β-Carotene–Lipid Interactions in Liposomes with Different Lipid Composition

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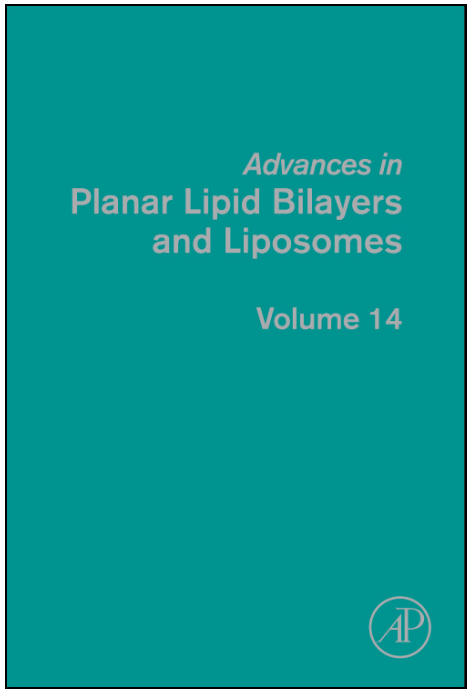

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CHAPTER TEN

β-Carotene–Lipid Interactions in Liposomes with Different Lipid Composition

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Contents

Abstract

Carotenoids perform light harvesting, photoprotection, electron transfer, and structural role in photosynthetic membranes. To unravel the β-carotene contribution to the stability of membranes, liposomes with different lipid composition (resembling the photosynthetic membranes, containing mainly galactolipids with a high degree of unsaturation, and egg phosphatidylcholine) were used. The aim was to gain insight into the mechanism of β-carotene–lipid interactions with a special focus on the fluidity of the bilayer. Data from absorption, pyrene fluorescence, and resonance Raman spectroscopy revealed that the degree of lipids' unsaturation regulates the penetration of β-carotene molecules into the membrane, thus modifying the lipid–pigment interactions.

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1. Introduction

Carotenoids are wide spread natural molecules, with over 600 structurally related compounds, both in plant and animal kingdom, which play multiple important physiological functions. From the spectroscopic and structural point of view the most typical feature of naturally occurring carotenoids is the long polyene chain with conjugated double bonds [1] that are responsible for the pigment properties of carotenoids, to absorb the electromagnetic radiation from the visual region. The double bond system constitutes a rod-like skeleton of the molecule that seems to play a key stabilization function of carotenoids, both in respect to lipid membranes and proteins [2].

In photosynthetic membranes, carotenoids perform different important functions as light harvesting, electron transfer, photoprotection, and structural role, stabilizing the membrane three-dimensional integrity [3–6]. They cover a spectral window lacking chlorophyll absorption (ca. 500 nm) [2,3] and play a protection role against high-light stress and reactive oxygen species, *via* quenching of electronic excited states of chlorophyll *a* molecules [5]. Carotenoids contribute also to the stability of the lipid molecules and preserve the three-dimensional integrity of bacterial and plant antenna complexes and the assembly of functional photosystem II under normal and potentially harmful environmental conditions [4,6].

The presence of carotenoids in the membrane influences, directly or indirectly, a vast range of physical and physiological processes. For investigation of the effects of various carotenoids on the membrane thermodynamic and mechanical properties, different model systems are used [7–10]. Carotenoids, being hydrophobic molecules, are predicted to be located within the hydrophobic core of the lipid bilayer. Their orientation within the membrane is dependent on the structure of the particular carotenoid and on the lipid composition of the host membrane. The orientational ordering of β-carotene, embedded in lamellar model lipid membranes and its effect on the membrane structural and dynamic properties have been investigated by variety of experimental methods: angle-resolved resonance Raman scattering, EPR, NMR, X-ray diffraction measurements, and computer simulation of molecular dynamics [7,9,11–13]. Localization of β-carotene, lacking polar groups in its molecule, in the lipid membrane environment is governed by van der Waals interactions with the hydrocarbon fatty chains of the lipids. The alterations in the angle-resolved Raman data have been used to monitor the phospholipid phase behavior in dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine, one-component systems and binary mixtures [7]. The found orientational distribution functions have shown that β-carotene is oriented parallel to the bilayer plane (dioleoyl lecithin) or perpendicular to it

(soybean lecithin). For dimyristoyl lecithin, egg-lecithin, and digalactosyldiacylglycerol, two maxima have been found in the orientational distribution: one parallel and one perpendicular to the bilayer surface. In contrast, it has been shown that in carotenoid-egg phosphatidylcholine liposomes, β-carotene is randomly distributed in the hydrocarbon interior of the bilayer, without any preferred, well defined orientation and retains a substantial degree of mobility increasing motional freedom of both lipid headgroup and alkyl chains in liquid crystalline state [9]. EPR investigations have shown that β-carotene tends to fluidize the interior of phosphatidylcholine membranes and to decrease the penetration barrier to small molecules to the headgroup region [2,11]. X-ray diffraction measurements have confirmed that β-carotene disordered the packing of phospholipid acyl chains in a manner that correlated with its pro-oxidant actions [12]. The results obtained by a molecular dynamics simulation of the fully hydrated bilayer made of palmitoyl oleoyl phosphatidylcholine (POPC) and containing β-carotene molecules indicated that the β-carotene rings were located in the region occupied by the carbonyl groups of the lipids [13]. These results suggest two pools of the preferential orientation of β-carotene: a slightly bent structure corresponding to a small chain tilt angle and a rather stretched structure that corresponds to a higher chain tilt. All these data, contradictory to a certain extent, revealed that the orientation and conformation of β-carotene molecules in model membranes are strongly dependent on its lipid content.

Majority of investigations reporting on the effect of β-carotene on physical properties of model lipid membranes have been performed on phospholipid bilayers [2,7–11]. As β-carotene represents an important pigment component of the photosynthetic thylakoid membranes, where the photosynthetic processes take place, it was interesting to investigate its role on model membranes, resembling the lipid composition of photosynthetic membranes.

Thylakoid membranes of photosynthetic organisms contain various lipid species, the main being galactolipids, such as monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG) [14,15]. MGDG comprises about half of the total thylakoid membrane lipid, the second most abundant lipid is DGDG which accounts to about 30% of the thylakoid lipids and SQDG that is found in a lower amount. The only phospholipid in the thylakoid membranes is phosphatidylglycerol (PG). MGDG and DGDG are neutral lipid molecules with a high degree of unsaturation of the fatty chains (predominantly 18:2 and 18:3). MGDG is a non-bilayer lipid forming hexagonal structures in aqueous medium. It has been shown that in native thylakoid membranes the non-bilayer lipids are arranged in a bilayer [16]. The other two components—PG and SQPG—are anionic lipids, providing negative charges to the thylakoid membrane [14,15].

The aim of the present work was to gain some insight into the mechanism of β-carotene–lipid interactions in liposomes with a special focus on the degree of unsaturation of the fatty chains. To investigate this effect, we used liposomes with lipid composition resembling that of photosynthetic thylakoid membranes, containing mainly galactolipids with a high degree of unsaturation of the fatty chains, and egg phosphatidylcholine liposomes. Absorption, pyrene fluorescence, and resonance Raman spectroscopy were applied to study the effect of β-carotene incorporation into the liposomes. The observed changes in β-carotene absorption and Raman spectra may be regarded as a result of the lipid–pigment interactions leading to a polyene geometry distortion, different in distinct lipid environment.

2. Materials and Methods

2.1. Materials

β-carotene, Tris-hydroxymethylaminomethane (TRIS), POPG, and pyrene were obtained from Sigma, EPC—from Avanti Polar Lipids. The chloroplast glycolipids SQDG, MGDG, and DGDG were purchased from Lipid Products (Redhill, Surrey, UK) and used as obtained.

2.2. Liposome Formation

The pigment was added to the lipids, before formation of liposomes, from a stock solution in chloroform to obtain following concentrations: 0.05, 1, 2, 3, 4, and 5 mol%. Concentrations of applied β-carotene were calculated as mol% in respect to the lipid concentration. Lipids equivalent to 1 mM (0.75 mg/ml) dissolved in chloroform, pure or mixed with different mol% β-carotene (1 mM in chloroform), were dried from the solvent under a gentle steam of N_2 to obtain a thin lipid layer on the bottom of a glass test tube. Thylakoid mix (TM) lipids were prepared as described in [17], 40% MGDG, 30% DGDG, 15% SQDG, and 15% POPG, on a weight base. Final traces of solvent were removed form the lipid film under deep vacuum over night followed by hydration with 50 mM TRIS buffer (*p*H 7.4). Small liposomes were formed by sonication for 40 s using ultrasound generator system. The residual, not integrated into liposomes β-carotene, was removed by two steps centrifugation at 15,000×g. Supernatant contained the β-carotene-doped TM or EPC liposomes and used for all experiments.

2.3. Determination of β-Carotene Concentration

The amount of β-carotene integrated into liposomes was determined by extraction of the pigment from the vesicles by ethanol and absorbance spectra were recorded on Specord 210 Plus in the spectral region

350–600 nm. Concentration of incorporated β-carotene was evaluated using molar extinction coefficient of β-carotene in ethanol at 453 nm, 141×10^3 M^{-1} cm^{-1} [18]. The results were used to determine the β-carotene incorporation yield (IY) as the ratio between the concentrations of integrated and applied β-carotene [19].

2.4. Steady-State Fluorescence Measurements

The fluorescent probe pyrene was added to TM and EPC liposomes, without and doped with different concentrations of β-carotene, at concentration 4 mol% in respect to lipids from a stock ethanol solution [19]. Care was taken the amount of added ethanol not to exceed 0.5%. Liposome–pyrene mixture was incubated at room temperature for 30 min and pyrene fluorescence was recorded with a Jobin-Ivon spectrofluorimeter. Fluorescence was excited at 332 nm and registered in the region 350–550 nm. Slit widths were 4 nm. After subtraction of the dark level, total fluorescence intensity (I) was determined as the total fluorescence area under the fluorescent contour. Pyrene, being apolar molecule, is completely buried within the hydrophobic region of the membrane [20]. Pyrene forms excimers (E) from monomers (M) by a diffusion-controlled process in fluid membranes that is directly related to membrane fluidity. In a monomeric form, pyrene gives a rise to a fluorescent peak at 393 nm (F393) and a broad one at 470 nm, emitted by the excimers (F470) [20].

2.5. Resonance Raman Spectra Measurements

Room temperature resonance Raman (RR) spectra were measured using a microRaman spectrometer (Jobin-Ivon, HR 800) with a grating 1800 g/mm. The excitation was provided by an argon ion laser (Innova 307, Coherent) at 514.5 nm. The laser intensity was 4 mW; the spectral resolution was 0.5 cm^{-1}.

All experiments were performed at room temperature, well above the phase transition of lipids.

3. Results

In Fig. 1, the chemical structures of the predominant lipid class of EPC (64% POPC)[A], of thylakoid mix lipids (MGDG) [B], and of β-carotene [C] are presented. DGDG and SQDG contain in the headgroup two or one galactose residue, respectively. Both galactolipids, MGDG and DGDG, contain highly unsaturated fatty chains, three double bonds per chain, which

determines the high degree of fluidity of the hydrophobic interior of the membrane [14]. The relative length of presented molecules is comparable.

Small liposomes were formed by sonication [17] without or in the presence of different concentrations of β-carotene. In Fig. 2A, the

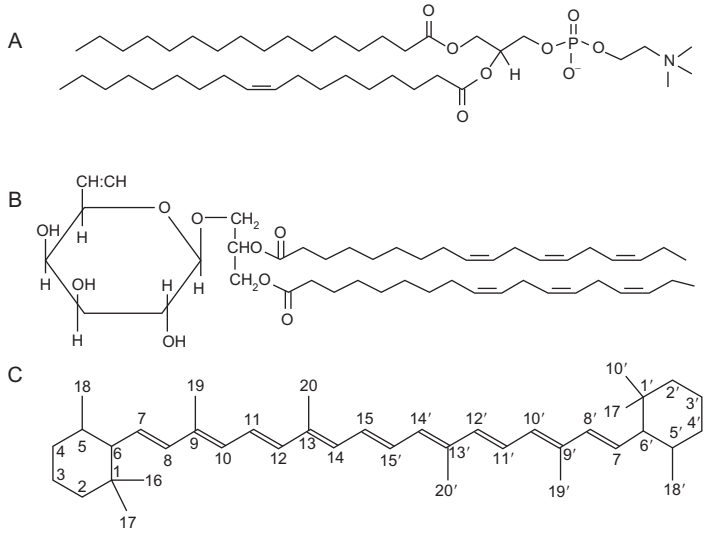

Figure 1 Chemical structure of predominant lipid species of EPC—POPC (A), of thylakoid mixture—MGDG (B), and of β-carotene (C).

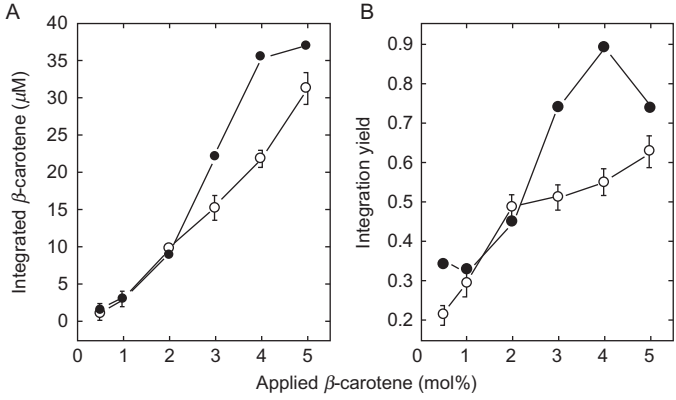

Figure 2 Concentration of β-carotene, integrated into small liposomes, EPC (-○-) or thylakoid mix ones (TM) (-●-) as a function of concentration of applied β-carotene (A). Integration yield of β-carotene into EPC (-○-) or into TM liposomes (-●-), determined as the ratio integrated/applied β-carotene (B). Mean values are determined from at least three independent experiments. Where SD bars are not seen, they are smaller than the value points.

calculated concentrations of integrated β-carotene in both types of liposomes as dependent on the concentration of applied pigment are presented. As expected, more β-carotene was integrated into liposomes with increase of concentration of applied pigment. For EPC liposomes, this dependence was nearly linear. For TM liposomes, integration of β-carotene at higher concentrations of applied pigment was higher than in EPC liposomes. The values of integrated and applied β-carotene concentrations were used to calculate the integration yield of the pigment (IY) (Fig. 2B). The obtained data for EPC liposomes were in a good agreement with the values obtained for IY of β-carotene in DPPC liposomes [21].

In Fig. 3, the absorbance spectra of β-carotene, extracted from EPC liposomes by ethanol, and the spectra of both investigated liposomes, dissolved in buffer and containing the highest concentration of integrated β-carotene, are shown. The spectra of ethanol extracts show a typical carotene contour, with two maxima, at 453 and 476 nm and a shoulder at 430 nm (Fig. 3, spectrum 1). The three spectra were normalized at 453 nm (0–1 transition).

In the spectra of EPC and TM liposomes, dissolved in buffer, three peaks were resolved, at 453, 480, and 518 nm. The shoulder at 430 nm (0–2 transition) was less expressed than in the ethanol extract of β-carotene. The peak at 480 nm (0–0 transition) in EPC and TM liposomes was higher than in the ethanol extract and its height was comparable with that at 453 nm.

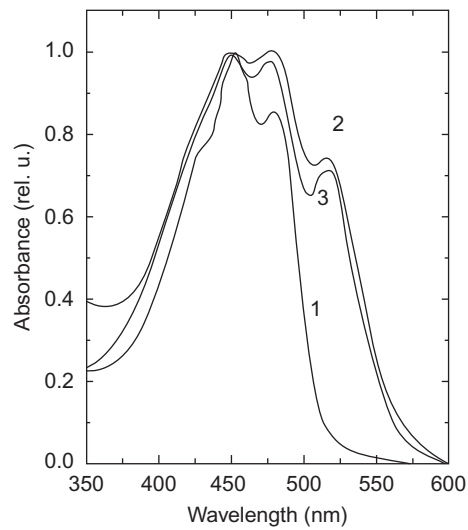

Figure 3 Absorbance spectra of β-carotene, extracted from liposomes by ethanol (1) and integrated into liposomes (EPC—2, TM—3), dissolved in TRIS buffer. Spectra are normalized to the intensity at 453 nm.

The most interesting difference in the spectra of ethanol extract and of liposomes, containing β-carotene, was the additional absorbance peak at 518 nm. Nevertheless that β-carotene was integrated into different liposomes and with different efficiency the resulting spectra were very similar. Here, we present only the spectra of liposomes with the highest concentration of integrated β-carotene because for the lower concentrations the shapes of the spectra were identical in respect to height and position of the peaks (data not shown).

The fluorescent probe pyrene is often used for determining membrane fluidity. Pyrene is an apolar molecule, composed of four fused benzol rings, completely buried into the hydrophobic interior of lipid membranes and forms excimers from monomers in fluid membranes by a diffusion controlled process [20]. The excimer formation is determined by the ratio of the excimer to monomer fluorescence quantum yield (E/M). In Fig. 4, the fluorescent spectra of EPC and TM liposomes, without and containing different concentrations of β-carotene, are presented. With increase of concentration of integrated β-carotene, the overall pyrene fluorescence for both types of liposomes was quenched. In Fig. 5, the total areas under the pyrene fluorescent spectra of EPC and TM liposomes containing increasing concentrations of β-carotene as a measure of the degree of quenching are presented. The observed decrease supposed that the pyrene fluorescence was quenched by β-carotene, due to an effective energy transfer from pyrene excimers to β-carotene since the excimer fluorescence

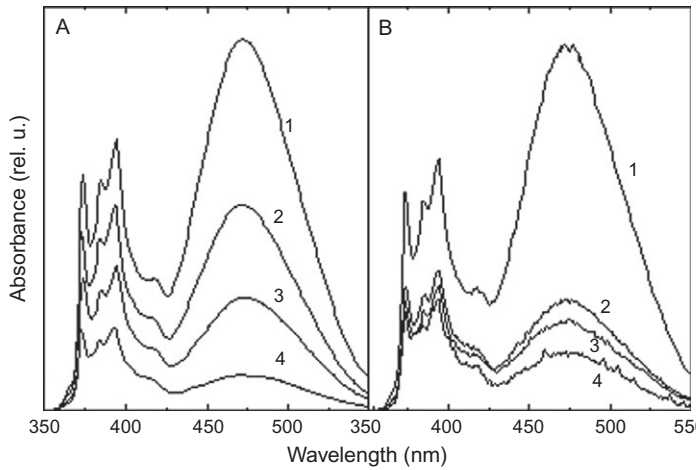

Figure 4 Fluorescence spectra of pyrene, integrated into EPC (A) or TM (B) liposomes, pure (1) and doped with different concentrations of β-carotene; concentration of applied β-carotene 3 μM (2), 9 μM (3) and 35 μM (4). Fluorescence was excited at 332 nm, slits—4 nm.

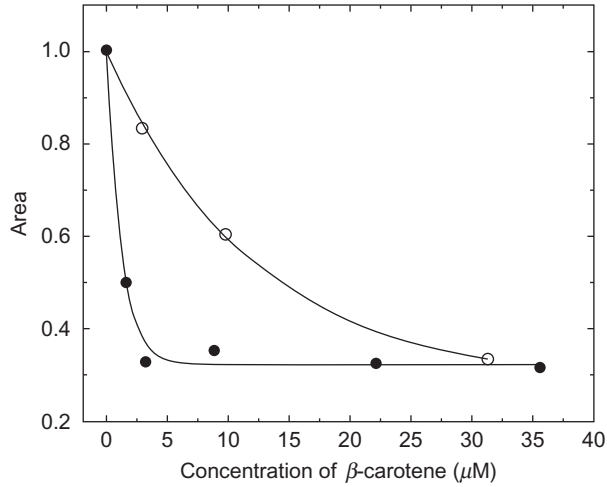

Figure 5 Area under the fluorescent spectra of pyrene in EPC (-○-) or TM (-●-) liposomes, without and containing different concentrations of β-carotene, in respect to concentration of integrated pigment. Area under the fluorescent spectra of pyrene in pure liposomes was taken as 1.

spectrum overlaps the carotene absorption. The quenching of pyrene fluorescence indicated that the pigment molecules were localized in the hydrophobic environment of the bilayer, in the vicinity of pyrene molecules, and the distance between them is estimated to be not higher than 7 Å in order the supposed energy transfer to take place. The more effective quenching for TM liposomes could be due to the higher motional freedom of the fatty chains of lipids that allows deeper penetration of β-carotene in the lipid bilayer and closer contact with the hydrophobic molecule of pyrene.

In an attempt to get more detailed information about β-carotene–lipid interactions in EPC and TM liposomes, we compared their RR spectra, excited at 514.5 nm, to the spectrum of β-carotene dissolved in pyridine. The comparison is presented in Fig. 6. Pyridine was chosen as a solvent as its refractive index ($n = 1.5092$) is close to that of membrane lipids. The excitation wavelength was near to the observed additional absorbance peak of β-carotene integrated into both types of liposomes (Fig. 3). The RR spectra manifest the characteristic for carotenoids' four main frequency bands in Raman spectrum (called from ν_1 to ν_4). The main bands have been assigned as follows: ν_1 to in-phase stretching vibrations of the C=C bonds, ν_2 to C–C stretching coupled to C_{15}–H in-plane (ip) bending, ν_3 to methyl CH_3 ip rocking vibrations, and ν_4 (around 960 cm^{-1}) have been attributed either to the out-of-plane wagging motions of the C–H groups of the carotenoid molecules and/or to the C–CH_3 stretching [22–25]. Differences are mainly observed in the region of ν_4 and ν_2 bands.

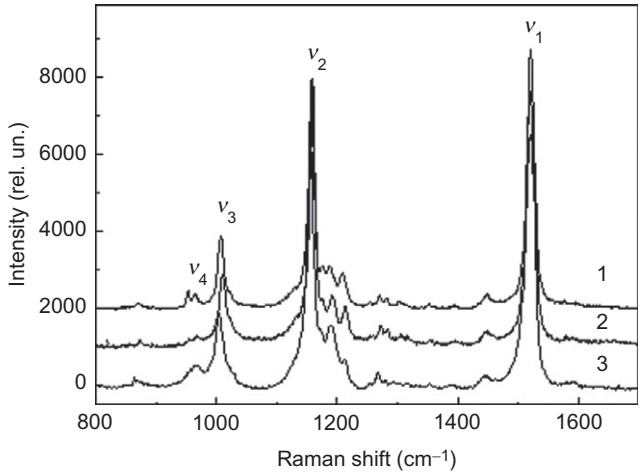

Figure 6 Characteristic resonance Raman spectra of β-carotene integrated into EPC (1), TM (2) liposomes and dissolved in pyridine (3) at room temperature excited at 514.5 nm. Spectra are up shifted for better visualization. Concentrations of β-carotene were 25, 24, and 100 μM, respectively.

In the region of ν_2 band (Fig. 6), we observed characteristic frequencies at 1175, 1190, and 1210 cm^{-1}. Their structure in the liposomes differed from that one in solution (pyridine). The 1175 cm^{-1} line could be assigned to methyl rocking at C_{18}. The other two lines have been attributed to localized stretching modes: 1190 cm^{-1} at C_8–C_9 and 1210 cm^{-1} at C_{12}–C_{13}, combined with C_{15}=$C_{15'}$, according to the recently published theoretical results for β-carotene Raman-active modes [26]. The relative intensity of the band at around 1210 cm^{-1} increased in both kinds of liposomes correlating with the changes in ν_4 band.

Structured ν_4 bands, indicative of out-of-plane distortions of conjugated backbone of the β-carotene molecule, were observed in both EPC and TM liposomes with incorporated β-carotene. They exhibited two transitions at 954 and 965 cm^{-1} becoming particularly clear from Fig. 7, where the bands were normalized to the intensity of the band at 965 cm^{-1} and compared to the unstructured band of β-carotene, dissolved in pyridine. Based on the normal coordinate analysis made for β-carotene by Saito and Tasumi [27], the mode around 950 cm^{-1} has been assigned to the torsion at C_7=C_8, whereas the mode at 965 cm^{-1} to the torsion at C_{11}=$C_{12'}$. These Raman-active modes have been recently predicted also by Tschirner *et al.* [26], at 974 and 982 cm^{-1} with low intensities, arising from methyl rocking $C_{16'}$ and CH out-of-plane wagging around C_{11}=C_{12}, but observed at 957 and 967 cm^{-1} [26].

The intensity of the band located at 954 cm^{-1} changed weakly with the pigment concentration in EPC liposomes (Fig. 7A), remaining lower than that at 965 cm^{-1}. In contrast, in TM liposomes, the 954 cm^{-1} intensity

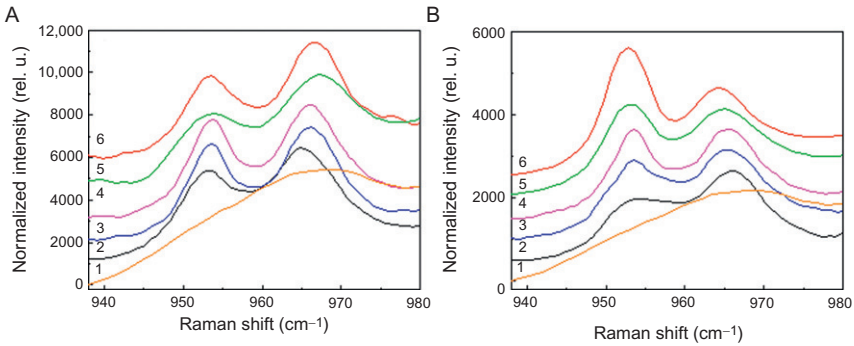

Figure 7 Resonance Raman ν_4 band of spectra of β-carotene integrated with different concentration into EPC (A) and TM (B) liposomes at room temperature excited at 514.5 nm. Spectra are up shifted for better visualization. Numbers next to the spectra correspond to the following concentration: 1–100 μM β-carotene in pyridine; in EPC liposomes: 2–3, 3–10, 4–15, 5–25, 6–31 μM and in TM liposomes: 2–3, 3–9, 4–12, 5–24, 6–38 μM.

increased gradually with the pigment concentration and became twice higher than that at 965 cm^{-1} at concentration 38 μM (Fig. 7B) indicating that the polyene geometry at $C_7{=}C_8$ was distorted stronger in TM liposomes with integrated higher concentration of the pigment. Thus, based on the observed different concentration, dependences of the intensity of these two modes in both EPC and TM liposomes, we can suppose that the out-of-plane distortions of conjugated backbones of the β-carotene molecules were different in respect to the type of liposomes.

4. Discussion

In order to study the β-carotene–lipid interactions in photosynthetic thylakoid membranes, we used liposomes, composed of lipids mimicking the lipid composition of plant chloroplast membranes, which are characterized with a high degree of unsaturation of their fatty chains. The alterations in absorption, pyrene fluorescence, and resonance Raman spectra, induced by β-carotene incorporation into TM and EPC liposomes, were compared to determine the role of lipid unsaturation for these interactions. When integrated into model membranes β-carotene can adopt various orientations, depending on the type of host lipids [7,9,11–13].

In majority of published data on the effect of carotenoids on the physicochemical properties of model membranes, only the concentration of the applied carotenoids is taken into account. It is uncertain how much of β-carotene molecules added to the sample during preparation can be

dissolved in the lipid bilayer in the form of monomers. Because of these solubility problems and uncertainties, the carotenoid concentration is discussed as the amount added to the sample during preparation of liposomes and not as the amount dissolved in the lipid bilayer [9,11–13,28]. To the best of our knowledge, there are only two reports on degree of incorporation of various carotenoids in different model membranes, phosphatidylcholine vesicles, finding high incorporation rate for xanthophylls and low incorporation for β-carotene [21,29]. In this study, we estimate the amount of integrated β-carotene into two different types of liposomes, composed of egg phosphatidylcholine and of lipids, found in photosynthetic thylakoid membranes. The results indicated that the integration rate of β-carotene was dependent on the fluidity of the hydrophobic interior of the bilayer. As expected, the amount of β-carotene integrated into TM liposomes was higher than in EPC ones, especially for high concentration of applied pigment (Fig. 2) due to the higher fluidity of the hydrophobic interior of TM liposomes. All presented results were given as a function of the real concentration of β-carotene, integrated into liposomes.

The higher degree of motional freedom of the hydrophobic interior of TM liposomes determined not only the higher concentration of integrated β-carotene but probably the molecules of the pigment were inserted deeper in the bilayer as evidenced by the accelerated quenching of pyrene fluorescence (Fig. 5). This quenching was realized by an effective energy transfer from the pyrene excimers to β-carotene molecules, situated in a close proximity, as the maxima of excimers' fluorescence emission and the β-carotene absorption overlap (see Figs. 3 and 4).

In the absorbance spectra of the two types of liposomes, containing β-carotene, we observed an additional peak at higher wavelengths, 518 nm (Fig. 3). We consider that this peak should be rather due to lipid/pigment interactions than to a possible formation of J-aggregates, characterized by a new absorption band at 530 nm [30,31]. The arguments in favor of this conclusion were the following. One of the key factors controlling the formation of interval J-aggregates has been shown to be the high initial concentration (100 μM), allowing the excitonic interactions between carotenoid molecules [30]. All used concentrations in this work were well below these values to induce aggregation. Another argument was the inverse correlation between the position of the absorption maximum of the aggregation band with the catotenoid's polarity, found by Ruban *et al.* [31]. Since the polar molecules of zeaxanthin had an aggregation maximum at 534 nm, the expected aggregation band for apolar β-carotene should be at higher wavelengths. The observed position of the additional absorption band in liposomes at 518 nm shows that the assignment of this peak to the formation of β-carotene aggregates is not feasible. So, we can attribute this band to lipid/pigment interactions.

Resonance Raman spectroscopy is a nondestructive method providing precise information on the type and conformation of carotenoid molecules

[22–25]. All presented resonance Raman spectra, excited by a narrow laser line coinciding with a new carotenoid absorption band, 514.5 nm, we assigned to the all-*trans* β-carotene. The following two reasons support this assignment. Firstly, we did not observe an upshift of the ν_1 band induced generally by *cis* isomerization [24,25,32]. Secondly, our experimental data (Fig. 6) showed a relatively very weak intensity around 1134 cm^{-1} in the so-called *cis*-isomerization fingerprint region ν_2 (1100–1300 cm^{-1}) [24,32]. It led us to the conclusion that the presence of other geometrical conformers in liposomes is not likely.

The structured ν_4 bands [22,25,27], observed in both types of liposomes containing β-carotene, indicated that the pigment incorporation led to out-of-plane distortions of conjugated backbone of the β-carotene molecules. Moreover, the degree of lipids' unsaturation exhibited pronounced difference in their effect on the molecular planar structure of all-*trans* β-carotene molecules during their integration. Induced stronger deformation of the carotenoid molecular geometry in the fluid environment of TM liposomes can be due to the deeper and easier insertion of pigment molecules between TM lipids as compared to the EPC ones. The more effective quenching of pyrene fluorescence in TM liposomes supported this notion. The deeper insertion should lead to the exhibited stronger distortion of carbon backbone of pigment molecules at $C_7{=}C_8$ in TM liposomes. The different penetration deepness of β-carotene molecules in liposomes suggested that the molecule orientation could be in different directions: parallel or perpendicular to the bilayer surface as it was concluded for dimyristoyl lecithin, egg-lecithin, and digalactosyldiacylglycerol liposomes [7]. The two different orientations into the bilayer could impose the distinct extent of the deviation from the planarity of β-carotene molecules at two different places in the conjugated backbone generating the two Raman-active modes at 954 and 965 cm^{-1} (Fig. 7). The perpendicularly orientated molecules to the membrane surface (being parallel to the lipid alkyl chains) are subjected to a slighter deformation than those, oriented parallel to the surface in the interior of the bilayer. These considerations are in accordance with the suggested two distinct deformations of β-carotene molecules, resulted from a molecular dynamics simulation of the fully hydrated bilayer composed of POPC [13].

In conclusion, our data imply that the degree of lipids' unsaturation regulate the penetration deepness of β-carotene molecules in liposomes thus modifying the lipid–pigment interactions leading to the polyene geometry distortion, different in distinct lipid environment.

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