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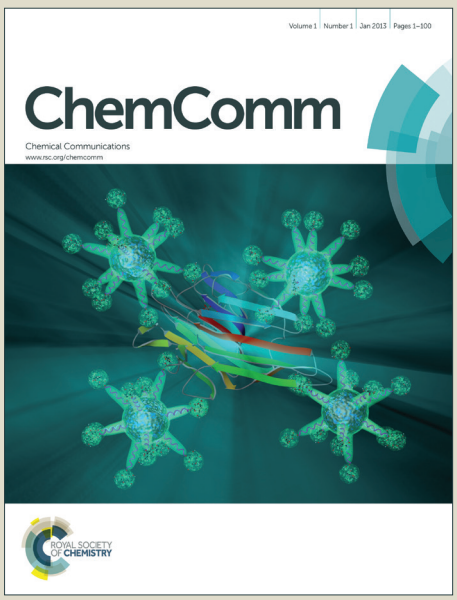

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Synergism of Antioxidant Action of Vitamins E, C and Quercetin Is Related to Formation of Molecular Associations in Biomembranes

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Vitamins E, C and polyphenols (flavonoids and non-flavonoids) are major natural antioxidants capable of preventing damage generated by oxidative stress. Here we show the capacity of these antioxidants to form non-covalent association within lipid bilayers close to the membrane/cytosol interface. Antioxidant regeneration is significantly enhanced in these complexes.

Over the last decades, natural antioxidants have attracted increasing interest, largely because they have been shown to exhibit preventive effects against various disorders caused by oxidative stress, including cardiovascular and neurodegenerative diseases, ageing and also certain cancers[1]. Despite recent progress in the field, there are still many open and fundamental questions concerning antioxidant mechanisms and biological targets, and the exact role in various pathologies is still under scrutiny[2]. A deep understanding of antioxidant action is mandatory for a safe and efficient usage in nutrition, health prevention, cosmetics and food preservation. Most of the known antioxidants are efficient scavengers of reactive oxygen species (ROS), which are overproduced during oxidative stress. Oxidation of lipids (namely lipid peroxidation, LPO) is a major process in oxidative stress, which is initiated by various endogenous (e.g., inflammation, enzymatic processes) or exogenous (e.g., radiation, smoking, pollution) effects. The propagation stage of LPO[3] can be inhibited by lipophilic or amphiphilic antioxidants sufficiently incorporated in lipid bilayers[4]. In addition, hydrophilic and polar antioxidants are able to scavenge ROS that diffuse toward membranes, thus inhibiting the initiation stage of LPO. Vitamin E (α-tocopherol, henceforth referred to as vitE)[5], vitamin C (ascorbic acid, vitC) and natural polyphenols are major antioxidants found in food. Depending on their bioavailability[2,6], these antioxidants are known to be highly efficient ROS scavengers in different phases, namely vitE in membranes[7,8], vitC in plasma or cytosol[9] and flavonoids at the membrane/water interface[4,10]. When acting simultaneously, their overall antioxidant activity is synergistically enhanced[3,11,12]. Free radical scavenging by vitE yields the corresponding α-tocopheroxyl radical by hydrogen atom transfer (HAT), which in turn can be regenerated back to vitE by vitC[3,11,12]. This synergistic effect has been shown enhanced by flavonoids[11-13], which are efficient hydrogen atom donor antioxidants[14,15].

Here, we present a molecular description of the interaction between vitE, vitC and a representative flavonoid antioxidant, namely quercetin[16] (Figure 1), in lipid bilayer membranes. Using both *in vitro* and *in silico* models, the formation of mutual associations at the membrane/water interface is described for the first time. This description enables better rationalization of vitE regeneration by vitC, which is often enhanced in the presence of flavonoids.

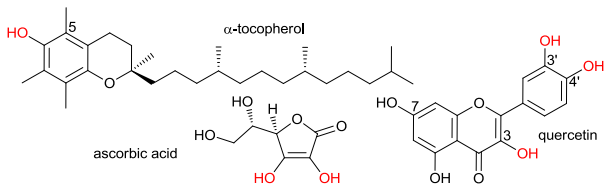

Figure 1. Antioxidant compounds evaluated in this study. The active antioxidant OH groups (prone to HAT) are shown in red.

The penetration and positioning of vitC, vitE and quercetin in membrane was evaluated using a lipid bilayer model comprising DOPC molecules, as phosphatidylcholines are major components of biological membranes in plant and animal cells[17]. Molecular dynamic (MD) simulations were used, which have been repeatedly shown to predict the positioning of small molecules in lipid bilayers in agreement with experimental data[4,18-20]. The behavior of those three (non-interacting) antioxidants was evaluated by placing a single molecule in the lipid bilayer model during the MD simulations.

The simulations showed that vitE localizes below the membrane/water interface and can penetrate through the membrane center. The peak position of the C5-methyl group of vitE was found to be 1.5 ± 0.3 nm from the bilayer center (Figure 2A), which agrees with recent experimental data in DOPC bilayers (1.7 ± 0.4 nm)[8]. The OH group of vitE, which is responsible for free radical scavenging by HAT[21], was mainly located close to the lipid polar head groups, i.e., at the lipid/water interface suggesting inhibition of both the LPO-initiation (directly) and LPO-propagation (if the lipid chains adopt a transient snorkel-like shape[8,22]). Moreover, flip-flops may occur with an energetic barrier of 0.65 kcal.mol^{-1} as obtained by COSMOmic (Figure S1). This roughly corresponds to an occurrence of 1 flip-flop event every 1 µs at a 10^{-6} µM concentration, in agreement with observations from our MD simulations. The flip-flop process is accompanied by the transient presence of the active OH group inside the bilayer (Figure 2A) hence scavenging the deeply buried peroxy radicals and playing a direct role in inhibition of LPO-propagation.

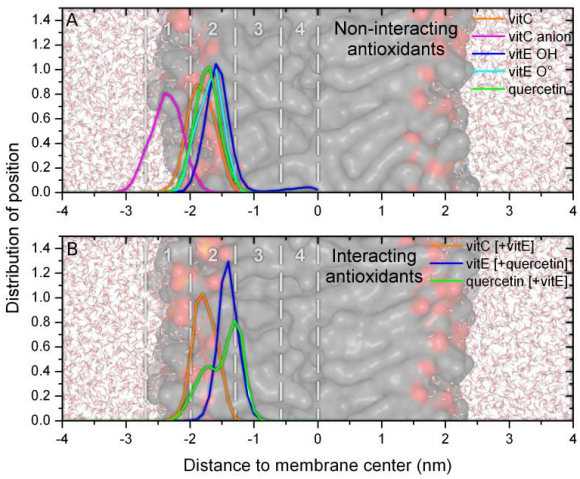

Figure 2. Position of center of mass of vitC and quercetin, and the antioxidant OH group of vitE in DOPC. (A) individual molecules, (B) close contact pairs.

VitC is less buried in the lipid bilayer than vitE and resides in the outer layer close to the water phase (1.9 ± 0.3 nm) because of the lower lipophilicity of vitC with respect to vitE. Interestingly, the average location of quercetin and its aryloxyl radical formed under oxidative stress (1.7 ± 0.3 nm) was found to lie between that of vitC and vitE (Figure 2A). The flip-flop of quercetin is much less efficient than that of vitE, due to higher energetic barrier of 10.2 kcal.mol^{-1} (Figure S1), corresponding to a 1 s time-scale occurrence at 10^{-6} µM.

Under physiological conditions (pH 7.4) and in an aqueous environment, vitC and quercetin are deprotonated (first pK_a equal 4.2 and 5.7 in water for vitC and quercetin, respectively). As expected[23] the corresponding anions lies outside the membrane (Figure 2A) i.e. 2.5 ± 0.3 nm and 2.4 ± 0.2 nm for ascorbate and the phenolate form of quercetin (deprotonated at C-7), respectively. Acid-base equilibrium is likely to occur in the overlapping regions with the protonated forms (Figure 2A).

The lateral (*x*,*y*-plane) diffusion coefficients of vitC, quercetin and vitE were 17 ± 2, 17 ± 2 and 22 ± 5 x 10^{-8} cm^2.s^{-1}, respectively, as obtained from averaging MD trajectories (Table S1). These values are in agreement with the experimental self-diffusion coefficients of DOPC at 313 K (14.10^{-8} cm^2.s^{-1})[24], confirming that the MD simulation time was sufficient to allow correct sampling of all intermolecular motions. The diffusion coefficients along the *z*-axis were lower by one order-of-magnitude for the three antioxidants (Table S1), confirming rather extended residence time in the equilibrium locations.

According to the respective locations of the three studied antioxidants, quercetin may act i) by scavenging free radicals diffusing into the membrane like vitE, both quercetin and vitE being regenerated by vitC; and/or ii) as vitE regenerator, thus enhancing the regeneration by acting in synergy alongside vitC. The active OH group of vitE overlapped that of the center of mass of vitC and quercetin in the head group region (Figure 2A) highlighting the proximity of the three antioxidants, so that the formation of mutual complexes seems likely, in the membrane layer close to the surface.

To confirm that such intermolecular complexes can be formed in the membrane, a series of 300 ns free MD simulations of the lipid bilayer containing several vitC, vitE and quercetin molecules was performed. This procedure allowed sufficient sampling of all possible non-covalent rearrangements and interactions (see Methodology). During the MD simulations, long-lasting (> 90% of the time) and close-contact pairs were observed, namely hetero-association complexes quercetin:vitE, quercetin:vitC and vitC:vitE, and self-association complexes quercetin:quercetin and vitE:vitE (Figure S2, Table S2). An extensive set of one hundred of 100-ns-long MD simulations quantified formation of self- and hetero-association, amounting to 27:45:28% for quercetin:quercetin, quercetin:vitE and vitE:vitE, respectively (Table S3). This does not significantly differ from a random distribution (25:50:25%); however, this should be interpreted with care, as the sampling is still quite limited despite all the effort.

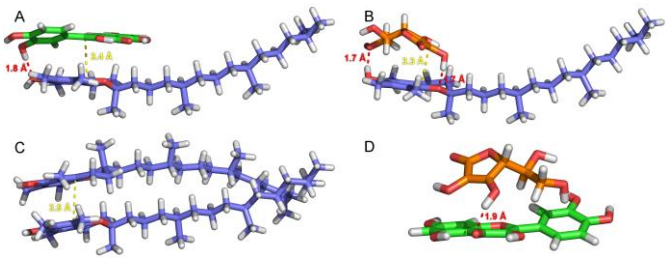

Figure 3. Most stable associations as obtained from DFT-D. (A) quercetin:vitE, (B) vitC:vitE, (C) vitE:vitE, and (D) quercetin:vitC.

The driving force of such non-covalent association was thoroughly analyzed with quantum chemical calculations. Quercetin:quercetin, quercetin:vitE and vitE:vitE pairs were mainly held together by π-stacking interactions, whereas pairs involving vitC were stabilized only by intermolecular H-bonding. The stability of these non-covalent interactions was confirmed with density functional theory (DFT) augmented by

an empirical dispersion term, namely B3P86-D2 recently re-parameterized to accurately evaluate stabilities of polyphenol non-covalent complexes[25]. Different intermolecular arrangements were predicted, namely head-to-head and head-to-tail, in which the importance of π-stacking (ring-to-ring distance of around 3.6 Å, as typical for π-stacking of aromatic rings[26]) and H-bonding was confirmed (see Figure 3 for the most stable geometries and Dataset S1 for all *xyz* geometries).

The *in vacuo* enthalpies of association ranged from -24.4 to -10.8 kcal.mol^{-1} (Table 1). The presence of aqueous environment lowered the absolute values of these association enthalpies by 10.0, 5.8, 8.0 and 14.2 kcal.mol^{-1} for quercetin:vitE, quercetin:vitC, vitC:vitE and vitE:vitE, respectively (Table 1). An entropy loss is expected accompanying formation of the non-covalent complexes, probably counterbalancing the strongly negative enthalpies of association. However, this entropy loss is most probably lower in the organized membrane phase with respect to vacuum[27] (see Methodology section).

Table 1: Association energies and enthalpies (kcal.mol^{-1}) calculated as the difference in energy (enthalpy) between the most stable complex and the isolated fragments, in the gas phase and in PCM-type benzene and water solvents. Negative values indicate that the association is thermodynamically favored compared to the pair of isolated fragments quercetin and vitE.

System	ΔE_{gas}	ΔH_{gas}	ΔH_{C6H6}	ΔH_{H2O}
quercetin:vitE	-15.8	-15.1	-9.0	-5.1
quercetin:vitC	-11.1	-10.8	-9.3	-5.0
vitC:vitE	-15.4	-15.3	-9.0	-7.2
vitE:vitE	-28.0	-24.4	-13.6	-10.2
quercetin:quercetin	-13.7[a]	-	-	-

[a] from ref. [25] with B3P86-D2/cc-pVDZ (BSSE corrected).

In any event, the quantum calculations confirmed that the associations are stabilized by a combination of intermolecular hydrogen bonding and π-stacking. According to this quantum evaluation, attractive forces definitely exist between the three antioxidants, favoring the formation of non-covalent (self- and hetero-) associations of antioxidants.

An experimental confirmation was obtained from the fluorescence quenching of vitE embedded in DOPC liposomes in the presence of quercetin, added at increasing concentrations. VitE-containing liposomes were formed by addition of vitE to DOPC prior to liposome formation. These liposomes were then pelleted and re-suspended in buffer by a double ultra-centrifugation/re-suspension procedure so that non-inserted vitE molecules were discarded (see Materials and Methods for details). Following this procedure, the measured vitE fluorescence (Figure 4A, condition: 0 µM of quercetin) was unambiguously assigned to vitE molecules embedded in the bilayer and not lying on the liposome surface.

With increasing quercetin concentration to the vitE-containing liposomes, a significant decrease in vitE fluorescence intensity was observed (Figure 4A). Quercetin did not exhibit any fluorescence when excited at 291 nm (excitation wavelength of vitE, Figure S3A) in both aqueous solutions and liposomes (Figure S3), therefore ruling out interference. The quercetin concentration-dependent fluorescence quenching thus suggests that i) quercetin molecules have the capacity to insert into the DOPC bilayer, and ii) quercetin:vitE complexes are formed.

The $I_0/I = f$([quercetin]) Stern-Volmer plot is clearly non-linear and follows a quadratic function (Figure 4B), which is unambiguously attributed to the presence of both static and dynamic quenching.[43,44] The linearity of $[I_0/I - 1]$/[quercetin] = f([quercetin]) also confirms this concomitant quenching (Figure S4). The confirmed occurrence of static quenching supports the results of MD simulations, indicating that quercetin penetrates the membrane and forms non-covalent complexes with vitE.

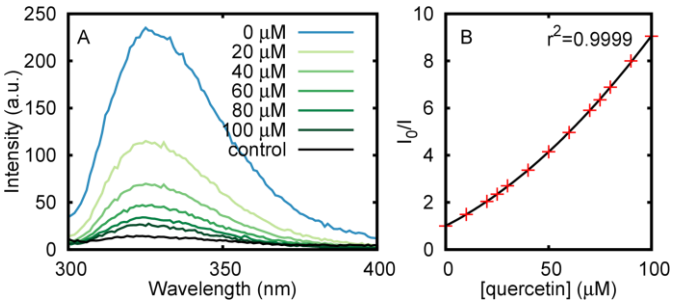

Figure 4: Fluorescence emission of vitE in liposomes with increasing concentrations of quercetin (0 to 100 µM). (A) Fluorescence spectra, (B) Stern-Volmer plot. VitE was excited at λ_{exc} = 291 nm after incorporation into liposomes. The control condition was performed by incubation of vitE (50 µM) with vitE-free DOPC liposomes. Prior to quercetin addition, the non inserted vitE molecules were eliminated from the liposome suspension by double centrifugation and resuspension.

Our findings help to rationalize the results of previous experimental studies showing that addition of flavonoids synergistically increases the antioxidant activity of a vitE and vitC mixture in membranes[11]. The existence of non-covalent complexes between these antioxidants explains how pairs can dramatically improve LPO inhibition by increasing intermolecular contacts between antioxidants, enhancing recycling and subsequent synergic effects.

Indeed, from a thermodynamic point of view, the capacity of regeneration is confirmed by comparing the bond dissociation enthalpies (BDEs) of the most labile hydroxyl group of each antioxidant (Figure 1). The BDEs were calculated as 75.5, 78.7, and 78.7 kcal.mol^{-1} for vitE, quercetin (4'-OH group) and vitC, respectively. These low values agree with previous experimental data that have been strongly supported theoretically[15,29], showing that these three compounds have a strong capacity to scavenge free radicals by HAT. The BDE values were similar for all three compounds, which indicates that HAT between the different antioxidants (native or oxidized) is thermodynamically allowable i.e., enabling the regeneration process. The only limitation to this process is the capacity of two antioxidants to come into contact. Here, we have shown that non-covalent interactions (mainly π-stacking and hydrogen bonding) drive this association process and put in close contact the active OH groups (Figures 3 and S2). This

geometrically and statistically enables quercetin undergo HAT towards vitE to regenerate it. Because the BDEs of both compounds are rather close in energy, the reverse process (regeneration of quercetin by vitE) is likely as well, despite being less preferred. Due to π-stacking interactions between aromatic rings in a given complex, electron transfer between the two π-conjugated antioxidant partners is also likely to occur.

These effects would be even more enhanced in larger aggregates, e.g., in nanodomains (lipid rafts). VitE has already been experimentally shown to preferentially localize in lipid rafts[30]. Aggregation and formation of domains have also been evidenced at the membrane surface for catechin derivatives[31], but also inside the bilayer for quercetin[23] and curcumin[19].

The average position of the non-covalent associations in the membrane was also evaluated. No significant location difference was detected between the antioxidants in the complexes and their respective individual partners, except for quercetin:vitE. Indeed, in these pairs, quercetin exhibited a probability density with two peaks (Figure 2B). Although 50% of the quercetin molecules remained at a similar location to the individual molecules (1.7 ± 0.2 nm), 50% were pulled deeper into the membrane (1.3 ± 0.1 nm). This latter location allows the quercetin:vitE pair to span a larger part of membrane with respect to the non-interacting quercetin. This shift towards the center of the membrane may increase the capacity of quercetin to directly inhibit the propagation stage of LPO by scavenging lipid peroxy free radicals, which may also contribute to the synergetic effects.

We have presented a molecular insight into the synergism of vitE, vitC and polyphenols. Our results showed that vitE can reach vitC in the polar head group region of the membrane and form associations that favor its recycling. Quercetin can readily form non-covalent associations with vitE and vitC in membranes, therefore enabling regeneration of vitE and mediating vitE regeneration by vitC. The occurrence of such associations should be systematically considered to support the research in new cocktails of collaborative antioxidants.

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Notes and references

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