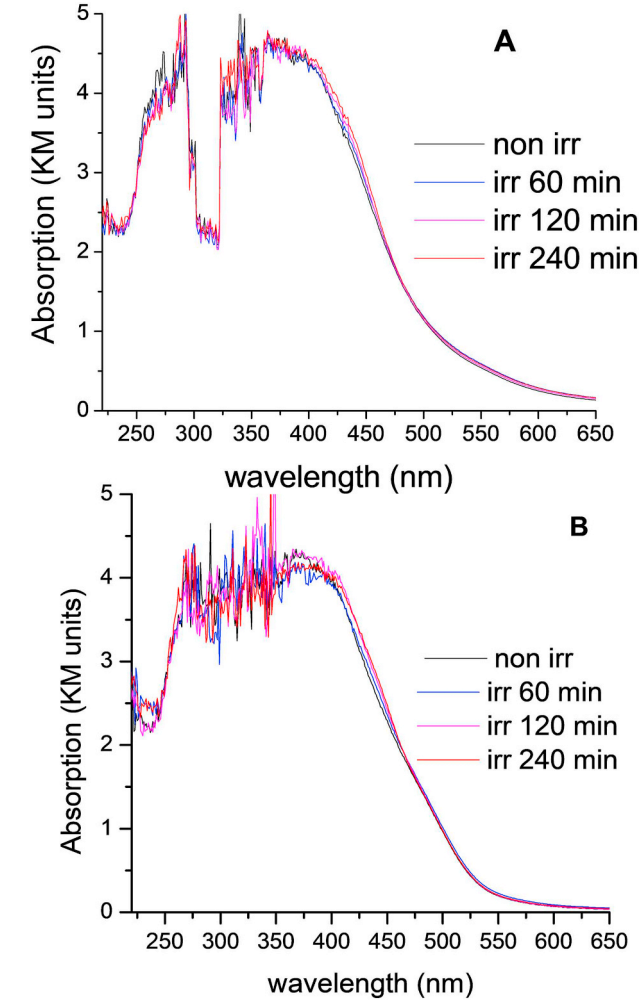

Fig. 2. Absorption spectra in Kubelka-Munk units of A) MgAl-LDH-FA and B) ZnAl-LDH-FA powders recorded after 0 (black line), 60 (blue line), 120 (pink line), 240 (red line) min of irradiation at 440 nm (λ_{exc} = 440 nm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

behavior is strongly affected by the environment; thus, it deserves a detailed investigation.

The absorption spectra, in the 200–650 nm region (Fig. 1), were recorded at different irradiation times, to examine the role of the inorganic matrix in preventing FA photodegradation. Furthermore, the comparative analysis of the spectra, before irradiation, can give qualitative information on the chemical nature of the chromophore.

The spectrum of crystalline FA (Fig. 1, Fig. SI3, Supplementary material) presents a broad band centered at 350 nm with a shoulder at 420 nm. The latter documents the close π-π packing arrangement of the molecules organized as crystals. For this reason, in the solid state the photodegradation processes are mitigated. ZnAl-LDH-FA and MgAl-LDH-FA (Fig. 2A and B, respectively, Fig. SI4 and SI5, Supplementary material) showed, similarly to FA, the maximum absorption at 350 nm, while the red-shifted shoulder at 420 nm is not observed in the hybrid samples. In the crystalline FA (Fig. 1, Fig. SI3, Supplementary material), the chromophores might experience π-stacking interactions, while in the hybrids (Fig. 2A and B) the FA units are more dispersed in monomeric form. Thus, the use of crystalline FA as reference is not appropriate. To better evaluate the photoprotective effect of LDH, it is most suitable to use a FA solution as reference sample.

FA degradation is influenced by different factors such as irradiation wavelength, temperature, concentration, oxygen level and pH (Gazzali et al., 2016); some of these effects can have a synergistic action, giving rise to a complex mechanism of photodegradation. Literature data document an interesting debate about the photostability of FA in aqueous media at different pH's, although in many cases different irradiation sources and excitation wavelengths have been used making the comparison difficult (Dántola et al., 2010; Liang et al., 2013; Bellavinha et al., 2014; Gazzali et al., 2016).

In order to evaluate the photoprotection capacities of the matrices, we exposed the FA solutions, at two different pH values, 1.2 and 8.0, to stationary irradiation to evaluate the impact on the spectral features so that we can discuss the effect of the matrices. The absorption spectra registered for FA in water solutions at pH = 1.2 (Fig. 3A) and pH = 8.0 (Fig. 3B) showed that in both cases FA photodegrades since spectral changes are observed. A qualitative analysis of the spectra might suggest that the degradation is very efficient in alkaline conditions (Fig. 3B), although the higher fraction of light absorbed cannot be neglected. These findings agree with the literature data; excitation of FA in air-equilibrated solutions leads to cleavage and oxidation of the

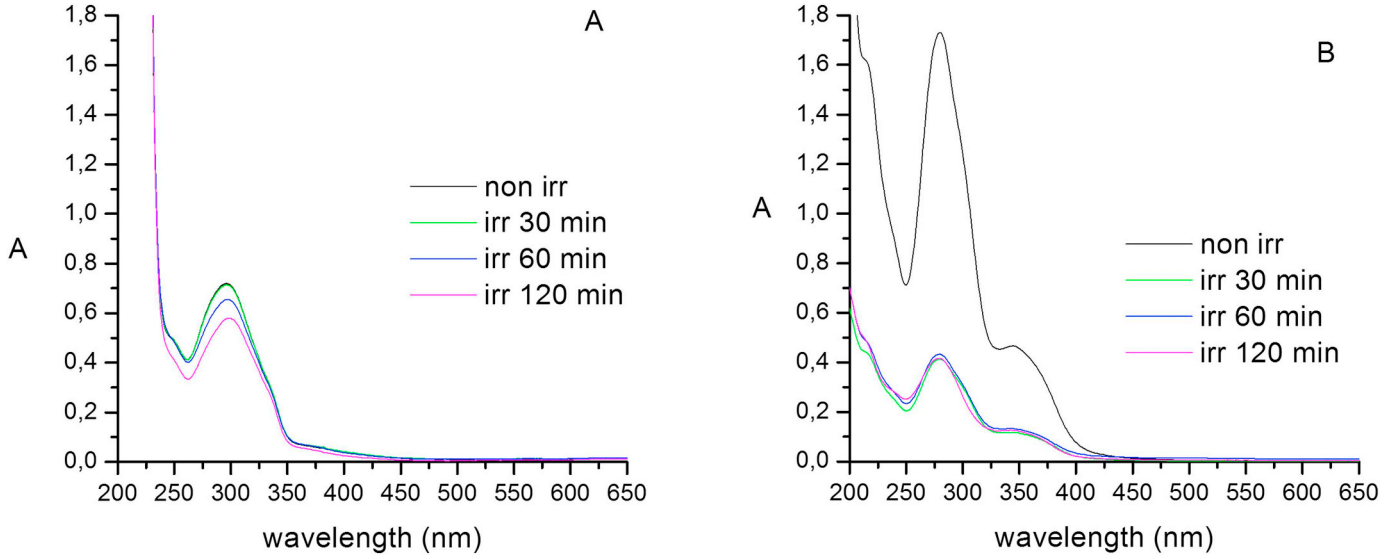

Fig. 3. Absorption spectra of FA in H_2O at A) pH = 1.2 and B) pH = 8.0 recorded at different irradiation times: 0 (black line), 30 (green line), 60 (blue line) and 120 (pink line) min (λ_{exc} = 340 nm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

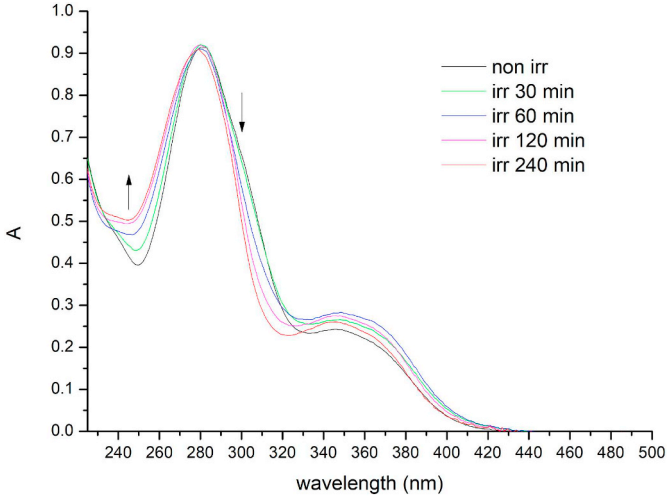

Fig. 4. Absorption spectrum of FA in synthetic sweat pH 5.5 recorded at different irradiation times: 0 (black line), 30 (green line), 60 (blue line), 120 (pink line) and 240 (red line) min (λ_{exc} = 340 nm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

molecule, yielding 6-formyl pteridine and p-aminobenzoyl-L-glutamic acid as photoproducts (Dántola et al., 2010). It must be underlined that a similar photochemical behavior is observed in a synthetic sweat phosphate buffer medium. Indeed, a solution of FA in this medium, simulating skin-like pH and composition, was irradiated and used as control (Fig. 4). The sample was irradiated for four hours, in the presence of molecular oxygen and the resulting spectra showed evident modifications in the 250–400 nm range (Fig. 4) attributable to FA photolysis (Dántola et al., 2010). Particularly, the spectra in Fig. 4 show that the absorption below 275 nm increases upon irradiation, while the absorption between 280 and 330 nm decreases.

The spectra obtained from the hybrids (Fig. 2, Fig. SI4 and SI5, Supplementary material) show the same characteristics of those obtained from FA in solution at two different pH values. This suggests that in the hybrid samples the absorption band could be due to the contributions of different FA species. Indeed, the broad absorption band observed for ZnAl-LDH-FA and MgAl-LDH-FA could be attributed to different forms of FA; in fact, different interactions can be established between the molecule, as mono (Fig. 2A, Fig. SI4, Supplementary material) or bicarboxylate (Fig. 2B, Fig. SI5, Supplementary material) anion, with LDH metals.

Using this sample as reference, the hybrids ZnAl-LDH-FA and MgAl-LDH-FA did not show significant changes in the absorption spectra upon four hours irradiation at λ_{max} = 440 nm (Fig. 2A and B, Fig. SI4 and SI5, Supplementary material). This behavior is ascribable to the spatial confinement imposed to the organic molecules by the matrices, which likely limits the efficiencies of cleavage and of oxidation photoreactions; once intercalated, FA is photoprotected from UV cleavage.

The complex state in which FA is present in the hybrid is more stable than the free form, as observed in previous works for other molecules (Perioli et al., 2006; Perioli et al., 2008; Perioli et al., 2015).

3.3. Emulgel composition optimization studies

The next step of our study was the choice of the most appropriate formulation suitable for hybrids topical application; it was chosen taking into account their properties and stability as well as the application site and its characteristics. To avoid any chemical alteration or modification of the starting material, the use of acidic or alkaline solutions was avoided. LDH are pH sensitive and undergo solubilization at pH values smaller than 4.0 and > 10.0, as well as in saline solutions. The anions can in fact promote the de-intercalation of FA molecules (Miyata, 1983; Jobbágy and Regazzoni, 2011).

With these considerations in mind, ZnAl-LDH-FA and MgAl-LDH-FA were introduced in O/W emulgels, in which the viscosity of the external water phase was increased by the addition of a suitable polymeric gelling agent.

Emulgel was selected as suitable formulation because of its degree of elegance and ready washability; this kind of formulation is very interesting also because of its thixotropy, easy spreadability, easy removability, moisturizing effect suppleness, water-solubility, longer shelf-life, bio-friendliness, aesthetical appeal and high ability to penetrate skin (Ajazuddin et al., 2013).

To obtain a rapid FA release after application, the hybrids were loaded in the external water phase (W). The choice of the most appropriate internal lipophilic phase came from the necessity to form a thin film able to prevent evaporation of water from the skin after application with a consequent enhancement of FA penetration. Many attempts were made to find the most suitable composition, starting from the hydrophilic base cream (O/W emulsion), reported in the Farnacopea Ufficiale Italiana (F.U. XII Ed. 2008, cetomacrogol base cream monograph):

Oil phase (O): vaseline (15%), liquid paraffin (6%), cetostearyl alcohol (7.2%);

Water phase (W): cetomacrogol (1.8%), water (70%) using an O/W ratio of 28.2/71.8.

The first modification consisted in the replacement of the water phase (cetomacrogol and water) with a hydrogel. The latter was prepared according to the indications for gel preparation reported in Ph. Eur. 9.0th Ed. modified by adding LDH to the composition: gelling agent (2%), glycerol (5%), LDH (5%), water (88%). At first, HEC (2%) was used as gelling agent because of its film-forming ability, swellability, biodegradability, lack of toxicity, and tolerability (Prini, 2014). The obtained emulgel was preliminarily evaluated considering its organoleptic properties; it must in fact satisfy the patient's/consumer's needs. Skin-feel attributes and physical appearance at different times (after preparation, 1, 7, 30 and 180 days) were evaluated keeping the sample at room temperature. With regard to skin feel, the emulgel resulted greasy and produced an unpleasant sensation. In addition, the formulation's physical characteristics resulted sub-optimal as phase separation occurred after one week of storage at room temperature. This was attributed to the high fluidity of the emulgel, which is due to HEC network inability to stabilize the internal oil phase. A new emulgel, in which HEC (2%) hydrogel was replaced by hydroxypropyl methylcellulose (HPMC, 2%) hydrogel, was thus prepared. HPMC in fact, is known to be a suitable thickening, dispersing and stabilizing agent, which is largely used as excipient in topical formulations (Singh and Tolman, 2013).

By a preliminary physical appearance analysis, the modification of the composition of the external phase (W) produced an improvement of emulgel texture as well as physical stabilization; no separation phase was observed along six months. However, after application, the emulgel was still feeling greasy due to the high lipophilic character of vaseline and liquid paraffin in the oil phase. Further modifications were carried out in order to obtain a pleasant and greaseless final product. The composition of the oil phase (O) was modified using the lipophilic excipients Gelucire® 50/13 and Transcutol®P, in place of Vaseline and paraffin, respectively. Gelucire® 50/13 is a non-ionic, water dispersible surfactant, which is composed of polyethyleneglycolates. It is able to self-emulsify on contact with aqueous media, forming a fine dispersion. This property makes Gelucire® 50/13 a suitable stabilizing agent for biphasic systems with no need for further ingredients. Transcutol®P is a diethylene glycol monoethyl ether, which is well known for its ability to enhance skin penetration. It can therefore be used to enhance FA penetration through skin. The organoleptic analysis showed that the obtained formulation was pleasant and provided for a good texture; it was homogeneous and physically stable, this further modification allowed

to define the final emulgels composition (see method section, Table 1).

3.4. X-ray analysis of emulgels

The emulgels containing the hybrids were submitted to X-ray analysis to evaluate if the original hybrids' structures have been maintained after the incorporation into emulgel.

Emulgel 3 and 4 (Fig. SI6, Supplementary material) showed the same pattern of the corresponding non-formulated hybrids (Fig. SI6, Supplementary material) In the case of emulgel 3, the intensity of the first reflection of the hybrid is very low. This is explainable considering that, due to the loading % (higher for MgAl-LDH-FA in comparison to ZnAl-LDH-FA) the amount of hybrid introduced in the formulation to obtain a final FA amount of 0.5%, is reduced in comparison to emulgel 4. This means that the limited amount of MgAl-LDH-FA in the emulgel 3 sample submitted to X-ray analysis is responsible for the weak reflection observed. The X-ray spectra of both emulgels do not show reflections attributable to FA crystals (Fig. SI6, Supplementary material).

We can therefore conclude that the incorporation of the hybrids in the emulgels did not alter their original structure; FA is therefore still stored into LDH lamellae in molecular form.

3.5. Photostability of emulgel

The absorption spectra of the control, emulgel 3 and 4 were recorded (Fig. 5A–C) in order to evaluate if the hybrid photoprotective effect is preserved also in the case of formulated products.

The obtained spectra showed a narrowing of the absorption band for the emulgel samples compared to the powder ones. Upon steady-state irradiation, no significant spectral changes were observed for the emulgels containing the hybrids (Fig. 5B–C), while the control (Fig. 5A) showed a clear increase of the absorbance at wavelength higher than 350 nm. These data indicate that the photoprotection action of the inorganic matrices is maintained in the formulations.

3.6. Rheological measurements

The rheogram reported in Fig. 6A highlights that the LDH presence improves the flow capacity of the formulations (emulgels 1–4). The control emulgel appeared to be the most viscous, with a high resistance to flow under mechanical solicitations, as supported by the very narrow range of shear rates measured, $5.0{\cdot}10^{-5}$– $1.3\ 10^{-3}\,s^{-1}$ (Fig. 6). Emulgels 1–4 showed pseudo-plastic behavior with some differences in the flow properties. Emulgel 3 is the less viscous as demonstrated by the measured range of shear rates $4.6{\cdot}10^{-4}$–$1.1\ 10^{-2}\,s^{-1}$, while emulgel 1, flowed in a slightly narrower range of shear rates $1.9{\cdot}10^{-3}$–$6.9\ 10^{-2}\,s^{-1}$. For emulgels 2 and 4, this range further decreased to $4.2{\cdot}10^{-3}$–$5\ 10^{-2}\,s^{-1}$ and $2.9{\cdot}10^{-3}$–$5.7\ 10^{-2}\,s^{1}$, respectively (Fig. 6). Taking into account these data, a flow attitude scale was drawn. Emulgel 3, containing MgAl-LDH-FA resulted to be less viscous than emulgel 4, containing ZnAl-LDH-FA (Fig. 6); emulgel 1 containing the physical mixture MgAl-LDH-Cl/FA resulted to be less viscous than emulgel 2 (based on ZnAl-LDH-Cl/FA) (Fig. 6). This is very important as it highlights the interesting effect of MgAl-LDH in the improvement of the formulation flow properties. These findings are in agreement with previous literature (Costa et al., 2006; Li et al., 2008; Perioli et al., 2013; Perioli et al., 2015).

The high viscosity of control emulgel can be ascribed to the thickness deriving from the presence of HPMC in the external water phase. Particularly, the presence of the HPMC's methoxy groups reduces the flexibility of the polymeric network with consequent high resistance to flow under mechanical stress. Relaxation of polymeric chains is limited, likely because of the cellulose methoxy groups' lipophilicity and of the presence of FA crystals, which reduce water molecules access to the inner spaces. For these reasons, the formulation is very consistent, highly viscous and not fluid.

The incorporation of FA crystals in the hydrophilic water phase was very difficult due to its lipophilicity. The intercalation into LDH can solve this problem by generating a new product (the hybrid) in which the lipophilicity of the active ingredient is reduced. The interlayer spaces provide niches in the hydrophobic regions, which are protected from water. Thus, the hybrid is a hydrophilic product that can be easily dispersed in the formulation and is able to improve its flow properties. The rheograms of emulgels 1 and 2 containing the physical mixtures MgAl-LDH-Cl/FA and ZnAl-LDH-Cl/FA, respectively, also confirm this finding (Fig. 6). These formulations, when compared to emulgels containing the hybrids appeared slightly more viscous; this is ascribable to the presence of FA crystals. On the other hand, emulgels 1 and 2 showed a reduced resistance to flow, compared to the control emulgel. Up to the shear stress value $6.1{\cdot}10^{1}$ Pa, the rheograms of both emulgels 1 and 2 are similar; emulgel 1 (range of shear rates $1.9{\cdot}10^{-3}$–$6.9\ 10^{-2}\,s^{-1}$) started to flow better than emulgel 2 (range of shear rates $4.2{\cdot}10^{-3}$–$5\ 10^{-2}\,s^{-1}$) and this behavior can be ascribed to the presence of Mg (MgAl-LDH-Cl), which works as rheological agent better than Zn (ZnAl-LDH-Cl) (Li et al., 2009).

Moreover, the observed differences can also be ascribed to the narrow particle size distribution of ZnAl-LDH-FA (Fig. S1, Supplementary material). ZnAl-LDH-FA exposes a greater surface area, per mass unit, to the polymeric network; a softer and more quickly flowing structure is observed, compared to the emulgel containing MgAl-LDH-FA (emulgel 4).

The yield stress measurement performed at 25 °C furnished information about the prepared emulgels extrusion attitude (Table 1). Extrusion from packaging, squeezing and spreading are related with the rheological characteristics of the formulation. The yield stress represents the shear stress variation vs viscosity of the formulation. Control emulgel showed a high yield value, defined as the minimum shear stress required to produce continuous deformation of the formulation. In fact, the high viscosity of this sample requires to achieve shear stress values of 27 Pa in order to observe the flow (Fig. 6B). Emulgels 3 and 4 resulted to be the most fluent formulations, starting to flow at very low shear stress values (4 Pa and 6 Pa for emulgel containing MgAl-LDH-FA and ZnAl-LDH-FA respectively, Fig. 6B).This result is in agreement with the viscosity measurements. Emulgels 1 and 2, containing the physical mixtures of FA/MgAl-LDH-Cl and ZnAl-LDH-Cl respectively, showed the highest yield value (Fig. 6B). The coexistence of crystalline FA and LDH in the formulations, dispersed as solid particles is responsible for this result. In fact, before the shear stress application, the solid particles are disarranged into the formulation. As the shear stress increases, the particles start to orientate in the direction of the applied force and the formulation starts to flow. After that, all the particles are aligned in the direction of the flow. In accordance with these considerations, the complete orientation of all the solid particles is obtained, reaching very high shear stress values (74 Pa).

The obtained results highlighted that the enhancement of the rheological properties of semisolid formulations in presence of LDH. Moreover, the use of hybrids instead of the physical mixtures (LDH/crystalline) is preferable.

3.7. Effect of hybrids on cells viability

The photostability studies highlighted that the intercalation procedure can prevent FA photodegradation. By this strategy, it is possible to improve the safety of FA exposed to UV rays preventing the formation of secondary toxic photoproducts.

In-vitro cytotoxicity experiments were carried out in order to better define the suitability of the proposed formulation, also with regard to the safety of hybrids use in topical formulations. Particularly, the effect of hybrids on the viability of immortalized keratinocytes and primary dermal fibroblasts was evaluated.

Keratinocytes are the first cells exposed to topical/dermatological formulations; thus, they can be used as a model cell system to study

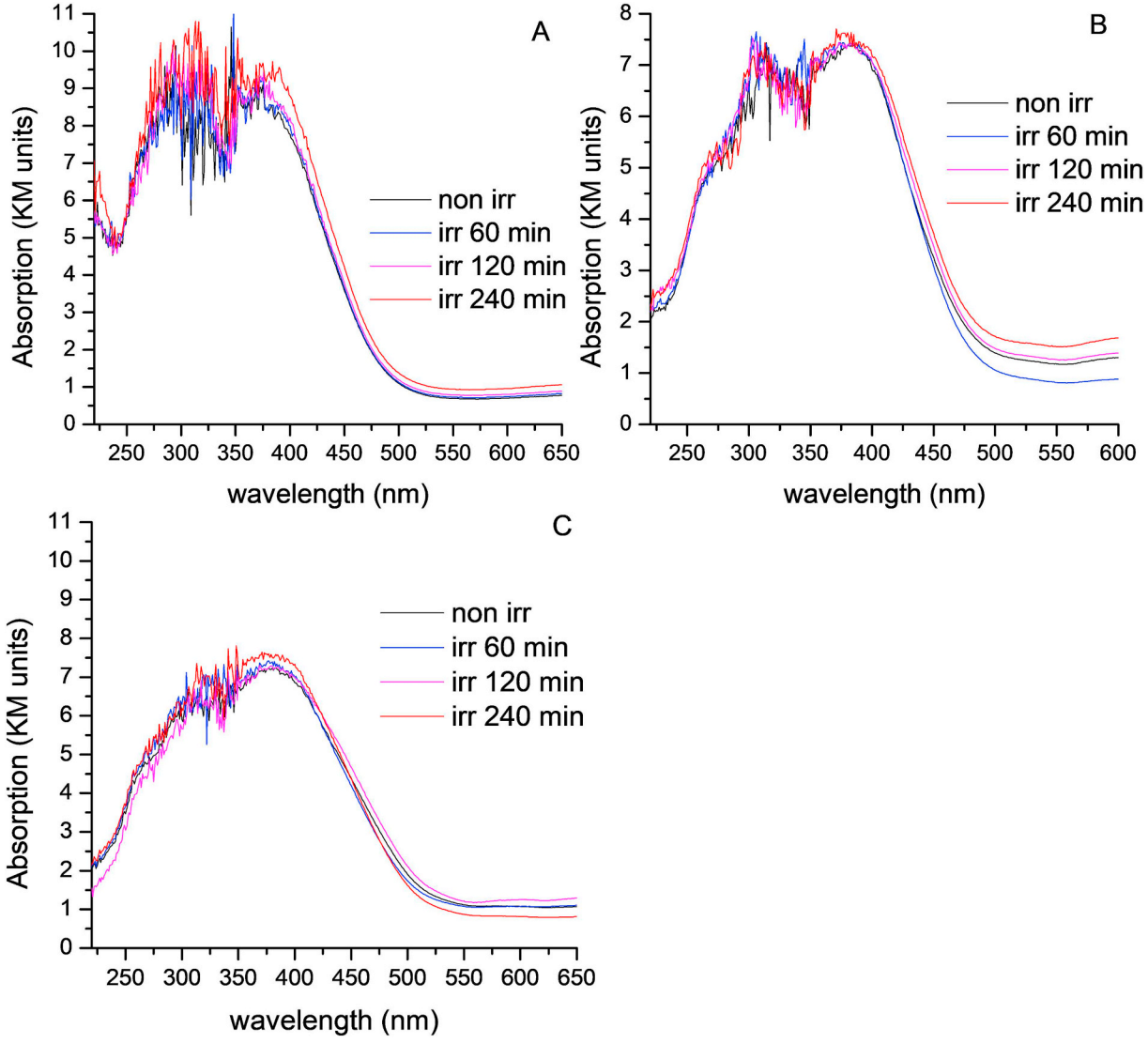

Fig. 5. Absorption spectra of: A) control emulgel (containing crystalline FA) B) emulgel 3 and C) emulgel 4 recorded after 0 (black line), 60 (blu line), 120 (pink line), 240 (red line) min of irradiation (λ_{exc} = 440 nm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

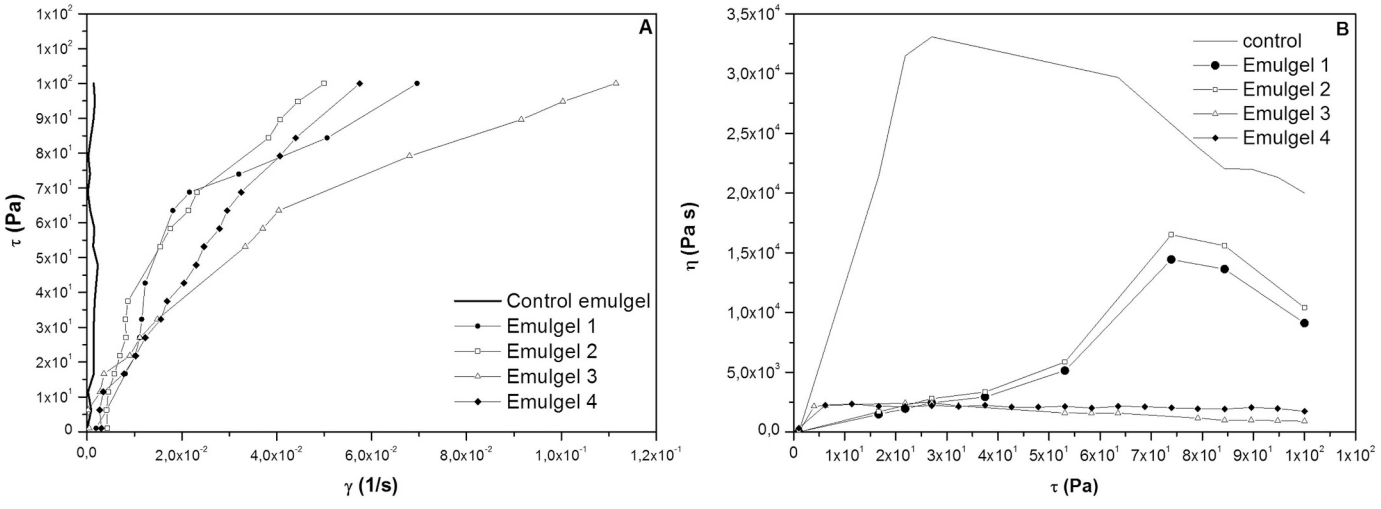

Fig. 6. A) Plots of the shear stress (τ) as a function of the shear rate (γ) for the different emulgels measured at 32 °C to simulate the skin application conditions; B) Plots of viscosity (η) as a function of the shear stress (τ) for the different emulgels as indicators of the formulation extrusion attitude.

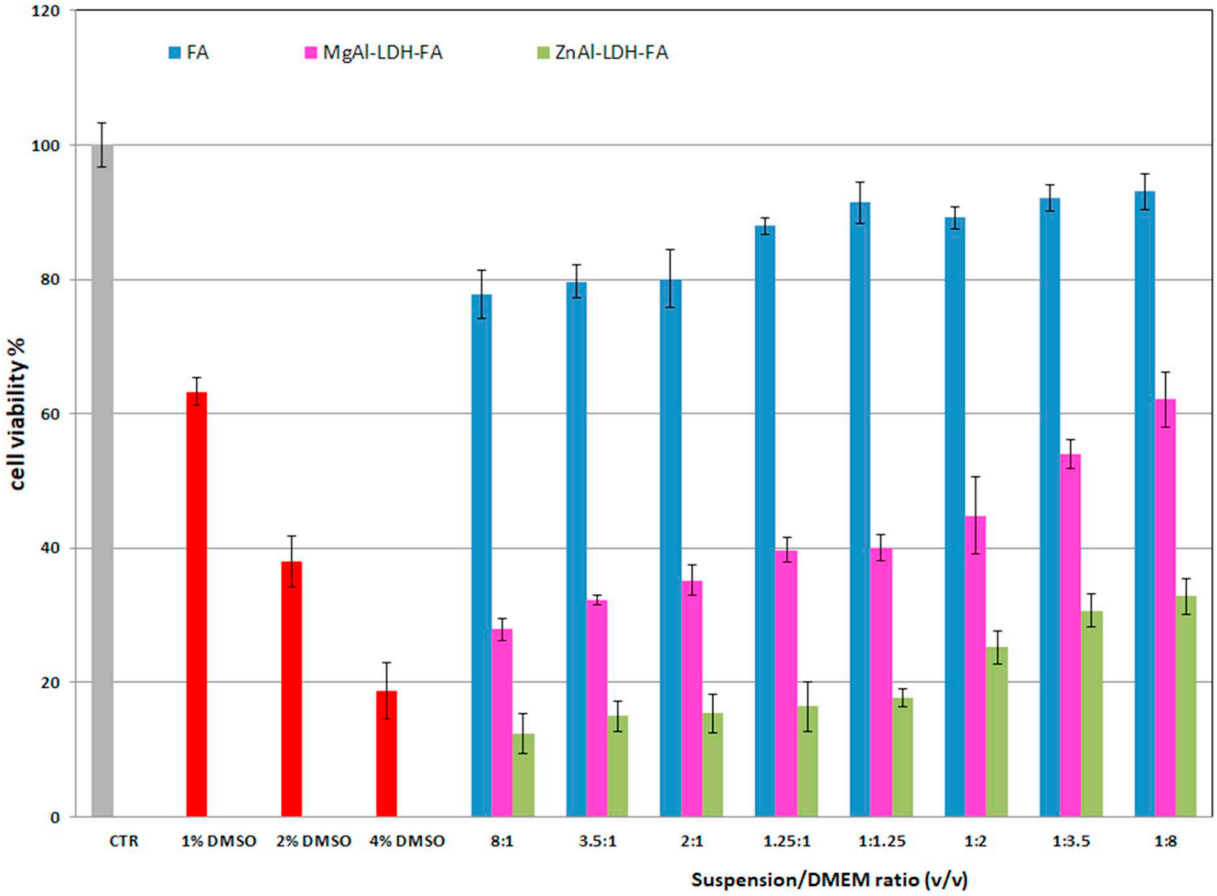

Fig. 7. Effect on HaCat keratinocytes viability incubated with different amount of suspension (diluted with DMEM) deriving from the contact between crystalline FA (blu), MgAl-LDH-FA (violet), ZnAl-LDH-FA (green) and DMEM (n = 3, ± SD). DMEM is used as negative control (CTR with 100% of cell viability) and DMSO, known to induce cell death, is used as a positive control with 3 different concentrations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

normal skin barrier development and response after different treatments. For this reason, it was important to evaluate the effect of hybrids on their vitality.

Firstly, the effect of LDH-FA particles on cells viability was evaluated. With this aim, cells were incubated with a suspension of LDH-FA particles in DMEM (for preparation see paragraph 2.8.2. Samples preparation). The obtained results highlighted that both hybrids are cytotoxic. Cells treated with crystalline FA particles showed a viability > 77% in all the tested dilutions (Fig. 7). ZnAl-LDH-FA is more toxic obtaining a viability of ~30% at the highest dilutions (1:8 and 1:3.5 suspension/DMEM *v*/v). This value decreases drastically increasing the suspension amount reaching 12% with the highest amount of suspension assayed (8:1 suspension/DMEM *v*/v) (Fig. 7). About MgAl-LDH-FA, cells viability resulted higher in comparison to cells treated with ZnAl-LDH-FA. However, just the cells treated with the highest dilution (1:8 suspension/DMEM v/v) showed a viability of 62% (Fig. 7). In all the other ratios the viability resulted < 60%, decreasing as supernatant content increases (Fig. 7), reaching 27.9% using the ratio 8:1 (suspension DMEM v/v).

These data suggest that, in the experimental conditions used, both LDH hybrids are toxic for cells. The obtained results can be the consequence of LDH particles interaction with cells and/or the products (metals) deriving from LDH partial solubilization.

In order to discriminate the factors responsible for cells death a new MTT test was carried out on HaCaT cells treated with the supernatant obtained from DMEM incubation with LDH-FA and then separated from the solid particles by centrifugation (for preparation see paragraph 2.8.2. Samples preparation).

The obtained results reported in Fig. 8 showed that the hybrid ZnAl-LDH-FA (green bars) impairs cells viability (< 60%) especially when the amount of supernatant prevails on the amount of DMEM. When the volume of supernatant deriving from ZnAl-LDH-FA suspension in DMEM is < 25% of the total amount (1:8 supernatant/DMEM *v*/v), as reported in Fig. 8, the cell viability is not compromised. On the contrary, the viability was maintained within acceptable limits in the case of cells treated with the supernatant deriving from MgAl-LDH-FA (violet bars) (Fig. 8). Data obtained both from the incubation of suspension and supernatant of ZnAl-LDH-FA suggest that this material is not safe for the user/patient.

One gram of the prepared emulgel (containing 17.76 mg of ZnAl-LDH-FA) can cover approximately an area of 8.5 cm^2 (as calculated according to the method described by Arvouet-Grand et al., 1995), and shown in Fig. 9. It is therefore reasonable to hypothesize that in the case of emulgel 4 (containing ZnAl-LDH-FA) the amount applied on skin, falling within the range of dilution ratios of 8:1–1.25:1 supernatant/DMEM v/v, would contain a percentage of hybrid expected to be toxic for skin cells.

In order to better understand these results further studies were carried out. In particular, the supernatant obtained from the incubation of both MgAl-LDH-FA and ZnAl-LDH-FA with DMEM was submitted to ICP-OES analysis in order to evaluate the presence of metal ions in solution (deriving from partial LDH solubilization, during the incubation time). DMEM was used as blank. The obtained results (Table 3) showed a considerable concentration of Zn ions (49.25 μg/ml) in the culture medium, generally devoid of this element. These results suggest that zinc ions exert toxic effect on cells as observed from other authors

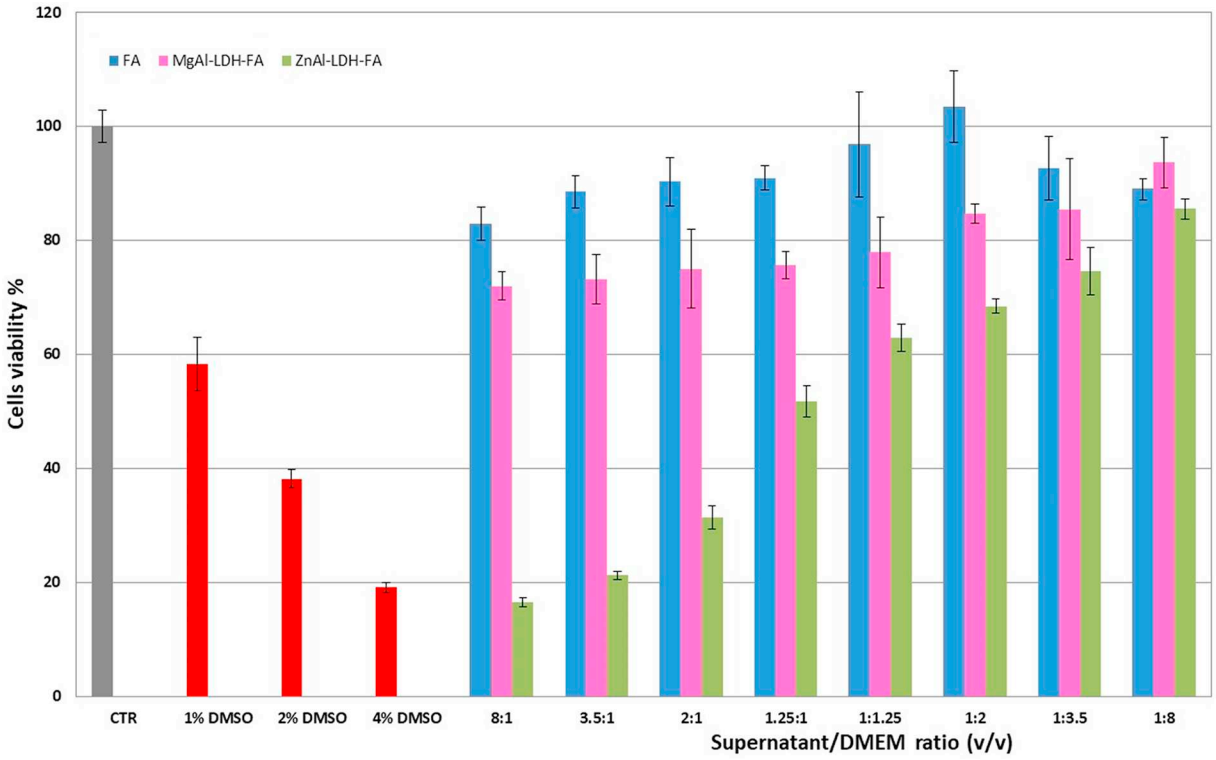

Fig. 8. Effect on HaCat keratinocytes viability incubated with different amount of supernatants (diluted with DMEM) deriving from the contact between crystalline FA (blu), MgAl-LDH-FA (violet), ZnAl-LDH-FA (green) and DMEM (n = 3, ± SD). DMEM is used as negative control (CTR with 100% of cell viability) and DMSO, known to induce cell death, is used as a positive control with 3 different concentrations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

as well (Choi and Choy, 2011).

In the case of the supernatant obtained after DMEM incubation with MgAl-LDH-FA, Mg ions concentration is twice in comparison to DMEM (blank). This modification does not compromise cells viability as observed in Fig. 8.

The concentration of Al ions increased in both supernatants compared to DMEM. However, the experimental conditions used and the concentrations obtained do not testify the absolute toxicity of Al ions. In fact, the viability of cells treated with MgAl-LDH-FA supernatant is always > 60% thought the Al ions concentration is similar to ZnAl-LDH-FA supernatant (Table 5).

Comparing the results reported in Fig. 7 (cells incubated with suspensions) to those in Fig. 8 (cells incubated with supernatants) a high difference of cells viability can be detected. In particular, in the experimental conditions used, the viability of cells treated with the supernatants is less impaired than that treated with the suspensions. Based on the obtained results it was hypothesized that in the case of MgAl-LDH-FA the toxicity is due to particles interaction with cells responsible for an alteration of the physiological homeostasis leading to death. Further studies will be necessary in order to clarify these mechanisms.

Table 3
Zn/Mg and Al amounts measured in DMEM incubated for 24 h at 37 °C with MgAl-LDH-FA and ZnAl-LDH-FA.

Sample	Metals concentrations (μg/ml)		
	Mg	Al	Zn
DMEM (blank)	18.45	0.02	–
DMEM after the incubation with MgAl-LDH-FA	32.87	1.63	–
DMEM after the incubation with ZnAl-LDH-FA	17.72	1.98	49.25

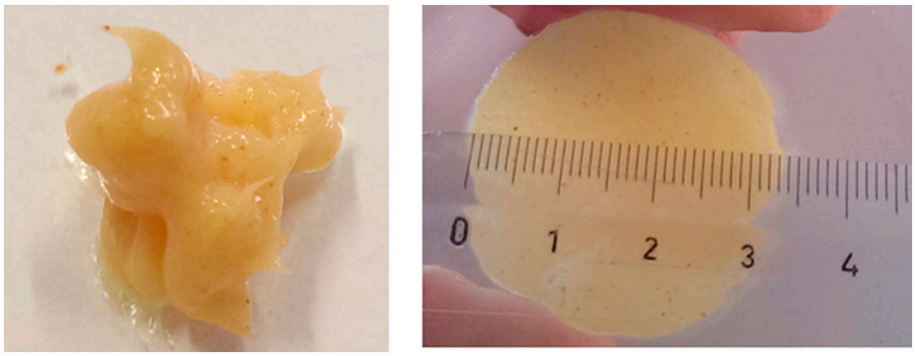

Fig. 9. Surface area covered by one gram of emulgel 4 containing the hybrid ZnAl-LDH-FA.

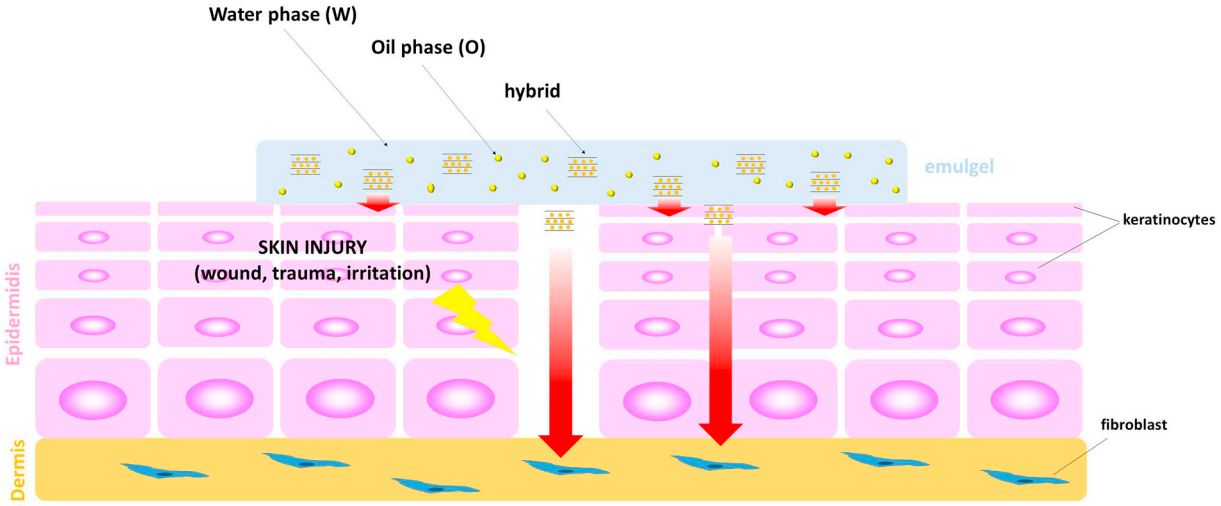

Fig. 10. Schematic representation of hybrids possible distribution after application on skin surface.

In the case of ZnAl-LDH-FA the toxic effect is a combination of the particles interaction with cells and of the high concentration of Zn ions in solution as cells viability measured both with the solution and the suspension is strongly reduced.

The MTT test was then carried out on fibroblasts with the aim to evaluate the effect of the hybrids on this cell line. The hybrid particles, dispersed in the formulation, could in fact cross the epidermis and reach the dermis (Fig. 10), particularly if the emulgel is applied on skin where the stratum corneum structure is compromised as in the case of aged or photo-damaged skin.

The results obtained incubating crystalline FA and LDH-FA hybrid suspensions with fibroblasts were comparable to that obtained with HaCaT cells (data not shown). Incubating the cells with the solution also in this case the viability of cells treated with the supernatant deriving from ZnAl-LDH-FA is seriously compromised (Fig. 11). After 24 h of incubation, cell viability resulted smaller than 60%, except for the highest dilutions of supernatant assayed (1:3.5 and 1:8 supernatant/DMEM *v*/v). Also in the case of fibroblasts, the viability was maintained within acceptable limits for the cells treated with the supernatant deriving from MgAl-LDH-FA (violet) (Fig. 11).

The results obtained from both incubation with keratinocytes and dermal fibroblasts allow to hypothesize that emulgel 4 ZnAl-LDH-FA

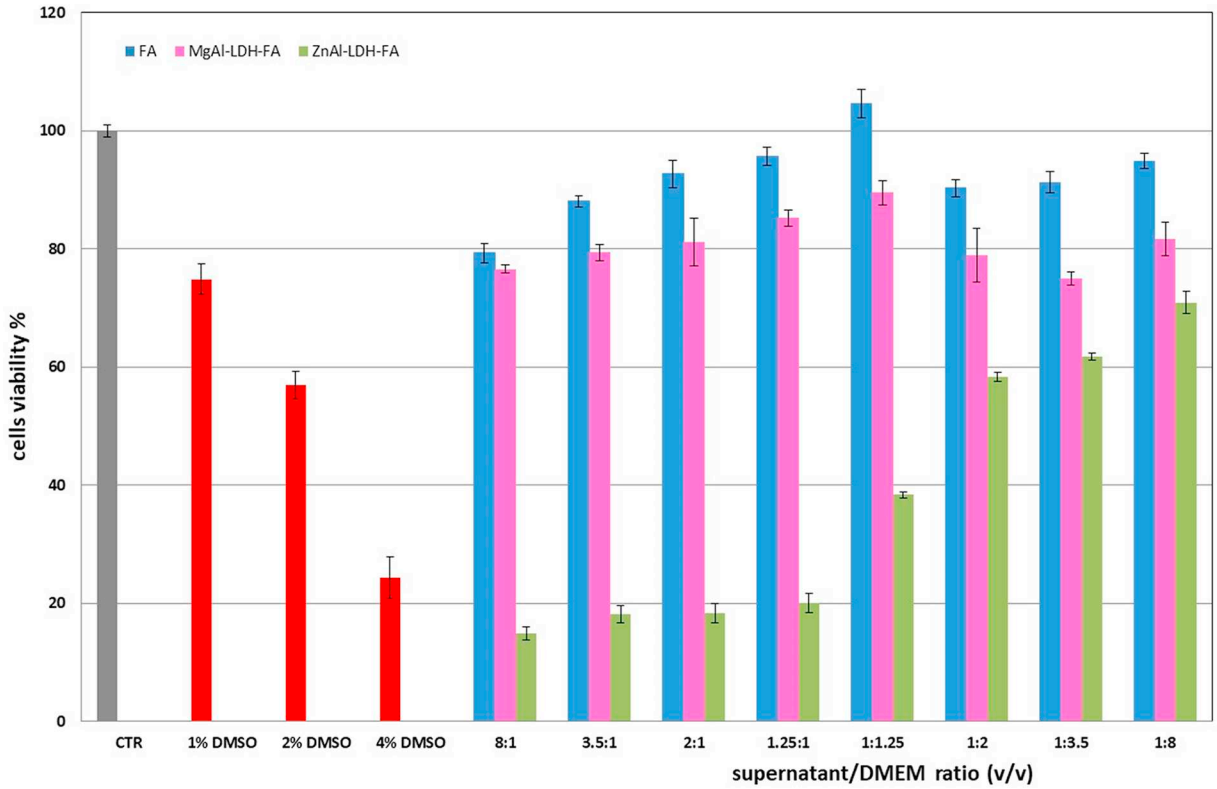

Fig. 11. Effect on primary dermal fibroblasts viability incubated with different amount of supernatants (diluted with DMEM) deriving from the contact between crystalline FA (blu), MgAl-LDH-FA (violet), ZnAl-LDH-FA (green) and DMEM (n = 3, ± SD). DMEM is used as negative control (CTR with 100% of cell viability) and DMSO, known to induce cell death, is used as a positive control with three different concentrations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

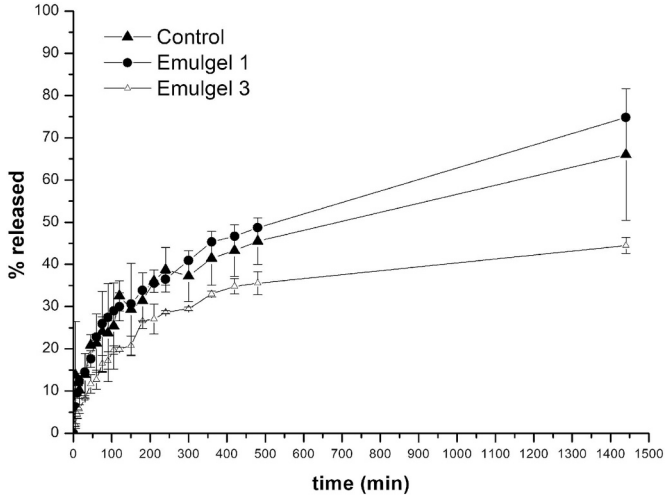

Fig. 12. In vitro release profiles of FA in synthetic sweat at pH 5.5 at 32 °C obtained from control emulgel, emulgel 1 and 3 (n = 3, ± SD).

does not satisfy the requirement of safety and its use must be avoided. Emulgel 3 MgAl-LDH-FA does not impair cell viability and it can therefore be assessed as safe to use.

3.8. FA quantification in DMEM

FA concentrations in the samples obtained incubating crystalline FA, MgAl-LDH-FA and ZnAl-LDH-FA with DMEM for 24 h at 37 °C were measured by HPLC method, obtaining the following values:

- 1.26 g/l from the sample crystalline FA in DMEM,
- 0.60 g/l from the sample MgAl-LDH-FA FA in DMEM,
- 0.65 g/l from the sample ZnAl-LDH-FA FA in DMEM,

These results can be explained considering that at pH 7.4 (of DMEM) FA release from the hybrids takes place by ion exchange mechanism obtaining a sustained release compared to crystalline FA.

3.9. In vitro release studies

The cytotoxic effect of ZnAl-LDH-FA observed in the previous studies suggested that this material could not be safe for use on skin, for this reason emulgels 2 and 4 were not considered for the in vitro release studies.

The profiles obtained from emulgels 1 and 3 showed that none of them reached the total release after 1440 min (Fig. 12). As observed in Table 4 and Fig. 12, FA is released from emulgel 3 in a sustained manner, yielding an incomplete release of 44.5% over a period of 24 h (1440 min). This result can be ascribed to two different factors: (i) the kinetic of FA release from the hybrid, and (ii) the effect of the polymeric network present in the water phase of the emulsion in which the hybrid MgAl-LDH-FA is dispersed, conditioning FA diffusion in the bulk solution.

With regard to the first aspect, it must be taken into account that FA intercalated into LDH is gradually released by ion exchange mechanism established with the synthetic sweat anions. This aspect, together to the possible influence of the polymeric matrix, was deeply investigated by the elaboration on the in-vitro release data by the Ritger and Peppas kinetic mathematical model $M_t/M_\infty = K^{tn}$, applied to swellable matrices (Ritger and Peppas, 1987) and based on the evaluation of the diffusional exponent value. An exponent value of 1 means that drug release occurs as an apparent zero-order mechanism. A value instead equal to 0.5 means that drug release is controlled by a pure Fickian diffusion mechanism. Values in the range of 0.5–1 indicate an anomalous non-Fickian mechanism; both liquid penetration rate and polymeric chain relaxation rate control the release.

The influence of LDH on the release was evaluated by the kinetic model proposed by Bhaskar et al. (1986) applied to exchangeable matrices.

Emulgel 3 showed the highest r value in a first order kinetic data fit ($r = 0.9965$), when Bhaskar's kinetic model was applied, an high $r = 0.9672$ value was obtained too (Table 5). The obtained results suggest that FA release from emugel 3 is influenced both by the ion exchange mechanism and by a diffusional gradient generated by FA molecules in solution.

In these cases of the control emulgel and emulgel 1 in which FA is dispersed in the external water phase as crystals, it can be hypothesized that FA dissolution from is faster than from the hybrid; for this reason they are able to produce, at the same time, higher concentrations than emulgel 3.

Emulgel 1 (Fig. 12), containing the physical mixture FA/MgAl-LDH-Cl, showed the highest amount released, reaching 74.8% in 24 h (Table 4). The application of the mathematical model showed the best fitting for $n = 0.5$ ($r = 0.9889$, Table 5) meaning that the release process follows a diffusional mechanism according to Fick's law. At time 0, the gradient between the crystalline surface (stagnant layer) and the bulk solution is the highest, representing the driving force responsible for FA diffusion.

The control emulgel, which produced a total amount released of 66% within 24 h (Fig. 12, Table 4), showed the best fitting for the first order kinetic ($r = 0.9857$, Table 5), meaning that FA release probably mainly depends on the diffusional gradient responsible for the release rate control.

4. Conclusions

An innovative formulation was studied and developed with the aim of providing an alternative to the currently available folic acid-based products.

Compared to free FA, the hybrids MgAl-LDH-FA and ZnAl-LDH-FA showed improved UV stability, preventing the formation of toxic photoproducts. The hybrids introduction in the external phase of O/W emulgels produced several benefits so that they can be considered multitasking agents. MgAl-LDH-FA and ZnAl-LDH-FA (i) are more easily workable than crystalline FA, (ii) improve formulation rheological properties, and (iii) maintain FA photoprotection even after introduction in the emulgel.

In-vitro studies performed on human immortalized keratinocytes and human primary dermal fibroblasts highlighted that ZnAl-LDH-FA is less safe than MgAl-LDH-FA. The latter seems to be the most suitable to be used on skin, however the amount of MgAl-LDH-FA to be introduced in the formulation must be established carefully in order to remain

Table 4
FA release % in synthetic sweat pH 5.5 at different time points.

Sample	15 min	30 min	60 min	120 min	240 min	360 min	480 min	1440 min
Control emulgel	10.0%	14.0%	21.3%	32.5%	38.7%	41.5%	45.5%	66.0%
Emulgel 1	12.3%	14.5%	22.7%	30.0%	36.5%	45.3%	48.7%	74.8%
Emulgel 3	5.7%	8.3%	12.7%	20.0%	28.6%	33.0%	32.5%	44.5%

Table 5
Ritger and Peppas's kinetic mathematical model, first order and ion exchange resins kinetics model fitting for emulgels.

emulgel	$M_t/M_\infty = kt^n$						$M_t/M_\infty = 1\text{-}e^{-kt}$	$M_t/M_\infty = 1\text{-}e^{k\ 0.65}$
	$n = 1$	$n = 0.9$	$n = 0.8$	$n = 0.7$	$n = 0.6$	$n = 0.5$	First order	Ion exchange resins
	Zero order							
Control	$y = 0.0377\times + 21.2$	$y = 0.08\times + 19.8$	$y = 0.1695\times + 18.1$	$y = 0.359\times + 15.9$	$y = 0.9372\times + 10.1$	$y = 1.6205\times + 9.3$	$y = -138.65\times + 7.1$	–
	$r = 0.8816$	$r = 0.9050$	$r = 0.9277$	$r = 0.9486$	$r = 0.9735$	$r = 0.9796$	$\boldsymbol{r = 0.9857}$	
1	$y = 0.0449\times + 20.7$	$y = 0.0951\times + 19.1$	$y = 0.2012\times + 17.0$	$y = 0.4256\times + 14.4$	$y = 1.0851\times + 8.0$	$y = 1.9194\times + 6.6$	$y = -124.65\times + 9.0$	–
	$r = 0.8942$	$r = 0.9166$	$r = 0.9385$	$r = 0.9587$	$r = 0.9821$	$\boldsymbol{r = 0.9889}$	$r = 0.9737$	
3	$y = 0.0283\times + 14.5$	$y = 0.0608\times + 13.4$	$y = 0.1306\times + 11.9$	$y = 0.2809\times + 10.1$	$y = 0.8815\times + 3.0$	$y = 1.3088\times + 4.4$	$y = -176.72\times + 2.1$	$y = -0.0024\times\text{-}0.04$
	$r = 0.7842$	$r = 0.8164$	$r = 0.8494$	$r = 0.8819$	$r = 0.9682$	$r = 0.9402$	$\boldsymbol{r = 0.9965}$	$\boldsymbol{r = 0.9276}$

within the limits of safety.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clay.2018.12.009.

Acknowledgements

Authors would like to acknowledge Gattefossè, France for the gift samples of Gelucire® 50/13 and Transcutol® P. We wish to thank Prof. Oriana Piermatti from Dipartimento di Chimica, Biologia e Biotecnologie – University of Perugia for the microelemental analysis.

Declaration of interest

The authors report no conflicts of interest. This research was funded by Regione Umbria (POR Umbria FSE 2007-2013).

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