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Original scientific paper

Investigation into the potential chemical mechanism of the pro-oxidant activity of carotenoids with liposomes under UV-irradiation

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Abstract: This study focuses on the behavior of *β*-carotene and lutein inside multilamellar liposomes under continuous UV-irradiation. The liposomes were obtained by the thin film method and carotenoids (Crts) were incorporated by mixing at various concentrations (0.005, 0.0075, 0.02, 0.07 and 0.5 mol %). Liposomes formation and the presence of Crts inside them were confirmed by SEM microscopy and FT-IR spectroscopy, respectively. The antioxidant/pro-oxidant activity of Crts inside liposomes was determined by the thiobarbituric acid–malondialdehyde (TBA–MDA) test. The investigated Crts acted more or less unexpected (as pro-oxidants) inside the lipid bilayers, interacting with the UV-produced lipid radicals and simultaneously suffering under the UV-irradiation. Their pro-oxidant activity with liposomes and under UV-irradiation could be explained by the formation of unstable adducts in the reaction with peroxyl radicals, or by Crts-cation radicals formation *via* the electron transfer mechanism. Such tentatively unexpected behavior of carotenoids should be taken into consideration in further carotenoids-based UV-filters projections in cosmetic formulations for skin protection.

Keywords: *β*-carotene; lutein; liposomes; UV-irradiation; anti/pro-oxidants.

INTRODUCTION

Carotenoids (Crts) are one of the most effective quenchers of singlet oxygen and/or the excited triplet states of photoactive molecules. They are usually classified as preventive antioxidants, since they quench singlet oxygen ($^1O_2$), thereby suppressing destruction of critical cellular biomolecules, such as lipids, proteins and DNA.[1–5] However, Crts are also very efficient chain-breaking antioxidants since they react with already generated free radicals, interrupting free-radical chain reactions.[6–10] However, the exact mechanism of Crts anti- or pro-oxidant act-

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ivity is still not fully understood although many authors have tried to explain their controversial behavior[11,12] *via* higher oxygen partial pressure in the system,[2,6,13–15] Crts concentration,[15,16] their instability to light and high temperature,[17,18] reaction with singlet oxygen and the formation of oxy-products of Crts.[19–23]

Since the investigation of mutual interactions between the main constituent of biomembranes is complicated *in vivo* due to there being many variables that have an influence on biomembrane functioning, they are very often investigated *in vitro* using lipid models produced from different types of phospholipids. Liposomes are one of the very common biomembrane models; they are artificially-prepared vesicles consisting of lipid bilayers often composed of phosphatidylcholine-enriched phospholipids. Depending on the number of bilayers, liposomes could be classified as unilamellar, multilamellar and multivesicular. Unilamellar ones are further classified by size as small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) and giant unilamellar vesicles (GUV).[24]

Different agents are recognized as initiators of harmful free radical- mediated processes leading to biomembranes destruction. One of the most effective is lipid peroxidation (LP). Lipid peroxidation is either a free radical chain reaction (Type I) or, it occurs through a non-radical pathway (Type II), by direct reaction with singlet oxygen, created in the presence of a photosensitizer.[25–27] Like every other free radical chain reaction, LP consists of an initiation step (formation of lipid radicals – L, by abstraction of allylic and doubly allylic H-atoms from the hydrophobic polyunsaturated moieties of lipids), a propagation step (where lipid radicals react with oxygen to form lipid peroxyl radicals, $LOO^{\bullet}$), and a termination step (formation of lipid hydroperoxides, LOOH, with diene-type structures).[6,13,27] Typical lipid peroxidation initiators are reactive oxygen species (ROS), such as hydroxyl radicals ($^{\bullet}OH$) or peroxyl radicals ($ROO^{\bullet}$). They can be created through a variety of chemical reactions and external stresses (including UV-irradiation).[27]

The goal of this study was to investigate the behavior of β-carotene and lutein with multilamellar liposomes under continuous UV-irradiation. The investigated Crts acted more or less unexpected (as pro-oxidants) inside the lipid bilayer, interacting with the UV-produced lipid radicals but simultaneously, suffering under UV-irradiation.

EXPERIMENTAL

Materials

Phospholipids (Phospholipon® 90, PL90) were gifted by Phospholipid GmbH, Köln, Germany. According to the accompanying declaration, the mixture content were phosphatidylcholine 98 %, lysophosphatidylcholine 2.1 %, and the fatty acid composition was: palmitic acid 12±2 %, stearic acid 3±1 %, oleic acid 10±3 %, linoleic acid 66±5 % and linolenic acid 5±2 %. The maximum peroxide value was 1.3. The phospholipids mixtures were kept in the dark to prevent at least photo-oxidation processes.

Liposomes preparation

Liposomes were prepared by the thin film method while Crts (β-carotene and lutein) were incorporated by the mixing method: 0.2 g of phospholipids was dissolved in 3 cm^3 of chloroform, in a 10 cm^3 test tube, and vortexed. The total volume was divided into three equal portions in 10 cm^3 test tubes. One portion was separated for the preparation of "empty" liposomes. In the remaining two portions, the exact volume of Crts chloroform solution was added*. All tubes were placed under nitrogen blowers until all the chloroform had evaporated and a dry thin film remained on the test tubes walls. The tubes with thin lipid film were placed in an desiccator connected to an oil vacuum pump for 3–5 h in the dark. Hydration was performed by direct addition of phosphate buffer (2.5 cm^3, 0.1 mol dm^{-3}, pH 7.4) inside the tubes, followed by heating in a water bath (40 °C) and vortexing. When the entire film from the vessel wall had dissolved and an opalescent dispersion obtained, freeze–thaw process was started (alternately immersion of the tubes in liquid nitrogen and a water bath at 40 °C at least 5 times, 5 min each time). After the freeze–thaw procedure, which was used in order to improve Crts incorporation, the liposomes were vortexed 10 min and diluted with phosphate buffer up to 12.5 cm^3 in 100 cm^3 round bottom flasks. The vessels were sealed with a rubber septum, filled with nitrogen and subjected to sonication for 30 min at room temperature. After sonication, the liposomes were again vortexed for 10 min and centrifuged for 5 min at 4000 rpm in order to precipitate agglomerates and eventually formed Crts aggregates. The supernatants were diluted with phosphate buffer up to final volume of 25 cm^3. The solutions were bubbled with nitrogen, tightly sealed and stored at 4 °C in the dark.

Thiobarbituric acid–malondialdehyde (TBA–MDA) test

Lipid peroxidation inside the liposomes, as well as its inhibition in the presence of Crts, was measured by the TBA–MDA test.[28] This method is based on the reaction of malondialdehyde (MDA, secondary product of lipid peroxidation) with thiobarbituric acid (TBA) to obtain a red colored complex with maximum absorption at 532 nm. Liposomes with incorporated carotenoids in concentrations of 0.005, 0.0075, 0.02, 0.07 and 0.5 mol % were prepared for the TBA–MDA test. The test was performed simultaneously in three quartz cuvettes: cuvettes "sample" and "blank" contained 0.5 cm^3 of liposome dispersion with incorporated carotenoids. "Control" cuvette contained 0.5 cm^3 of "empty" liposomes (without carotenoids). Lipid peroxidation has been initiated by continuous UV-irradiation (UV-C and UV-B) during increasing time periods. The cuvettes were irradiated simultaneously. One mL of aqueous trichloroacetic acid (5.5 %) and 0.5 cm^3 of methanolic solution of BHT (1×10^{-3} mol dm^{-3}) were added to all cuvettes immediately after irradiation. Thiobarbituric acid solution, 0.5 cm^3 (4.2×10^{-2} mol dm^{-3} in 5×10^{-2} mol dm^{-3} NaOH) was added to the cuvettes "sample" and "control". The "blank" cuvette contained 0.5 cm^3 of water instead of TBA solution. All cuvettes have been incubated for 10 min at 50 °C in the dark, and centrifuged for 10 min at 5500 rpm. The visible spectra of the TBA–MDA complex in the "sample" and "control" were recorded from 400 to 800 nm against "blank". The increase in the absorbance of the "sample" and "control" at 532 nm was monitored and graphically presented.

UV-irradiation

Continuous irradiations of samples were performed in cylindrical photochemical reactor "Rayonnet", with 14 symmetrically placed lamps (Southern N.E. Ultraviolet Co., Hamden, CN) with emission maxima in three different ranges: 254 (UV-C) and 300 nm (UV-B). The

* $\varepsilon_{\beta\text{-car}}^{\text{chloroform}} = 128500\ dm^3\ mol^{-1}\ cm^{-1}$ and $\varepsilon_{\text{lutein}}^{\text{ethanol}} = 172000\ dm^3\ mol^{-1}\ cm^{-1}$ – the extinction coefficients used for Crts concentration adjustment.

samples were irradiated in quartz cuvettes (1 cm) placed on a rotating circular holder. The total emitted measured energy flux (measured by UV-meter, Solarmeter, model 8.0, Solartech) was about 25 W m^{-2} for 254 nm and 21 W m^{-2} for 300 nm. The samples absorb most of the incident energy flux: in the case of UV-C, it was about 94 %.

SEM microscopy

A thin film of liposomes was fixed on a membrane filter (pore size 0.45 μm, 0.5 cm square) by immersion in 1 % osmium tetroxide solution for 60 min. Thereafter, the sample was immersed in phosphate buffer (0.1 mol dm^{-3}, pH 7.2) for 20 min, and one more time for 10 min in pure buffered saline (pH 7.2), in order to complete the removal of free osmium tetroxide. The samples were dehydrated by immersion in solutions of different ethanol concentrations: 30, 50, 70, 96 and 100 %, twice for 3 min. Each sample was air dried for 5–10 min, flushed with nitrogen and tightly closed. The dried samples were stacked on a metal carrier and coated with layer of gold for 5 min in a JEOL JFC-110E-Ion Sputter, imaging was performed by an incorporated photographic camera (The micrographs were taken on a JEOL JSM 5300 (Japan) microscope), while processing of photographs was realized by a VEGA--CAM system using OZARIA® program package.

UV–Vis and FTIR spectrophotometry

The UV–Vis spectra of samples before and after irradiation with UV-light were recorded on a Varian Cary-100 spectrophotometer in the wavelength range from 200 to 600 nm using 1 cm cuvettes.

The FTIR spectra of the investigated samples were recorded on a Bomem MB-100 spectrophotometer in the range 4000–400 cm^{-1}, with a resolution of 2 cm^{-1}. The samples were prepared by coating KRS-5 plates with 200 μl of liposomes and dried for 24 h in the dark under vacuum in a desiccator connected to an oil vacuum pump. Clean KRS-5 plate were used as a background.

RESULTS

Structures of carotenoids used in this work are presented in Fig. 1.

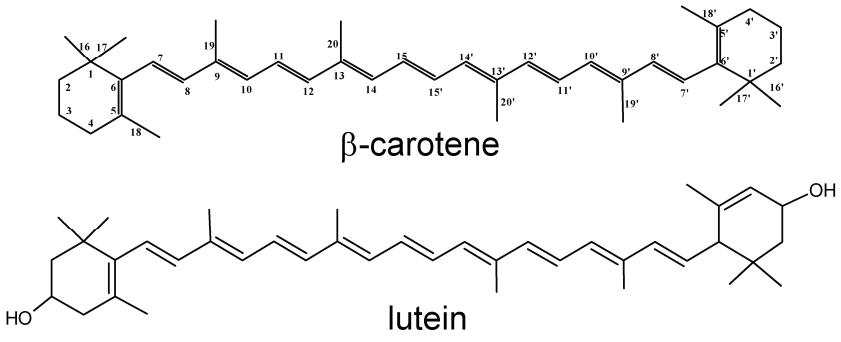

Fig. 1. Structures of the investigated carotenoids.

As an illustration and some kind of evidence that liposomes were really formed, their SEM micrographs are shown in the Supplementary material to this paper (Fig. S-1 of the Supplementary material to this paper).

The FTIR spectra of "empty" multilamellar liposomes as well as of those with incorporated β-carotene at a concentration of 0.07 mol % are shown in Fig. 2.

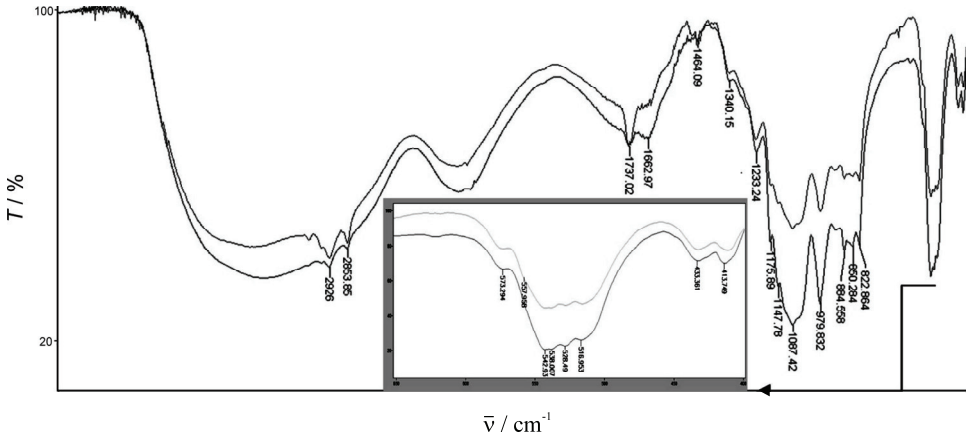

Fig. 2. FTIR spectra of "empty" liposomes (lower spectrum) and liposomes with incorporated β-carotene at a concentration of 0.07 mol % (upper spectrum). Magnified part 650–400 cm^{-1} is represented as an inlet.

The FTIR spectra of "empty" multilamellar liposomes as well as of those with incorporated lutein at a concentration of 0.07 mol % are shown in Fig. 3.

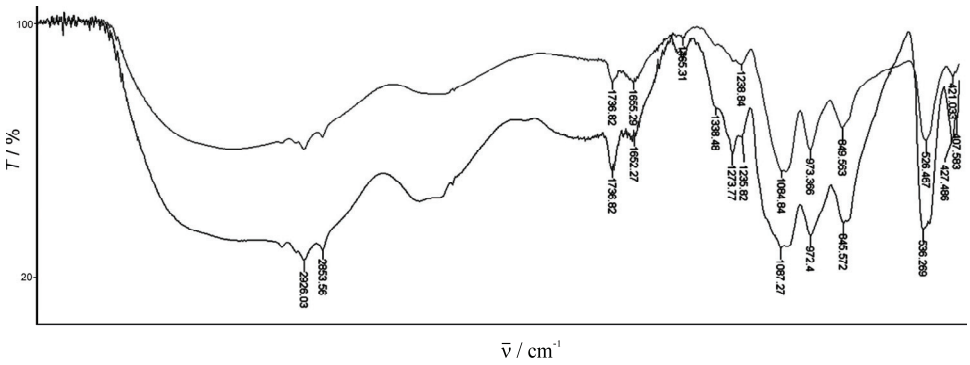

Fig. 3. FTIR spectra of "empty" liposomes (upper spectrum) and liposomes with incorporated lutein at a concentration of 0.07 mol % (lower spectrum).

Comparative representations of the absorbance changes of the "control" and "sample" at 532 nm are given in Figs. 4 and 5. The "control" is the TBA–MDA complex in liposomes without carotenoids and the "sample" is the TBA–MDA complex in liposomes with incorporated carotenoids (β-carotene in Fig. 4 and lutein in Fig. 5). Initiation of lipid peroxidation was realized by continuous UV--irradiation (UV-C, UV-B and UV-A range; not all data are shown).

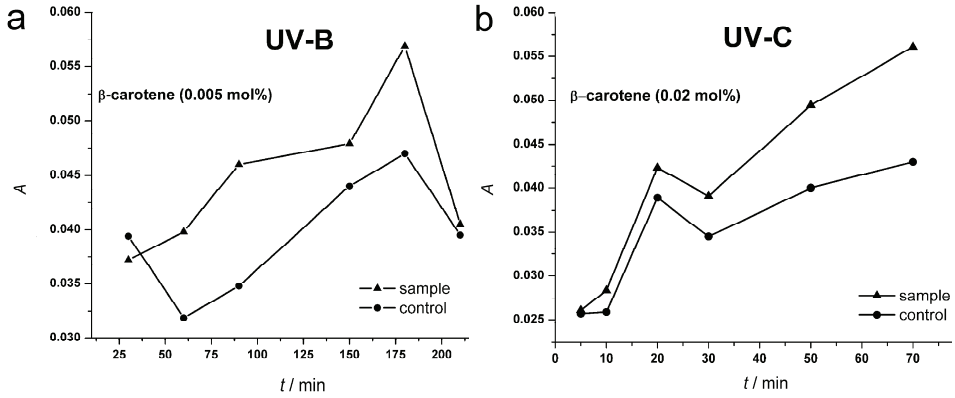

Fig. 4. Dependence of the absorbance at 532 nm for the "control" and "sample" on irradiation time, obtained by the TBA–MDA test, for β-carotene inside liposomes at a concentration of 0.005 (a) and 0.02 mol % (b). Lipid peroxidation was initiated by continuous UV-B (a) and UV-C (b) irradiation.

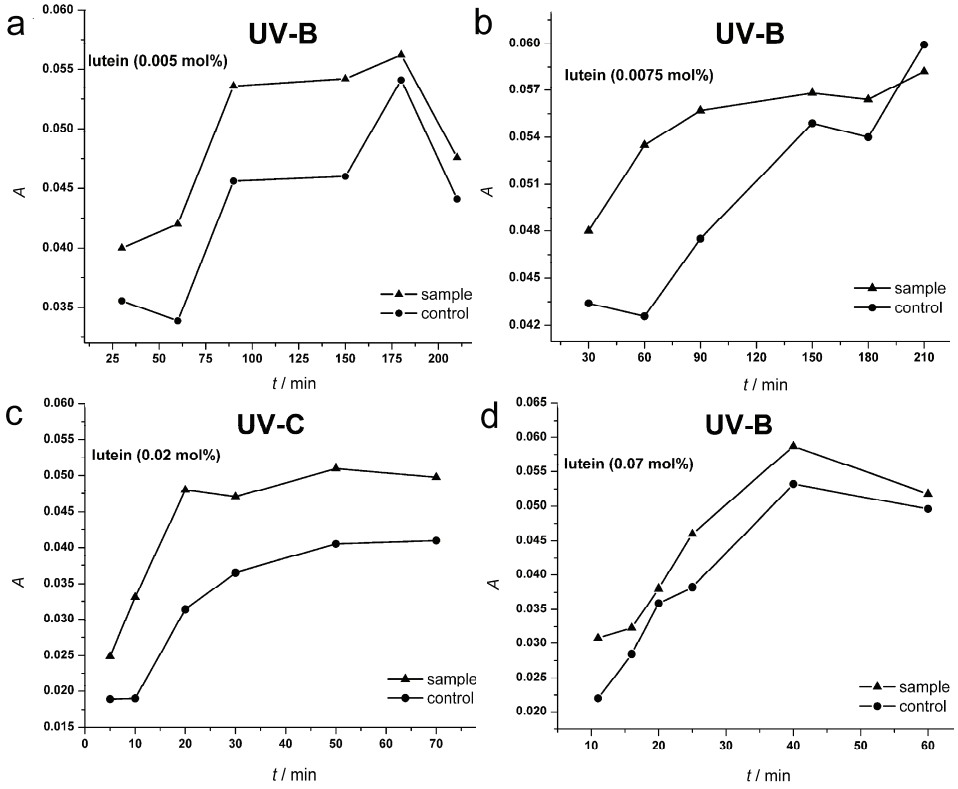

Fig. 5. Dependence of "control" and "sample" absorbance (at 532 nm) on irradiation time, obtained by TBA–MDA test, for lutein inside liposomes in concentration of 0.005 (a), 0.0075 (b), 0.02 (c) and 0.07 mol% (d). Lipid peroxidation was initiated by continuous UV-B (a, b and d) and UV-C (c) irradiation.

DISCUSSION

The presence of Crts inside liposomes was investigated by FTIR spectroscopy *via* monitoring the Crts–phospholipids interaction. The main spectral bands in the FTIR spectra originated from stretching of C–H bonds (around 3000 cm^{-1}), C=O bonds (around 1740 cm^{-1}) and C–O bonds (around 1160 cm^{-1}), as well as stretching vibrations of the PO_2^- group (around ν_{as} = 1250 cm^{-1} and ν_s = = 1090 cm^{-1}), and stretching vibrations of the N^+–$(CH_3)_3$ group (around 970 cm^{-1}). There were also wagging and rocking vibrations of $–CH_2$ groups, δ-vibrations at 1470 cm^{-1} and γ-vibrations at 1300 and 720 cm^{-1}.[29–33]

The FTIR spectra of liposomes with incorporated *β*-carotene (Fig. 2) and liposomes with lutein (Fig. 3) both at a concentration of 0.07 mol % were recorded as dry sediment on KRS-5 (TlBr–TlI) pellets. *β*-Carotene and lutein were incorporated from the beginning, *i.e.*, by the mixing method. There are a few characteristic regions in the FTIR spectra of organized phospholipids, *i.e.*, a band at around 1240 cm^{-1} and one at around 1090 cm^{-1} originating from PO_2^- asymmetric and symmetric stretching vibrations, respectively There is also a band arising from the stretching vibrations of PO_2^- group at 1060 cm^{-1}, as well as region of N^+–$(CH_3)_3$ asymmetric vibrations at around 970 cm^{-1}.[34,35] These bands have been used for investigations of the interactions of incorporated Crts with polar head-region of liposomes in lipid bilayers. On the other hand, the influence of Crts on the organization of the alkyl chains of phospholipids, which form the hydrophobic core of a membrane, could eventually be investigated by investigation of the interactions of the Crts conjugated polyenic system with the methyl groups of the alkyl lipid chains, by following changes in the bands at 2850 (stretching vibrations) and 1465 cm^{-1} (deformation vibrations).[34,35] It could be concluded that *β*-carotene (Fig. 2) and lutein (Fig. 3) influence the spectral structure at 2854 cm^{-1} in terms of increasing the intensity of the bands and shifting them slightly to lower wavenumbers, suggesting that incorporated Crts have an influence on the freedom of motion of the alkyl chains in the hydrophobic core of lipid membrane, *i.e.*, carotenoids probably interact with the methylene groups in the alkyl chains *via* van der Waals forces. The band at 1465 cm^{-1}, originating from deformation vibrations of the alkyl chain methylene groups, is very weak, but gains in intensity to some extent when the Crts were incorporated, especially lutein (Fig. 3). It is also evident that lutein has strong interactions with the PO_2^- groups based on the symmetric and asymmetric stretching vibrations at 1090 and 1240 cm^{-1}, respectively, which are particularly sensitive to hydrogen bonding.[36] Since lutein has hydroxyl groups on both sides, it interacts with PO_2^- groups through hydrogen bonds *via* its polar ends. According to this, it could be assumed that lutein "breaks through" the membrane, *i.e.*, its polar ends are located within the polar region of the liposomes phospholipids bilayer, and its conjugated chain interacts with the hydrophobic core of the lipid

bilayer (Fig. 6). The band at around 1060 cm^{-1} is assigned to the stretching vibrations of the PO_2^- group. Liposomes with incorporated lutein (Fig. 3) have a different structure of the 1200–1020 cm^{-1} band in comparison with that of the "empty" liposomes, confirming the relatively strong influence of lutein in the region of the polar heads of the lipid bilayer. Liposomes with incorporated *β*-carotene have a very similar structure of this band to that of the "empty" liposomes (Fig. 2), which is expected according to the non-polar nature of *β*-carotene.

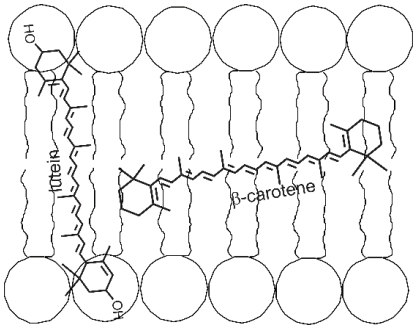

Fig. 6. Presumed positions of the investigated carotenoids inside a lipid bilayer.

Bands below 600 cm^{-1} probably originate from deformation vibration of the phosphate groups of phospholipids.[37,38] It is known that incorporation of PO_4^{3-} within a crystal decreases the symmetry of the bonds in this group, which can be investigated *via* the so-called ν_4 region of out-of-plane deformation vibrations in the 570–530 cm^{-1} region.[37,38] Since there were obvious changes in the shape and position of these bands for "empty" liposomes, and for those with incorporated carotenoids (Figs. 2 and 3), it could be concluded that this region (~570 and ~530 cm^{-1}) has great potential in the monitoring of the effects of carotenoids on the physical properties of membranes. In fact, *β*-carotene (Fig. 2) had no significant effect on the structure of the 516–542 cm^{-1} band, or on the position of the weak band at 570 cm^{-1}. Effect of lutein on structure and intensity of this band (around 536 cm^{-1}) was strongly expressed, since its incorporation (0.07 mol %) significantly increased the band intensity (Fig. 3) and pronounced structuring occurred. There was also a significant band shift to higher wavenumbers in comparison to the "empty" liposomes (526 cm^{-1}, Fig. 3). This shifting probably occurred due to interactions of the lutein hydroxyl groups with PO_4^{3-} of the phospholipids, through hydrogen bond formation, which cause ν_4 band shifting to higher wavenumbers. Hence, it could be concluded that lutein through its polar ends, *i.e.*, –OH groups, strongly interacts with phosphate groups of phospholipid molecules, since lutein probably penetrate the membrane like a rivet. Non-polar *β*-carotene, on the other hand, is probably immersed deep in the lipid bilayer (Fig. 6), and interacts substantially weaker with the phosphate group of the phospholipids molecules.

Besides FTIR spectra, the UV–Vis spectra of encapsulated Crts directly inside PL90 liposomes was also measured using an Olis Aminco DW2 spectrometer specialized for analyzing samples with a large scattering (Supplementary material, Fig. S-2). These spectra confirmed that encapsulated Crts were not aggregated suggesting that there was no contact of the Crts with water. Bearing this in mind, it could be concluded that the Crts chromophores were surrounded by hydrophobic fatty acid chains, and hence, it was assumed that they are completely encapsulated into the lipid bilayer of the formed liposomes.

TBA–MDA test. There is significant contradiction in the published results concerning the antioxidant activity of Crts. Crts are not universal antioxidants, one could be an antioxidant in some environment under certain conditions to some extent, but in other circumstances and in another environment could act in a diametrically opposite manner. Many researchers have tried to explain the mechanisms of antioxidant activity and the conditions under which Crts act as antioxidants or pro-oxidants. Two of the pioneering works in this field are the works of Burton and Ingold[6,39] in which an attempt was made to explain the behavior of β-carotene as an unusual antioxidant that cannot, according to its mechanisms of action, be classified into any group of known antioxidants. The authors studied the behavior of β-carotene in light of the experimental conditions – partial pressure of oxygen in the system (p_{O_2}), primarily. They concluded that β-carotene acts as an antioxidant at low p_{O_2} (ten times lower than atmospheric), and at very low β-carotene concentrations, by reaction with peroxyl radicals ($LOO^{\bullet}$), and carotenoid-radicals formation; or, much more likely, by the mechanism of peroxyl radical addition to the long conjugated chain of Crts, leading to the formation of resonance-stabilized carbon-centered radicals ($LOO–Crt^{\bullet}$). Thus, the formed radicals in further reaction with peroxyl radicals form non-radical products (adducts). In this way, peroxyl radicals are consumed and carotenoids act as chain-breaking antioxidants.[6,13,40–43] At higher oxygen partial pressures (atmospheric or higher), β-carotene shows pro-oxidant activity due to the reaction of carotenoid radicals with normal triplet oxygen, whereby carotenoid–peroxyl-radicals ($Crt–OO^{\bullet}$) are generated and they can act as pro-oxidants.[15] The generated adducts can further decompose (*e.g.*, *via* Crt–epoxides) and form radical species.[44–48]

Many authors have come to tentatively very contradictory results, which suggest the complexity of investigations of Crts antioxidant/pro-oxidant behavior, both *in vitro* and *in vivo*. First, differences in Crts behavior occur in light of analyzed system, *e.g.*, solution or organized biomembranes models,[13,14,41,49] differences in the lipid composition of the prepared liposomes,[50] the presence of other antioxidants, Crts concentration, partial pressure of oxygen in the system, method used to monitor the lipid peroxidation level and lipid peroxidation initiation (by free-radicals, autoxidation, photosensitized, UV-induced, *etc*.).[13,15–18,41,51]

Lipid peroxidation is usually initiated by azo-initiators[43] or UV-irradiation, in the presence and absence of a photosensitizer[51] in the liposomes. It was shown that in the reaction with peroxyl radicals, rapid bleaching of Crts occurs, caused by their degradation and the formation of oxy-products. Bleaching of Crts due to the formation of their oxidation products destroys their chromophores, *i.e.*, breaks (shortens) the Crts conjugated system.[40,42,43,45,46,52–54] Shortening of Crts conjugated chain leads to apo-carotenals and apo-carotenones formation, whereby the formation of carotenoid–epoxides is also very common. These compounds are also formed in the reaction of singlet oxygen with Crts. Singlet oxygen can add onto Crts, when labile auto-oxygenated products of Crts are formed, potentially reacting as pro-oxidants.[14,19–24,40] Some authors followed the pro-oxidant activity of Crts as a result of their instability to light and high temperatures, as a function of Crts concentration and the type of protective lipoidal targets.[17,18]

Results obtained by TBA–MDA test in this work for *β*-carotene and lutein at different concentrations are represented in Figs. 4 and 5, respectively. Initiation of LP is simplified since only UV-irradiation was used for this purpose. Namely, UV-light is, practically, always (based on literature data) used in combination with another radical initiator. In most cases that was an appropriate photosensitizer,[4,27,55,56] but salts of iron or other transition metal, Fenton systems,[57–59] *etc*. are as well. The primary products of UV-irradiated polyunsaturated phospholipids are lipid radicals obtained by removal of H-atoms from fatty acid chains. The lipid radicals formed react with the oxygen present in the system thus producing peroxyl radicals which initiate the chain reaction of lipid peroxidation, resulting in liposomes destruction.[51] Significant technical simplification of the TBA–MDA test is achieved by UV-initiation of LP (as is shown in this paper), since the number of components in the reaction mixture is reduced to a minimum. The antioxidant activities of *β*-carotene (Fig. 4) and lutein (Fig. 5) were followed by monitoring the control/sample ratio at 532 nm, at all the mentioned concentrations.

β-Carotene showed pro-oxidant activity at all concentrations (0.005 mol %, Fig. 4a; 0.02 mol %, Fig. 4b and at the higher concentration 0.5 mol %, data not shown), generally, the “control” absorbance was lower than the corresponding “sample” absorbance, especially when the samples were irradiated with UV-B light (Fig. 4a). Lutein also showed strong pro-oxidant activity at lower concentrations (0.005 and 0.0075 mol %, Fig. 5a and b, respectively) especially during UV-B irradiation but mainly pro-oxidant activity at higher concentrations (0.02 (Fig. 5c), 0.07 (Fig. 5d) and 0.5 mol % (data not shown)). Some of these experiments were repeated after the entire freeze–thaw procedure had been performed under high vacuum (in vacuum evaporator) in order to eliminate oxygen from the system. Immediately after liposome preparation, the TBA–MDA test was performed. The Crts showed exactly the same behavior (pro-oxidant activity) in

these repeated experiments, whereby the influence of oxygen in the system was considered. Other authors[6,14,15,39,41,42,44–46] obtained very similar results at p_{O_2} values much higher than atmospheric (19998 Pa, 20 % O_2), and even in an atmosphere of oxygen (101325 Pa, 100 % O_2). Namely, they explained the pro-oxidant activity of carotenoids at higher oxygen concentrations, usually in the solution, through Crts autoxidation.

However, pro-oxidant activity of Crts in the results presented herein cannot be explained by autoxidation of Crts since oxygen in the system was not present in high concentrations, on the contrary, it was even lower than atmospheric. So, pro-oxidant effect of Crts has to be connected to another parameters in the system, *i.e.*, to the Crts concentration. Many authors showed that increased concentrations of Crts in the system lead to their stronger pro-oxidant action.[15,17,18,41,51] The large amount of "lipid-originating" reactive radical species formed in liposomes by UV-radiation (in combination with the effect of UV-radiation on the Crts themselves) led to the pro-oxidant activity of the Crts in most experiments, at different concentrations (0.005, 0.0075, 0.02, 0.07 and 0.5 mol %). Haila[13] proposed a mechanism of Crts pro-oxidant action *via* the formation of reactive species leading to auto-oxidation of Crts or decomposition of adducts formed by the addition of lipid peroxide radicals to Crts. It is possible that UV-irradiation, generating a large amount of peroxyl radicals in liposomes and affecting Crts and their adducts, induces decomposition and consequently pro-oxidative actions of Crts. It seems that this effect is a function of the concentration of the system constituents, *i.e.*, lipid/carotenoid ratio. It should be kept in mind that the intensity and interval of UV-irradiation also play an important role in the behavior of Crts inside liposomes. There is no such experimental setup in the literature, *i.e.*, initiation of lipid peroxidation is rarely performed by UV--radiation only, *i.e.*, a photosensitizer is usually present (hence $^1O_2$), or lipid peroxidation was initiated by azo-initiators at high temperatures, by a Fenton system*, etc.* In the present system, in which all participants in the reaction suffered from UV-radiation, analysis of the Crts behavior is more complex. Based on previous studies of UV-radiation effects on Crts in *n*-hexane solution,[60] and antioxidant activity of Crts in solution under influence of UV-radiation without photosensitizer[61,62] and in the presence of benzophenone as a photosensitizer,[63,64] it was concluded that although Crts bleach, they have a certain antioxidant activity toward lipids. It was also concluded that intensive Crts bleaching in solution is most likely due to electron transfer, as well as the formation of Crts cation radicals ($Car^{\bullet+}$) or Crts anion radicals ($Car^{\bullet-}$).[60] When a lipid component was present in solution, it was proved that bleaching of Crts originated from their scavenging activities, *i.e.*, the antioxidant effect of Crts is a result of their ability to neutralize the created lipid radicals (chain-breaking activity). This is the main reason for the increased bleaching of Crts in solutions where benzophenone was

present.[63,64] The mechanism of adduct-formation was the least likely in this system (solution) because of the very rapid Crts bleaching. Bearing in mind that in liposomes, due to the low carotenoid concentration and large light scattering, monitoring of the bleaching of Crts is almost impossible, the mechanism of adduct-formation cannot be completely excluded. The movement of all reaction participants is strongly space-limited inside the liposomes, and a "cage effect" plays a very important role. Furthermore, it was proved[14] that these adducts, depending on the polarity of the environment, decay according to first order kinetics ($k \approx 10^3$ s^{-1}) thus creating species that could propagate lipid peroxidation. Liebler and McClure[65] concluded that β-carotene in reaction with peroxyl radicals gives unstable adducts that could be decomposed to carotene-epoxides and one alkoxyl radical, which could further propagate the chain reaction of lipid peroxidation. Simultaneously, there is evidence that the carotenoid-cation radical ($Car^{\bullet+}$), created by the electron transfer mechanism, is potentially very dangerous in biological systems, damaging proteins and causing their dysfunction. However, this radical is usually neutralized by vitamins C and E in biosystems.[14,47,66] Since there were no vitamins or other chemical species in the present investigated system, it could be concluded that there is another possible "trigger" mechanism for the pro-oxidant behavior of Crts.

On the other hand, the presence of Crts inside liposomes affects the structure of the lipid bilayer of the membrane. This can affect diffusion and enable easy movement of the lipid peroxyl radicals inside the liposomes, and thereby increase lipid peroxidation,[13] At higher concentrations, Crts could aggregate, which affects fluidity and permeability of membrane (increasing them) leading to their pro-oxidant activity.[14,15] Hence, beside the chemical reactions of Crts with a radical species (lipid–peroxyl radicals primarily), steric (spatial) effects and position (location) of Crts also play important roles in their pro-oxidant activity. Polar lutein, which penetrates the membrane as a "nail" with its polar ends (Fig. 6) could react with radicals that tend to accumulate near the phospholipids polar heads of the lipid bilayer of the membrane, while non-polar β-carotene could react with radicals deep inside the lipid bilayer.[2,7,14,41,67] One of the major problems in the investigation of the antioxidant/pro-oxidant activity of Crts is the fact that reaction products are generated in much lower concentrations than the concentration of the Crts themselves, which is also very low at the beginning. Moreover, the primary products of these reactions usually have a very short lifetime and cannot be isolated. Thus, secondary products have to be analyzed, which significantly complicates the detection of reaction mechanisms.

CONCLUSIONS

The potential chemical mechanism of Crts pro-oxidant activity inside liposomes under UV-irradiation could be explained by the formation of unstable

adducts in reaction with peroxyl radicals, or by the formation of Crts-cation radicals *via* the electron transfer mechanism. Steric effects, orientation and location of Crts in liposomes bilayer also play important roles in their antioxidant/pro-oxidant action. Antioxidant behavior of Crts *in vivo*, where they are naturally positioned inside lipid bilayer, suggests that the "right" (natural) carotenoids/lipids ratio is very important. The presented results indicated that Crts could be inefficient UV-protectors under certain conditions. Hence, defining the appropriate conditions and lipid/Crts ratio seems to be important step in projecting cosmetic formulations.

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SUPPLEMENTARY MATERIAL

SEM micrographs and UV–Vis spectra of the investigated materials are available electronically at the pages of the journal website: http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

ИЗВОД

ИСПИТИВАЊЕ ПОТЕНЦИЈАЛНИХ МЕХАНИЗАМА ПРООКСИДАТИВНЕ АКТИВНОСТИ КАРОТЕНОИДА УНУТАР ЛИПОЗОМА ПОД ДЕЈСТВОМ UV-ЗРАЧЕЊА

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Ова студија се бави понашањем β-каротена и лутеина унутар мултиламеларних липозома под континуалним UV-озрачивањем. Липозоми су добијени методом танког филма док су каротеноиди (Crts) инкорпорисани методом мешања у различитим концентрацијама (0,005, 0,0075, 0,02, 0,07 и 0,5 mol %). Формирање липозома и присуство Crts унутар њих су потврђени SEM микроскопијом и FT-IR спектроскопијом, редом. Антиоксидативна/прооксидативна активност каротеноида је праћена TBA–MDA тестом. Испитивани каротеноиди се понашају мање–више неочекивано (као прооксиданси) унутар липидног двослоја, интерагујући са UV-продукованим липидним радикалима, где и сами каротеноиди трпе дејство UV-зрачења. Прооксидативно понашање каротеноида, унутар липозома и под дејством UV-зрачења, може бити објашњено формирањем нестабилних адуката у реакцији са пероксил радикалима, или формирањем Crts катјон-радикала преко електрон-трансфер механизма. Овакво, условно речено, неочекивано понашање каротеноида треба узети у обзир у будућим планирању UV-филтера у козметичким формулацијама за заштиту коже од штетног дејства UV-зрачења.

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