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Research paper

The examination of skin lipid model systems stressed by ultraviolet irradiation in the presence of transition metal ions

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Abstract

In this study, we investigated the effects of ultraviolet (UV) radiation on lipid peroxidation in the presence of ionised iron as a transition metal. Fatty acids as important intercellular stratum corneum lipids and liposomes were used to model skin lipid systems for our experiments. A UV-A laser and a broad spectrum UV lamp were used to create high-level radiation. UV-related damage was quantified by the thiobarbituric acid assay detecting malondialdehyde. Electrospray mass spectrometry was used to characterise peroxidation products following UV exposure. We have shown that hydro- and endoperoxides are long stable intermediates deriving from lipid peroxidation. The incorporation of unsaturated fatty acids into phospholipid liposomes increased the average liposomal diameter and enhanced sensitivity to UV radiation. By comparing our data from laser induced monochromatic UV-A radiation and broad-spectrum UV irradiation, we have demonstrated that UV-A radiation can also induce lipid peroxidation in lipid model systems.

Keywords: Lipid peroxidation; Stratum corneum; Liposomes; UV radiation; Thiobarbituric acid assay; Mass spectrometry; Particle size determination

1. Introduction

Fatty acids are essential components of natural lipids, which determine the physiological structure and function of the human skin. They exist in the hydro-lipid skin surface film, intercellular stratum corneum lipids, and cell membranes [1].

As a part of the epidermal stratum corneum lipid matrix, intact free fatty acids play an essential role in the barrier function of the skin [2].

UV, oxygen and air pollutants together can severely damage biological structures like DNA, lipids and proteins [3]. Through the formation of free radicals, reactive oxygen species exert their toxicity by direct reaction with these biomolecules [4,5]. The human skin is of particular interest because it is constantly exposed to environmental radicals and sunlight. These factors accelerate premature skin ageing [6], mutation [7] and reduce the skin barrier function. Malondialdehyde is a classic secondary product of fatty acid lipid peroxidation, which can react with DNA forming harmful adducts, an alternative pathway to direct DNA oxidation [8]. Consequently, the protection of skin lipids from environmental hazards is of high interest in pharmaceutical research [9].

With liposomes closely resembling membrane structures, it renders them an excellent model system for in vitro investigations [10,11] and modern vehicle systems [12] to monitor cross-skin transport of drugs and cosmetics [13]. In this study, fatty acids were encapsulated inside phospholipid liposomes to distinguish between the action of UV on free fatty acids and phospholipid bilayers, possibly deriving from cosmetical formulations for topical application.

Transition metal ions are cofactors and electron suppliers, which mediate oxidation [14]. Iron is the most ubiquitous transition metal and an essential trace element in the human diet. Sun exposure of human and animal skin causes significant accumulation of non-heme iron in the epidermis and dermis compared to non-exposed areas (53–18 ppm, dry weight). Since iron can catalyse the generation of reactive oxygen species, the process seems to be a self catalysing danger for human skin [15].

Several methods have been described to study the effect of UV-radiation and oxygen on human skin. They involve either artificial models or cell cultures and in vivo systems [16,17]. To analyse peroxidation products in artificial systems, a chemical detection, electron spin resonance

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[18], and absorption spectroscopy to identify breakdown products with conjugated double bonds can be used. Indicators of the radiation effects in biological tissues include changes in cell and tissue morphology, sensitivity, proliferation rate, and cell growth [19]. UV lamps with a broad emission spectrum were employed for the study of skin cells [20]. However, an exact pathway for UV radiation effects on biological structures remains controversial because multiple parameters such as wavelength, morphology, and individual cell variation are involved.

We have therefore examined the effects of the two noxious stimuli, UV and oxygen, in the presence of iron and compared the scale of lipid peroxidation in different systems. The systems consist of fatty acids as stratum corneum lipids on one hand and several phosphatidylcholines as liposome generators and/or cholesterol as another important participant in the lipid domain of the stratum corneum and fatty acids on the other hand.

We designed liposome-lipid model systems for this study, which were characterised by mass spectrometry, photon correlation spectroscopy and the thiobarbituric acid assay. For radiation treatment two UV sources were used: (A) a frequency triple-pulsed Nd:YLF laser (pulse duration 5×10^{-12} s, wavelength 349 nm) and a broadband OSRAM-VITALUX lamp emitting a solar radiation spectrum (B).

2. Materials and methods

2.1. Sample preparation

Liposomes were used as artificial models for cell membranes. Stock solutions of all compounds in double-distilled water (lipids 1 mM, ferrous sulphate 500 μM) were stored at 2–8°C. The preparations were stable for at least 30 days as demonstrated by the thiobarbituric acid assay.

During the sample preparation, the ingredients were diluted in double-distilled water to the concentration required and transferred to test tubes. The tubes were wrapped with tin foil and placed on a flask shaker (GFL 3006, Gesellschaft für Labortechnik, Burgwedel, Germany) and rocked at 300 movements/min for 900 min. Prior to UV exposure, ferrous sulphate (10 μM) was added to all samples except a blank control. All stock solutions were stored under the same conditions. The intervals between sample make-up and UV treatment or between irradiation and evaluation were less than 720 min. We used simple systems which contained only fatty acids (100 μM), cholesterol (100 μM), or phospholipids (100 μM) in double-distilled water. Complex systems comprised mixtures of fatty acids (100 μM) and/or cholesterol (100 μM) as well as phospholipids (200 μM). The fatty acids, cholesterol, phospholipids, egg yolk lecithin and ferrous sulphate were of analytical grade and obtained from Sigma (Deisenhofen, Germany) (Fig. 1).

2.2. Radiation treatment

An OSRAM-VITALUX lamp was used as standard UV source to produce a sun-like spectrum (OSRAM, Hamburg, Germany). The distance between lamp and sample was 500 mm.

Prior to irradiation, 5.0 ml of each sample were transferred to open glass dishes of 55 mm diameter. The liquid depth was 2.1 mm and a homogeneous exposure can be assumed.

We further applied a pulsed UV laser with a wavelength of 349 nm (Physics Department, Martin-Luther-University Halle-Wittenberg, Halle, Germany). The pulse duration was 5 ps with a repetition frequency of 80 Hz. Providing a pulse energy of 20 μJ, we achieved a peak power of 4 MW and an average power of 1.6 mW.

The primary radiation source was a mode-locked Nd^{3+}: YLF laser, optically pumped by flash lamps with a wavelength of 1047 nm. The primary laser pulse frequency was partly doubled to yield two beams at 1047 and 523.5 nm. These beams were merged in a BBO crystal. After filtering stray frequencies, a sum frequency of 349 nm was obtained.

Two millilitres of each sample were exposed to laser irradiation in quartz cuvettes without mechanical treatment during irradiation. The beam diameter was 5 mm^2 in unfocused beams and 0.01 mm^2 in focused beams. Hereby, peak intensities of 800 GW/m^2 or 400 TW/m^2 (corresponding to a mean intensity of 320 W/m^2 or 160 kW/m^2) were used.

2.3. TBA-Reaction

Thiobarbituric acid reacts with aldehydes through an aldol condensation. The reaction with malondialdehyde, a secondary product of lipid peroxidation, is widely used to determine oxidation-related noxious effects on lipids [21–23].

In this study, the Buege–Aust method of the assay was used [24]. Two millilitres of a stock TBA reagent, consisting of 15% (w/v) trichloroacetic acid in 0.25 M HCl and 0.37% (w/v) thiobarbituric acid in 0.25 M HCl, were given to 1 ml of the UV treated sample. After heating at 90°C for 15 min and cooling down, a red TBA:MDA-complex (2:1) appears, which can be detected by fluorescence measurement. HPLC was used to quantify the pigment. A reversed phase HPLC column LiChrospher® 100, RP 8, particle size 5 μm (Merck, Darmstadt, Germany) was used with a mobile phase of methanol in water 30:70. The excitatory wavelength was 515 nm and the measurements were carried out at 555 nm. A calibration curve was established by preparing an aqueous solution of malondialdehyde-*bis*-dimethylacetal. At a low pH, the acetal groups are hydrolysed and malondialdehyde is released.

All mass readings refer either to the calibration line or 1.0 ml sample solution. By knowing the molecular weight of the calibration substance (M = 164.2), it is possible to extrapolate molar concentrations of malondialdehyde equivalents (MDE). Therefore, the following equation for 1.0 ng/ml

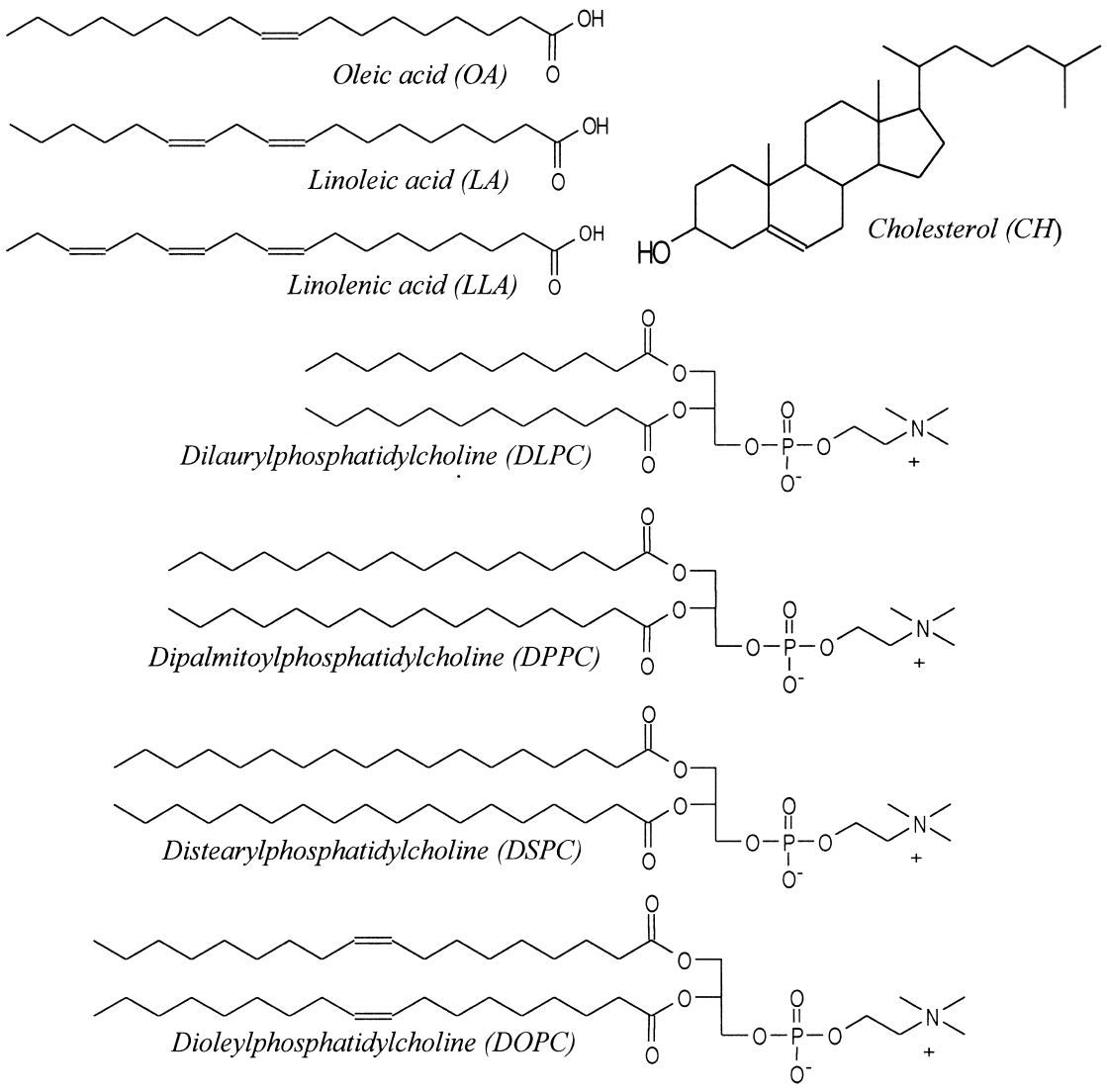

Fig. 1. Structures of the compounds used. Additionally egg yolk lecithin (LEC) as a phospholipid mixture was used (all substanced were of analytical grade)

sample was used

$$c(\mathrm{MDE}) = \frac{1\ \mathrm{ng}}{1\ \mathrm{ml}} \times \frac{1}{\mathrm{M}} = \frac{1\ \mu\mathrm{g/mol}}{1 \times 164.2\ \mathrm{g}} = \frac{1}{164.2}\,\mu\mathrm{M} \approx 0.0061\ \mu\mathrm{M} \quad (1)$$

The malondialdehyde equivalent concentration c(MDE) has to be referred to the molar concentration of the reagents, e.g. unsaturated fatty acids and phospholipids (c_0), to obtain a relative measurement c_{TBArel} (%) of the molecular breakdown (m_{TBA} is the weight of the thiobarbituric acid equivalents in 1.0 ml sample in ng) [25]

$$c_{\mathrm{TBArel}}(\%) = \frac{c\ (\mathrm{MDE}) \times m_{\mathrm{TBA}} \times 100}{c_0} \quad (2)$$

For the TBA reaction, a Merck-Hitachi HPLC system (Merck-Hitachi, Darmstadt, Germany) equipped with an auto-sampler AS-4000A, interface D-6000A, pump L-6200A, UV-VIS-Detector L-4250, and fluorescence detector F-1080 was used. Malondialdehyde-*bis*-(dimethylacetal), 2-thiobarbituric acid and trichloroacetic acid for TBA reaction were obtained from Sigma (Deisenhofen, Germany). Methanol for chromatography was purchased from Merck (Darmstadt, Germany).

2.4. Particle size determination

Particle diameters were determined using an Autosizer 2c with series 7032 Multi-8 Correlator by Malvern Instruments (Malvern, Worcester, UK).

2.5. Mass spectrometry

The mass spectrometric analysis was carried out using a Finnigan LCQ ion trap mass spectrometer with ESI interface and integrated syringe pump (ThermoQuest, San Jose, CA, USA).

Electrospray mass spectrometry was performed in the negative mode with an ESI voltage of 4.5 kV and a capillary

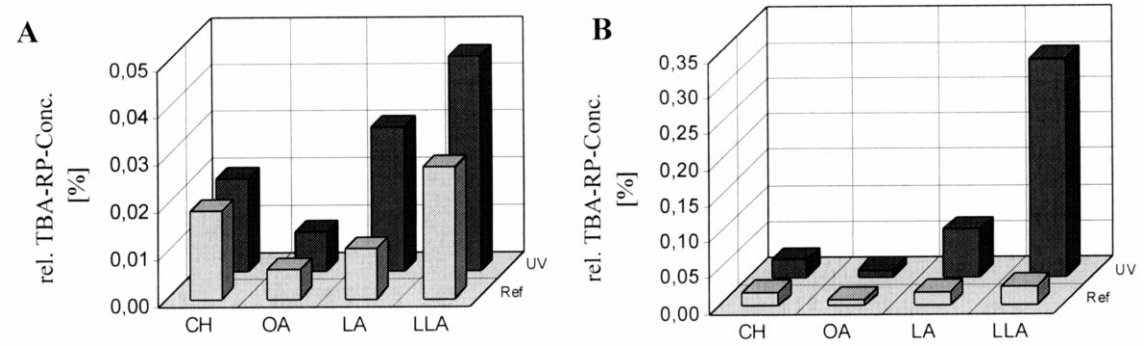

Fig. 2. TBA results of Laser irradiated samples without Fe(II) ions (A) and with 10 μM Fe(II) ions (B), CH, cholesterol; OA, oleic acid; LA, linoleic acid; LLA, linolenic acid; UV – the irradiated specimen and Ref the untreated reference sample.

temperature of 200°C. The aqueous samples were mixed with methanol (final concentration of sample for MS 1 μg/ml) to guarantee a stable ESI spray during injection via a syringe pump (10 μl/min). Collision induced dissociation (CID), Tandem-MS and MS^n -experiments were used to identify the primary and secondary products of lipid peroxidation.

The methanol, gradient grade for mass spectrometry, was purchased from Merck (Darmstadt, Germany).

2.6. Data analysis

All analytical data represent the mean values of sextupled measurements. The standard deviation always was below 5% (SD < 5%).

3. Results and discussion

3.1. TBA-Reaction results

3.1.1. Simple systems

3.1.1.1. Fatty acids and cholesterol Monochromatic irradiation at 349 nm laser and UV light covering the entire solar spectrum produced similar effects (data not shown). Both UV treatments were rather ineffective unless iron was present. A drastic increase of TBA product levels was observed in system containing linolenic acid instead of linoleic acid (Fig. 2). This augmentation is certainly caused by the additional double bond of linolenic acid. The UV absorption shifts to higher wavelengths and, therefore, a direct stimulation of linolenic acid via UV radiation may account for the high effect.

To establish whether the extent of lipid peroxidation depends on the dose of radiation or on a partially chemical reaction, we carried out irradiation-time dependence experiments and studies with changing iron concentrations (Fig. 3). As shown in the Fig. 3, chemical peroxidation appears to be rate limiting, because after 1440 min the TBA reaction level has only doubled compared to 30 min irradiation time.

The dependence between the iron concentration and TBA product levels appears to be non-linear (Fig. 3B).

In conclusion, the rate-limiting step of lipid peroxidation is neither a direct result of UV irradiation dose nor a chemical reaction catalysed by transition metal ions.

3.1.1.2. Phospholipids. As hypothesised, UV irradiation of saturated phosphatidylcholines such as DLPC, DPPC and DSPC does not yield any TBA products even in the presence of iron. Unsaturated compounds, which are

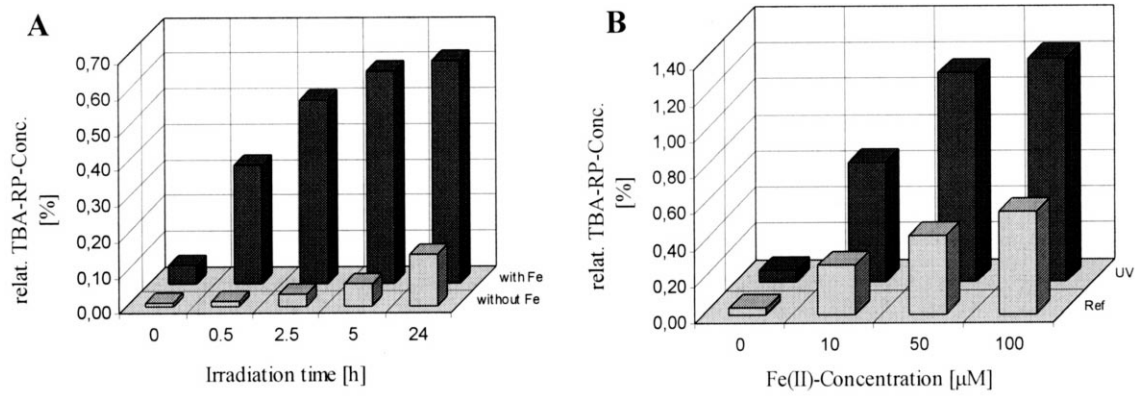

Fig. 3. LLA Radiation time dependence (A) and iron concentration dependence (B).

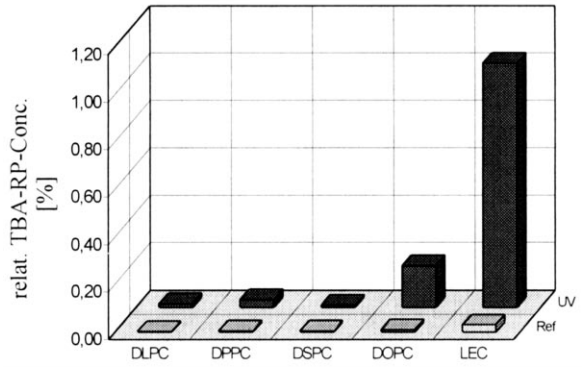

Fig. 4. Phosphatidylcholines irradiated by OSRAM lamp with 10 μM Fe(II) ions.

present in commercially available lecithins, react similar to the pure unsaturated fatty acids (Fig. 4).

3.1.2. Complex systems

The increase in lipid peroxidation is remarkable in liposomal samples consisting of saturated phosphatidylcholines and linolenic acid instead of pure linolenic acid (Fig. 5). This effect contributes to the different absorption behaviour of linolenic acid when encapsulated in liposomes. Bathochrome effects for the randomised distribution of unsaturated fatty acids in the structure of liposomal bilayers have been described in the literature [26].

The considerably elevated peroxidation of liposome-encapsulated linolenic acid could be explained through an increased reduction of Fe(III) ions. Phosphatidylcholines are known to act as complexators for Fe(III) ions. Thus, with more Fe(II) ions as Haber–Weiss catalysts available in solution, the formation of TBA product is accelerated. By comparing samples containing iron and iron-free samples, it was shown that the oxidative damage by UV-A radiation in unsaturated lipids depends on the presence of Fe(II) ions.

The UV absorbance of liposomes shifts to higher wavelengths compared to the single components. This is similar to the process observed during the generation of crystals.

The results show that neither UV radiation nor the presence of transition metal ions alone can trigger lipid peroxidation, but only the combination of both starts the reaction. There are three hypotheses to explain the results:

1. Primarily electron system activation occurs in the presence of sensitising Fe(II). This activation generates oxygen radicals (superoxide and hydroxyl radicals) which react with the lipids.
2. UV radiation generates reactive oxygen species directly. Transition metals catalyse the radical chain reaction and accelerate lipid peroxidation.
3. The lipids absorb UV light which again starts the chain reaction involving Fe(II).

Considering the widespread use of liposome formulations

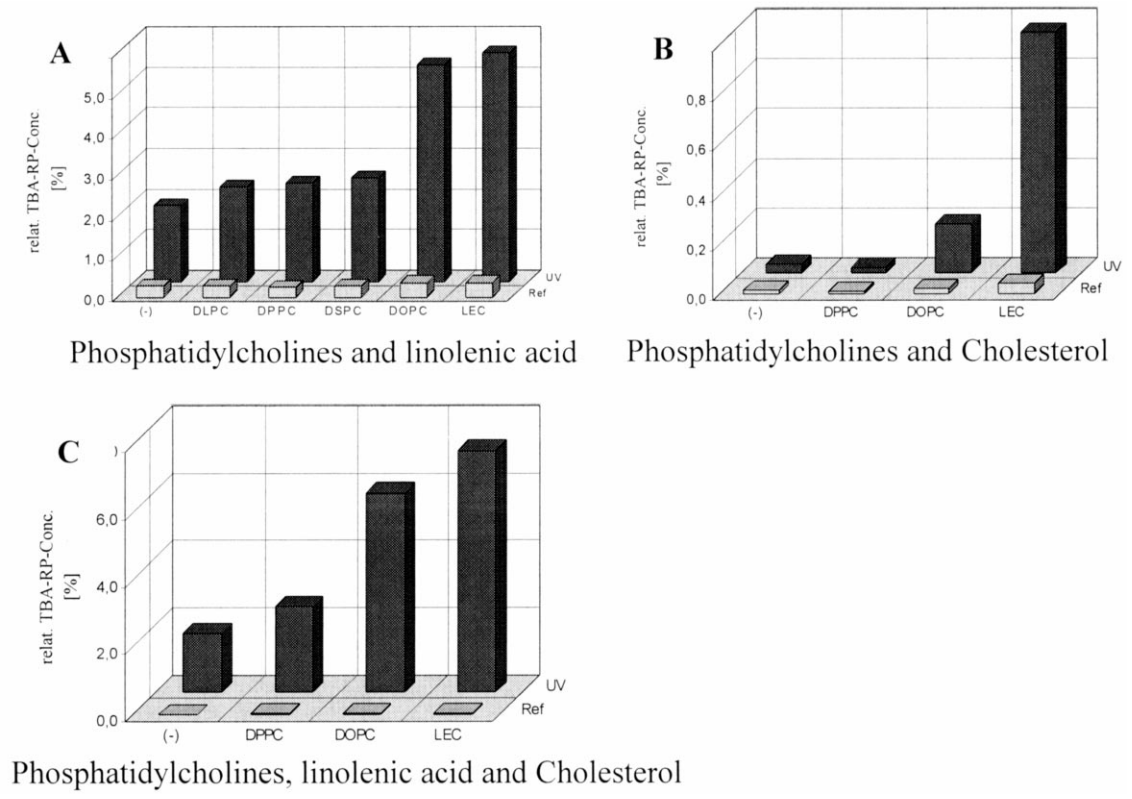

Fig. 5. (A–C) Irradiated by OSRAM lamp with 10 μM Fe (II) ions.

Table 1
Size classes of the liposomes

	Size class									
	1	2	3	4	5	6	7	8	9	10
nm	100–150	150–200	200–400	400–700	700–1000	1000–1500	1500–2000	2000–4000	4000–7000	7000–10 000

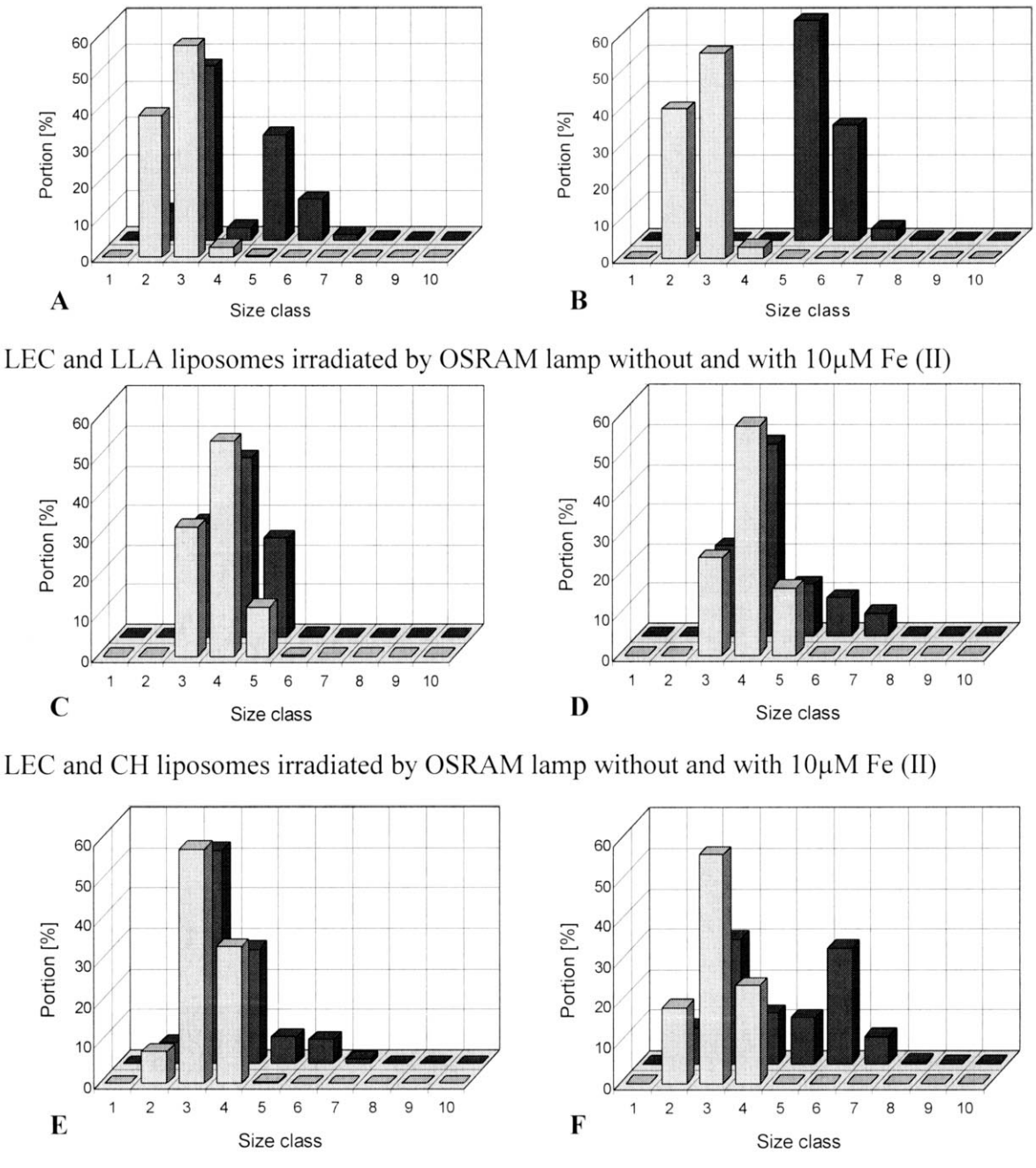

Fig. 6. (A–F) Complex systems irradiated by OSRAM lamp. (A,C,E) without Fe (II); (B,D,F) with 10 μM Fe(II); light grey columns show sample data before irradiation; the dark grey columns represent the data of the UV irradiated samples.

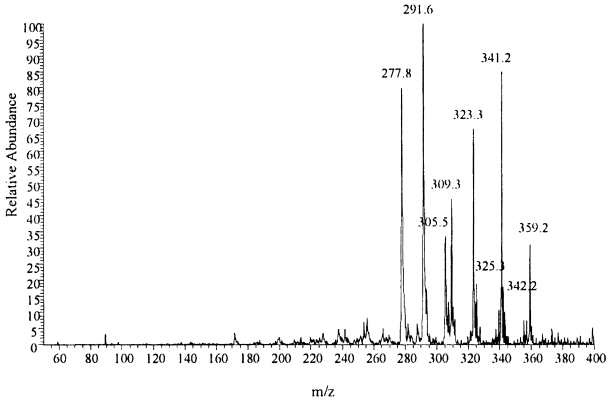

Fig. 7. Negative ion mode mass spectrum of linolenic acid irradiated 120 min by OSRAM lamp.

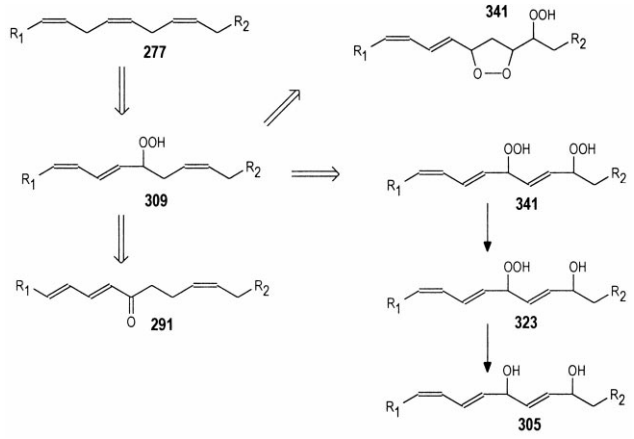

Fig. 8. Ways of linolenic acid peroxidation caused by UV-radiation.

for dermal application in cosmetics, the increased sensitivity of fatty acids in the present of ionised iron and UV light seems to be an alarm signal.

3.2. Particle size determinations

For better understanding, the sizes of the liposomes are divided into ten classes of different nanometer diameters as determined by the Autosizer (see Table 1).

3.2.1. Complex systems

The particle size distribution of the liposomes is not affected by the addition of iron ions as one can see by comparing the light grey columns of the different systems before and after the addition of ionised iron (Fig. 6).

UV radiation causes either liposome degradation or increases the liposomal diameter. The UV-related increase in particle diameter results from hydroperoxide generation of unsaturated fatty acids and unsaturated side chains in phospholipids. When peroxidised, fatty acid chains require more space within the liposomes. The hydrophobic van-der-Waals interaction between the lipophilic chains seems to be hindered by polar peroxide groups.

Increasing diameters apparently alter the permeability and the fluidity of the bilayers.

3.3. Mass spectrometry studies

ESI mass spectrums were recorded from all samples including blanks. There were significant changes in the spectrums only after irradiation of systems containing only linoleic and linolenic acid.

3.3.1. Linolenic acid

Fig. 7 shows the spectrum of linolenic acid after a 120-min UV irradiation.

The mass spectrums, and the pattern of the possible reactions show M-H peaks or masses of the compounds. The mass spectrum confirms the existence of hydro- and endoperoxides. It is interesting that, regarding to their mass spectra, the samples are stable for days. They were stored at room temperature and protected from light. Hence, hydro- and endoperoxides are stable primary products of LLA lipid peroxidation. Both mono-peroxidised compound at m/z 309 and di-peroxidised fatty acids at m/z 341 were detected.

Fig. 8 shows the proposed pathway of LLA lipid peroxidation.

The result suggests a long half-life of the primary products of peroxidation of lipids like LLA. It can be assumed that there are no substrates in the samples to react with these compounds. Considering the long-term stability of these products in living tissues and their potential to penetrate into deeper regions of human skin, further research remains necessary to study adverse reactions of lipid peroxides in viable cells of the epidermis.

4. Conclusions

1. Unsaturated fatty acids like oleic, linoleic and linolenic acid in methanolic solution absorb UV light at wavelengths below 280 nm.
2. Aqueous preparations of these substances do not show significant lipid peroxidation after UV radiation (UV lamp or UV-A laser) as demonstrated in the TBA assay.
3. In the presence of Fe(II) ions (10 μM), lipid peroxidation is clearly increased, but requires double bonds in the fatty acids.
4. Hydro- and endoperoxides are intermediates of lipid peroxidation with long-term stability.
5. Encapsulation of unsaturated fatty acids into phospholipid liposomes increases their sensitivity to UV radiation.
6. Peroxidation of these fatty acids increases the average liposome diameter.
7. Monochromatic irradiation at 349 nm and normal UV radiation induce similar lipid peroxidation.

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