Antioxidant activity of vitamin E homologues in model systems

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Abstract

The effect of tocopherols, tocotrienols and plastochromanol-8 in the inhibition of lipid peroxidation of liposomes prepared from natural chloroplast lipids, initiated by both water-soluble and lipid soluble azo-compounds, has been studied. In the case of tocopherols, α -tocopherol showed nearly no effect in the inhibition of lipid peroxidation, while β -, γ - and δ -tocopherols inhibited the reaction completely when it was initiated by lipid-soluble AMVN. Similar effects were observed for tocotrienol homologues. In the investigated reaction plastochromanol-8 was as effective as β -, γ - and δ -tocochromanols. When peroxidation was initiated by water-soluble AIPH in liposomes, the order and extent of inhibition was similar to those of AMVN initiator. However, in this case, α -tocopherol and α -tocotrienol showed more pronounced inhibition. When the prenyllipids were investigated in DPPH test, when incorporated into soy lipid liposomes, mixed micelles and micelles, DPPH oxidation was most pronounced in liposomes, followed by mixed micelles and micellar system. When the reaction of α -tocopherol, α -tocotrienol, plastochromanol-8 and α -tocopherol phosphate was examined in niosomes, the oxidation was most pronounced for α -tocopherol and plastochromanol-8, followed by α -tocotrienol. α -tocopherol phosphate showed no activity in this respect.

The obtained results were discussed in light of the prenyllipid structures and their localization in the investigated lipid systems.

Introduction

Vitamin E stands for a complex of naturally occurring tocochromanols¹⁻³. This group encompasses tocopherols, tocotrienols, plastochromanol-8 (PC-8), and their derivatives⁴. The number of methyl groups in the chromanol ring defines four naturally occurring α , β , γ -, δ -tocopherol and tocotrienol homologs^{1,5}. These compounds are lipid-soluble antioxidants which play a pivotal role in human health and nutrition³. They are generally found in high concentrations in vegetable oils such as almond, safflower, or canola oil or in other high-fat sources such as nuts, seeds, or grains⁶. The growing consumption of plant-derived oils rich in polyunsaturated fatty acids (PUFA) that are taken up into membrane phospholipids underscores the role of tocochromanols in antioxidant protection.

Tocochromanols are composed of two moieties: chromanol head group with a hydroxyl group and a prenyl side-chain, which can be saturated (tocopherols) or unsaturated (tocotrienols, PC-8) (Fig. 1). Tocochromanols differ in the methylation pattern of the chromanol ring at the position R1 and R2. Plastochromanol-8 is a γ -tocotrienol homologue with a longer side chain^{7,8} (Fig. 1). Moreover, the occurrence of a water-soluble α -tocopherol phosphate has been reported in biological systems⁹. A tocochromanol molecule usually resides in a membrane with the hydroxyl group near the aqueous interface, whereas the prenyl side-chain extends toward the center². α -Tocopherol as a synonym of vitamin E for humans, is the most examined tocopherol form, but many recent studies have indicate that other forms of vitamin E may exhibit more potent biological activity^{10,11}.

The primary function of tocochromanols is to protect polyunsaturated lipids from oxidation. This process is initiated by the free radicals, whereby lipid peroxy radicals play a chain-carrying role and thus propagate lipid peroxidation¹². Chain propagation occurs when a hydrogen atom from the target lipid is abstracted by the peroxy radical and further production of lipid hydroperoxide (LOOH) and a lipid radical (L[•]) as shown in Fig. 2. After reaction of L[•] with molecular oxygen another peroxy radical is generated, which propagates chain reaction. Tocochromanols scavenge the lipid peroxy radicals by donation of hydrogen before they react with lipids and therefore they are terminating the chain peroxidation^{12,13} (Fig. 2). When donating the hydrogen, vitamin E becomes a relatively unreactive free radical. Nonenzymatic lipid peroxidation leads to destruction of the membrane bilayer which is built of PUFA. Thus, it causes disturbances of cell structure and functionality^{5,12}, followed by the initiation or/and propagation of various chronic diseases¹⁴⁻¹⁶.

The efficiency of vitamin E antioxidant action depends on two factors: i) the mobility of the molecule within membranes, which is determined by the chemical nature of a prenyl chain, as well as the type of lipids; ii) the number of methyl group on the chromanol ring, where each methyl group confers additional antioxidant capacity in view of the electron donor properties^{17,18}. In addition, the proximity of the methyl groups to the hydroxyl group also plays an important function. Thus, α -homologues, which have the fully substituted chromanol ring, where methyl groups flank the hydroxyl group, are thought to be more effective than the other homologues^{5,18}. On the other hand, it has been shown that tocotrienols display higher bioactivity than tocopherols^{19,20}, because of the presence of conjugated double bonds in the isoprenoid tail. The unsaturated side chain of tocotrienols has a stronger disordering effect on membranes than tocopherols, leads to a greater mobility and more uniform distribution within the membranes. NMR studies have also proved that the chromanol head group of α -tocotrienol is situated closer to the membrane surface. These features may allow a greater ability of tocotrienols to interact with free radicals and may contribute to better recycling of the molecule to its active reduced form^{19,20}.

In the present study, we have compared the antioxidant activity of side-chain saturated (tocopherols) and unsaturated tocochromanols (tocotrienol and PC-8) in different model systems. First, we used liposomes prepared from a mixture of chloroplast lipids as a plant membrane model for the study of the inhibition of lipid peroxidation by tocochromanols. Lipid peroxidation was generated inside and outside the membrane using two azo-initiators: AMVN (2,2'-azobis(2,4-dimethylvaleronitrile)) and AIPH (2,20-Azobis-[2-(2-imidazolin-2-yl)propane] dihydrochloride)²¹. The azo-compounds have been successfully applied in many *in vivo* as well as *in vitro* studies, since the rate and site of free radical formation can be easily controlled by choosing the appropriate azo-compound and concentration. Taking this into account we used AMVN which is hydrophobic, and AIPH which is hydrophilic, thus two initiators localize the inside and outside of the membrane, respectively¹¹. Thereby homolysis of these initiators results in free radicals release in different membrane compartments (Fig. 3). Taking into account the structural differences between the investigated compounds we were going to study whether they influence the size of liposomes. Thus, we performed the dynamic light scattering (DLS) experiments. In a second part, using

the DPPH assay we compare the antioxidant activity of analyzed compounds in different nanocarriers: liposomes, micelles, mixed micelles and niosomes.

Results And Discussion

Inhibition of lipid peroxidation in liposomes using lipid-soluble AMVN

Inhibition of lipid peroxidation in liposomes was initiated both in hydrophobic and hydrophilic phase. For this experiment, we used liposomes composed of the plant-specific glycolipids: monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), and phosphatidylglycerol (PG). These diacyl lipids contain no phosphate in their headgroups, but instead one (MGDG) or two (DGDG) galactose residues. SQDG has a sulfated glucose moiety as a headgroup and is therefore negatively charged. While DGDG and SQDG are bilayer-forming lipids, pure MGDG forms hexagonal II or cubic phases, depending on experimental conditions²². Using HPLC we monitored simultaneous, time-course changes of lipid peroxides content and tocopherols decay. In the case of AMVN, a lipid-soluble initiator, the formation of peroxides was the most pronounced for α -tocopherol, whereas for other tocopherols their increase with time was slight. Moreover, α -tocopherol exhibited the most pronounced decay in this experimental setup. Its content showed a fast decrease after 2 h reaching 40% of the initial amount and after 4 h it decreased to 10% of the initial level (Fig. 3a). Such kind of effect was observed earlier by Nowicka et al¹¹. They confirmed that under these experimental conditions α -tocopherol plays a prooxidant role, which is mediated by its oxidation product of α -tocopheroxy radical. In the system, where α -tocopherol was present together with another antioxidant – plastoquinol-9 (PQH₂-9), its prooxidant effect was inhibited. Without PQH₂-9, the oxidative stimulation appeared. This indicates that in a presence of PQH₂-9, the oxidation product, α -tocopheroxy radical, is converted to α -tocopherol, and in the absence of PQH₂-9, α -tocopherol shows prooxidative action¹¹. Several *in vitro* studies have also shown a prooxidative effect of α -tocopherol in isolated human LDL^{23–25} as well as in other studies^{26,27}. These experiments clearly suggest that under low flux of radicals, α -tocopherol can play a prooxidative role, and this reaction is mediated by α -tocopheroxy radical. Upston et al.²⁸ have named this process as *tocopherol-mediated peroxidation*. After scavenging of a lipid radical, α -tocopherol is oxidized to α -tocopheroxy radical, which cannot easily find another radical to terminate. Under such conditions, its lifetime is prolonged and stimulates the lipid peroxidation, where it acts as a chain-carrying prooxidant²⁸. Such a mechanism could be similar in our study. In this simple model, with α -tocopherol as a single antioxidant molecule, the regeneration of α -tocopheroxy radical by other radicals is not possible, thus it starts to act as a prooxidant. Under the applied conditions, β - and γ -forms showed similar responses, both in the case of lipid peroxides formation as well their content decay, which account approx. 70% after 4 hours. During the reaction, δ -tocopherol consumption was the less pronounced (30% after 2 hours, 45% after 4h). Moreover we did not observed changes in lipid peroxides content, even after 4 h (Fig. 3a). It was shown previously that γ -tocopherol is less effective than α -tocopherol in hydrogen donation, so it reacts less pronounced with free radicals²⁹. Moreover, hydrogen donation capacity depends on the chromanol group structure²⁹, which is in agreement with our study, where we observed

the following relation in free radical scavenging $\delta > \gamma = \beta > \alpha$. It is possible, that the oxidation products of γ -, β -, δ -tocopherols could also play an antioxidant role, whereas α -tocopherol quinone (α -TQ), the oxidation product of α -tocopherol is ineffective against free radicals¹¹. Overall, the obtained results suggest that in our experimental setup, tocopherols beyond α -form play a significant role in lipid protection. Furthermore, the mechanism of their action seems to be different.

Figure 3b shows the effect of unsaturated tocotrienols on lipid peroxidation in liposomes during the reaction generated by AMVN. The effect for α -tocotrienol was similar to that of α -tocopherol, where this homolog seems to also play a prooxidant role. For α -tocotrienol, this effect was even more pronounced, because after 4 h it was fully consumed (Fig. 3b). This phenomenon was observed in a comparative study, in which both tocopherol and tocotrienol were added, and where the α -tocotrienol level decreased faster. Their higher activity was explained by its better mobility in lipid membrane than saturated α -tocopherol²⁷. In comparison to β - and γ -tocopherols, the corresponding tocotrienols were slightly less active in the inhibition of lipid peroxidation (Fig. 3b). Tocotrienols are more hydrophobic than tocopherols, thus they can penetrate deeper into the membranes and can be less effective in hydrogen donation. The prooxidative effect of γ -tocotrienol was observed in osteoblast culture in *in vitro* study³⁰. While its low concentration exhibit protective effect, the higher doses (100 μ M) had prooxidant and proapoptotic effect.

On the other hand, the most obvious lipid peroxidation inhibitory effect was observed for δ -tocotrienol. In our experiment, its level decrease most slowly (of about 50% after 4 h), moreover the lipid peroxide content did not increase (Fig. 3b). The fact that its response is the same as that of δ -tocopherol, it indicates that the difference in chromanol structure between tocopherols is responsible for different orientations within the membrane which is manifested in different antioxidant action. Our results are in agreement with a study performed earlier¹⁸. They showed that individual tocotrienols displayed different antioxidant potency in rat liver microsomal membranes, as well as in RAT-1 immortalized fibroblasts. The most effective homologue was δ -tocotrienol, followed by γ - and α -homologue. They explained this effect by the lowest methylation of δ -tocotrienol which allows it to be easily incorporated into the membrane.

In liposomes with PC-8, the level of lipid peroxides did not changed with time (Fig. 4). The level of PC-8, showed a slow decrease during the first 2 h, but after 4 hours its content reached $\sim 30\%$ of initial value (Fig. 4). Inhibition of lipid peroxidation in plant lipids liposomes by PC-8 was observed earlier¹¹, but its activity was similar to that of tocopherols. Our results showed that PC-8 has much better antioxidant properties than α -tocopherol and similar to the other tocopherols in a case of AMVN-induced lipid peroxidation. PC-8 was also a better antioxidant as compared to α -, β - and γ -tocotrienols (Fig. 3b and Fig. 4). During antioxidant action, PC-8 forms many different oxidation products, among them several play effective antioxidant roles, i.e. hydroxyl-plastochromanol (PC-OH)^{31,32}. This could explain the 70% loss of its content and effective inhibition of lipid peroxidation. PC-8 differs from the other tocopherols by the longer prenyl side-chain, having more double bonds. A study on FeAox-6, a synthetic form of α -homologue of vitamin E with four conjugated double bonds in the prenyl chain,

showed its better antioxidant potency than all investigated tocotrienols¹⁸. Thus, the higher hydrophobicity could lead to more uniform distribution within the cell membrane, as well as better mobility. Taken together, our results clearly indicate that structural differences of the examined tocochromanols leads to their different locations in the membrane as well as different protection mechanisms of free radical-initiated lipid peroxidation in plant-lipid liposomes.

Inhibition of lipid peroxidation in liposomes using water-soluble ALPH

Lipid peroxidation in liposomes was also initiated in the water phase by ALPH. We chose this azo-compound instead of commonly used AAPH^{11,33}, because under the same conditions ALPH gives 3.8 times more free radicals²¹. In our experimental set-up, under the same concentration, AAPH did not oxidize the lipids in a control sample (*data not shown*). The results of inhibition of ALPH-initiated lipid peroxidation by tocopherols are shown in Fig. 5. Similar to the effect observed for liposomes containing α -homologues in AMVN-dependent lipid peroxidation, here we also observed a time-course increase of lipid peroxides and α -forms decay which confirm its prooxidative role. During the reaction, the content of α -tocopherol decreased by 35% after 1 h, while α -tocotrienol more than 40% (Fig. 5a, b). We did not observe the formation of lipid peroxides in liposomes containing the remaining tocopherols and tocotrienols (β -, γ -, δ -). The decay of β - and δ -tocopherols content was 20% after 1 h, whereas γ -tocopherol was the same as for α -homologue (Fig. 5a). During the ALPH-generated peroxidation, all unsaturated tocochromanols decreased gradually during the reaction time.

We performed the additional experiment, where we compared the inhibition of lipid peroxidation activity of α -tocopherol and PC-8 (Fig. 6). In the case of PC-8, we observed inhibition of lipid peroxide formation together with a decrease of its content (20% of initial value after 1 h) (Fig. 6), similarly to those observed for AMVN-initiated peroxidation. We compared also the antioxidant activity of α -tocopherol phosphate in this experiment. In reaction mediated by ALPH in liposomes, the level of lipid peroxides was the same as that for α -tocopherol and α -tocopherol phosphate (*data not shown*). Taken together, the obtained results indicate that the chromanol head group of investigated molecules must be localized close to membrane interface, which facilitates their antioxidant action in ALPH-mediated free radicals lipid peroxidation.

Dynamic light scattering analysis of tocochromanols containing liposomes

The intensity based size distribution of the tocochromanol-loaded liposomes (Fig. 7) as obtained by DLS shows a well-defined peak with a mean of 200 nm and a polydispersity index (Pdl) of 0.16–0.24. The size achieved from distribution by intensity is the most reliable because it is a result of direct measurement. Figure 7 have shown the average hydrodynamic diameter of the plant-lipids liposomes containing the selected tocochromanols. The DLS analysis has indicated that liposomes loaded with saturated tocochromanols (α - and γ -tocopherols) are slightly smaller than those containing unsaturated compounds: α -tocotrienol, γ -tocotrienol and PC-8. The average diameter of liposomes with tocopherols was under 200 nm (186 nm for α -form and 194 nm for γ -tocopherol). The diameter of liposomes loaded with tocotrienols and PC-8 is above 200 nm (Fig. 7). This can be explained by the higher disorder causing by unsaturated tocochromanols in lipid membranes. In general, the effect of tocochromanols on particle

size is small because their shape is very close to cylindrical. Similar effect was observed by Massey and Pownall³⁴ in model human high-density lipoproteins loaded with α -tocopherol.

Antioxidant activity of tocochromanols in different nanocarriers - DPPH assay

In order to compare the antioxidant activity of representative tocochromanols (α -tocopherol, α -tocotrienol, γ -tocopherol, α -tocopherol phosphate, and PC-8) depending on different lipids, we performed additional measurements based on reduction of DPPH radicals. Tocochromanols were incorporated into different nanocarriers, which included: i) liposomes made of different soy-originated lipids: phosphatidylcholine (lecithin; PC), phosphatidylglycerol (PG), and phosphatidylinositol (PI); ii) mixed micelles prepared as a mixture of PG, PC or PI and 10 mM sodium cholate; iii) micelles build of a mixture of PG, PC or PI and 30 mM sodium cholate; iv) niosomes made of Tween 60 and cholesterol in a 1:1 ratio. As shown in Fig. 8, tocochromanols exhibited different antioxidant activity depending on the lipid used for liposome preparation. Among PC-based nanocarriers, α -tocopherol and α -tocotrienol exhibited higher antioxidant activity in liposomes (55% and 45% of DPPH inhibition, respectively), while PC-8 inversely ($\sim 25\%$). When incorporated into micelles, their activity was lower (Fig. 8A). In PG-nanocarriers the analyzed compounds showed the most pronounced antioxidant activity (80% for α -tocopherol and plastochochromanol-8, $> 50\%$ for α -tocotrienol) among all the investigated lipid systems (Fig. 8B). All compounds exhibited the most pronounced activity in liposomes, followed by mixed micelles and micelles (Fig. 8B). The most interesting results were obtained for PI-nanocarriers. PI-liposomes with α -tocopherol were less effective than micelles and mixed micelles (Fig. 8C). α -Tocotrienol in all the tested PI-carriers was not active (less than 10% of DPPH inhibition). PI-liposomes containing PC-8 showed more than 80% inhibition of DPPH which was 3 times higher than α -tocopherol and more than 8-times higher for α -tocotrienol (Fig. 8C). PC-8 antioxidant activity was lower in PI-mixed micelles, but DPPH inhibition was still higher than that observed for α -tocopherol. The antioxidant activity of α -tocopherol phosphate in liposomes was very low and did not change depending on the lipid used (*data not shown*). In order to check the tocochromanol antioxidant activity in nanocarriers, which are devoid of phospholipids, we incorporated them into niosomes. Figure 9 have shown the ratio of DPPH radical inhibition by the α -tocopherol, α -tocotrienol, PC-8, and α -tocopherol phosphate in niosomes. We observed a similar response for α -tocopherol and PC-8, while α -tocotrienol activity was more than 2-fold lower (Fig. 9). In niosomal system, α -tocopherol phosphate was not active (*data not shown*).

It has been many times shown that the nature of liposomes strongly influences the behavior of incorporated molecules^{35–38}. There are many reports showing the effect of tocopherols in pure lipid systems^{11,21,39}. It is clear that tocochromanols exert serious effects on lipid membrane properties, such as phase behavior and lipid dynamics. In general, the presence of α -tocopherol in phospholipid bilayers decreases the motional freedom of the lipid fatty acyl chains in the liquid-crystalline state, which is the predominant state of lipids in biological membranes under physiological conditions. In membranes made from non-bilayer lipids such as phosphatidylethanolamine, on the other hand, non-bilayer structures such as hexagonal II phases are promoted by the presence of α -tocopherol²². The general features of the location and orientation of α -tocopherol in phospholipid bilayers are also reproduced in the few atomistic

molecular dynamics (MD) simulations. Leng et al.⁴⁰ revealed that high disorder facilitates the probability that a polyunsaturated chain comes into close proximity with the hydroxyl group on a chromanol head group that resides at the surface of the bilayer. Additionally, they observed fast *flip-flop* movement of α -tocopherol, which suggests that the hydroxyl group can also easily penetrate into the interior of a polyunsaturated membrane to intercept lipid peroxy radicals. This scheme could also concern the other tocopherols.

Several studies suggested that α -tocopherol forms a complex with unsaturated fatty acids and in which chromanol ring is mainly involved^{41,42}. In complex with linoleic acid, a hydrogen bond links carboxyl of the fatty acid and the hydroxyl of the tocopherol, while the double bonds of the fatty acid conform a complementary structure to the methyl groups on the chromanol moiety⁴³. Atkinson et al.¹⁷ hypothesized that α -tocopherol preferentially incorporates into PUFA-rich domains, which is the most susceptible for oxidation. This indicates that the tocopherols distribution within the biological membranes is not uniform. These molecules could be oriented within the unsaturated lipids or domain. Taking into account chromanol head group involvement in such complex forming, the effect for remaining homologues would be different. Possibly, the strength of lipid-tocopherol complex in γ - or δ -form is lower, that facilitates their higher mobility within bilayer. That kind of interactions would depend not only on the numbers of methyl groups in the chromanol ring, but also on the type of membrane lipids/phospholipids.

Based on several biophysical techniques, it is found that PI causes packing defects and allows deeper water penetration in the DMPC bilayer⁴⁴. The orientation of the head group of PI in the model membrane has been shown to project perpendicularly from the membrane surface into the aqueous environment and this allows maximum hydration of the inositol ring⁴⁵. The surface area of this highly hydrated head group is expected to be larger than the cross-sectional area of the acyl chains, and this might lead to the looser packing of the acyl chains as compared to lipids with matching head group and acyl chain cross-sectional area⁴⁴. Thus permitted deeper penetration of water as well as biologically active molecules, i.e. proteins by altering both hydrophilic and hydrophobic interactions⁴⁶. It is worth to know that PI have biological implication in tocopherol metabolism in human body, demonstrated by Kono et al.⁴⁷ under experiments concerning α -tocopherol transfer protein (α -TTP) which binds and transfer α -tocopherol. They have shown that interactions with phosphoinositides are also critical for α -TTP function by mediating the binding of α -TTP to PI(3,4)P₂ or PI(4,5)P₂ in the membrane. Indeed, they have observed direct exchange of PI(4,5)P₂ for tocopherol upon incubation of α -TTP with lipid vesicles *in vitro*. Recently, Chung et al.⁴⁸ have shown that the presence of 5-phosphoinositides at the plasma membrane is essential for α -TTP-mediated transport of α -tocopherol.

The results obtained in the present study suggest that tocopherol-lipid interactions strongly influence the antioxidant activity of vitamin E and depends on the type of membrane lipids. On the other hand, the presence of the tocopherols influences the properties of the membrane itself. In this context, tocopherols could play a non-antioxidant functions. As natural components of lipid membranes they strongly influence the bilayer structure and dynamics and thus can modulate the membrane-dependent

processes, such as enzymes activity, signaling cascades, active and passive transport or gene expression. The data concerning this issue is scarce. The further experiments, which will show not only location of tocopherols in the membrane, but also show the phospholipids environment at the same location are needed.

Materials And Methods

Materials. Tocopherol standards of the high purity (α -99.9%, β -99.5%, γ -99.5%, δ -99.9%) were purchased from Merck (Darmstadt, Germany), while tocotrienol standards (purity of 99.5%) were obtained from Calbiochem. α -Tocopherol phosphate standard was purchased from Sigma Aldrich (Poland). The PC-8 standard was purified from linseed oil³⁹. Chloroplast lipids: monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulphoquinovosyldiacylglycerol (SQDG) were obtained from Lipid Products (England), while soy-derived lipids: phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylinositol (PI) from Avanti Polar Lipids Inc. Cholesterol, sodium cholate, and Tween 60 was obtained from Merck Milipore (Sigma Aldrich). 2,2'-azobis[2-(2-imidazolin-2-yl)propane]-dihydrochloride (AIPH) was from TCI Chemicals, while 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) from Sigma Aldrich. Solvents used for high performance liquid chromatography (HPLC) were of HPLC-grade purity and were purchased in Chemland (Poland).

Methods

Lipid peroxidation of tocopherol-containing liposomes

Lipid peroxidation was initiated in the water phase by AIPH and in the lipid bilayer by AMVN. In these experiments, liposomes were prepared from a mixture of chloroplast lipids MGDG:DGDG:SQDG:PG, 4:2:1:1 (mol/mol), respectively. In experiments where lipid peroxidation was initiated with AIPH, a tocopherol was mixed with 20 mM ethanol solution of chloroplast lipids and injected into 25 mM HEPES buffer (pH 6.5) followed by the addition of AIPH in water. The final concentration of tocopherols and chloroplast lipids was 50 μ M and 0.5 mM, respectively, and that of AIPH was 100 μ M (for antioxidant activity of tocopherols and tocotrienols comparison) or 250 μ M (for PC-8 and α -tocopherol phosphate). Where lipid peroxidation was initiated with AMVN, a tocopherol was mixed with ethanol solutions of chloroplast lipids and AMVN. Then, the mixture was injected into 25 mM HEPES buffer (pH 6.5). The final concentration of tocopherol and chloroplast lipids was 50 μ M and 0.5 mM, respectively, and that of AMVN was 100 μ M (for antioxidant activity of tocopherols and tocotrienols comparison) or 250 μ M (for PC-8 and α -tocopherol phosphate).

Incubation of liposome suspensions was carried out at 37°C. During incubation, samples were taken at 0, 2, and 4 h in the case of AMVN-generated lipid peroxidation or at 0, 0.5, and 1 h, in the case of AIPH-generated lipid peroxidation. Afterwards, 300 μ L of each sample was extracted with 600 μ L of ethyl acetate using Eppendorf mini-rotator (2 min, 2000 rpm), 400 μ L of organic phase was evaporated under

stream nitrogen and dissolved in 130 μ L of HPLC eluent. Progress of the peroxidation was followed by measurement of lipid peroxides level and tocopherols content by HPLC.

HPLC analysis

Analysis of lipid peroxides was performed in acetonitrile:methanol:water (72:8:1, v/v), at a flow rate of 1.5 ml/min and absorbance detection at 234 nm. The tocopherols and tocotrienols were analyzed in acetonitrile:methanol:water (72:8:1, v/v), whereas PC-8 in methanol:hexane (340:20, v/v), all at a flow rate of 1.5 ml/min using fluorescence detection ($\lambda_{\text{ex}} = 290$ nm, $\lambda_{\text{em}} = 330$ nm). For the experiment, a reverse-phase C18 column (5 μ m, 25 cm x 0.4 cm) (MZ-Analysentechnik, Mainz, Germany) was used.

Dynamic light scattering (DLS) measurements

Particle size of chloroplast lipid liposomes with incorporated compounds were determined using Malvern Zetasizer Nano S (Malvern Instruments, Worcestershire, UK) particle size analyzer. Each sample was measured five times and the results were expressed as the average value.

Preparation of tocopherol-containing nanocarriers

In order to prepare liposomes suspension, a tocopherol was mixed with ethanol (PC, PG) or ethanol/chloroform (1:1, v/v) (PI) solution of soybean lipids. In the case where ethanol/chloroform solution of PI was used, solvents were evaporated before the next steps. Then, the appropriate volume of water was added, and the mixture was shaken using laboratory vortex (Omni Inc., Atlanta, USA) for 2 min, incubated in 37°C for 30 min, shaken again (2 min), and centrifuged (10,000 g x 3 min) using a benchtop centrifuge (Eppendorf, Hamburg, Germany). The final concentration of tocopherol and lipids was 50 μ M and 0.5 mM, respectively.

To prepare mixed micelles and micelles suspensions, the procedure described previously by Zhang and Wang⁴⁹ was used, with some modifications. For micelles preparation water solution of sodium cholate was added to the initial mixture of tocopherol and soybean lipids solution. The samples were stabilized at the room temperature and used for measurements. The final concentration of sodium cholate in mixed micelles and micelles was 10 mM and 30 mM, respectively.

Niosomes suspensions were prepared by mixing tocopherol with solutions of cholesterol in ethanol:chloroform (1:1, v/v) and Tween60 in ethanol. The mixture was incubated at 45°C for 15 min and evaporated under a stream of nitrogen to obtain lipid film. Then, the lipid film was solubilized in 1 mL of water, followed by vortexing for 2 min and centrifugation (10,000 g x 3 min). The final concentration of cholesterol and Tween60 was 10 mg/ml and that of tocopherols was 50 μ M.

DPPH scavenging assay

The antioxidant activity of the tocopherol-containing nanocarriers was determined by the DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging assay. Briefly, 0.5 mL of prepared nanocarrier samples was mixed with 0.5 mL of ethanol solution of DPPH (0.2 mM) and vortexed shortly (15 s). Then, the mixture was incubated at room temperature for 30 min in the dark. The absorbance was measured at 529 nm, using a

Bio Cary UV-VIS spectrophotometer (Varian Inc., USA). The DPPH radical scavenging activity was calculated according to Eq. ⁴⁹:

$$DPPH \text{ oxidation (\%)} = \frac{(A_0 - A_1)}{A_0} \cdot 100\%$$

where A_0 is absorbance of the control and A_1 is absorbance of the sample after 30 min.

Statistical analysis

All results are expressed as the mean $\pm$ standard deviation (SD). Data were analyzed using MS Office Excel 2016 and Origin 8.6 (OriginLab, Northampton, Massachusetts, USA). One-way analysis of variance (ANOVA) with Tukey's or Duncan's post hoc tests were used to determine significant differences between means (Statistica 13.3; TIBCO Software Inc., USA). *P* values below 0.05 were considered statistically significant.

Declarations

Competing interests

The authors declare no competing interests.

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Authors Contributions

RS: conceived and designed the research interpreted data, wrote the manuscript; AT-M conducted HPLC and DPPH experiments, analyzed data, conducted statistical analysis, prepared all graphs; AZ: conducted DLS experimntes; JK reviewed the manuscript. All authors read and approved the manuscript.

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Figures

Figure 1

Chemical structure of tocochromanols.

Figure 2

Function of tocochromanols in lipid peroxidation (A); Schematic illustration of an experimental setup used in a present study (B).

Figure 3

A) The effect of tocopherols on the rate of lipid peroxides formation in plant lipid liposomes initiated by AMVN (bars). Time-course of tocopherol level during the peroxidation reaction (lines). The reaction was performed at 37°C. B) The effect of tocotrienols on the rate of lipid peroxides formation on plant lipid liposomes initiated by AMVN (bars). Time-course of tocotrienol decay during the peroxidation reaction

(lines). The reaction were conducted at 37°C. The data are means + SE (n = 3). Different letters on bars of the same colors indicate a statistically significant difference (P<0.05; Duncan's post hoc test).

Figure 4

The effect of plastoquinone on the rate of lipid peroxides formation on plant lipid liposomes initiated by AMVN (bars). Time-course of plastoquinone decay during the peroxidation reaction (lines). The reaction were conducted at 37°C. The data are means ± SE (n = 3). Different letters on bars of the same colors indicate a statistically significant difference (P<0.05; Duncan's post hoc test).

Figure 5

A) The effect of tocopherols on the rate of lipid peroxides formation on plant lipid liposomes initiated by AIPH (bars). Time-course of tocopherol decay during the peroxidation reaction (lines) (B) The effect of tocotrienols on the rate of lipid peroxides formation on plant lipid liposomes initiated by AIPH (bars). Time-course of tocotrienol decay during the peroxidation reaction (lines). The reaction were conducted at

37°C. The data are means $\pm$ SE (n = 3). Different letters on bars of the same colors indicate a statistically significant difference (P<0.05; Duncan's post hoc test).

Figure 6

The effect of PC-8 on the rate of lipid peroxides formation on plant lipid liposomes initiated by AIPH (bars). Time-course of PC-8 decay during the peroxidation reaction (lines). The reaction were conducted at 37°C. The data are means $\pm$ SE (n = 3). Different letters on bars of the same colors indicate a statistically significant difference (P<0.05; Duncan's post hoc test).

Figure 7

Intensity based size distribution of the tocopherol loaded liposomes (averages of 5 measurements).

Figure 8

DPPH radical scavenging activity of α -tocopherol, α -tocotrienol, and PC-8 incorporated in different nanocarriers: liposomes, mixed micelles, and micelles. Samples with different capital letters (A-C) indicate significant differences between the same nanocarriers for the different tocochromanol. Samples designated with different lower-case letters (a-c) were significantly different between different nanocarriers for the same tocochromanol ($P < 0.05$; Tukey's post hoc test).

Figure 9

DPPH radical scavenging activity of α -tocopherol, α -tocotrienol, α -tocopherol phosphate, and plastochromanol-8 incorporated into niosomes. Different letters indicate statistically significant difference ($P < 0.05$; Tukey's post hoc test).