Spectrophotometric Studies on Antioxidants-Doped Liposomes

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Abstract: Antioxidants are the effective modulators of physical properties of model and natural membranes as a scavenger of what called free radical. To demonstrate the relationship between the structure of antioxidants and their effect on the molecular dynamics of membranes, UV spectroscopy is applied to investigate the influence of three structurally different antioxidants: Ascorbic acid (vitamin C), vitamin E and Zinc, on the behavior of dipalmitoylphosphatidylcholine (DPPC) multilamellar vesicles. The results obtained indicate that positively charged liposomes doped with each of vitamin C and vitamin E or negatively charged liposomes doped with zinc contribute to membrane fluidity changes as they have been shown to decrease membrane fluidity. The results suggest that the hydrophobic core of the membrane is poorly affected by these three structurally different antioxidants molecules and, consequently, they cannot insert deeply into the bilayer and the interaction is mainly localized at the polar head level which strongly influences membrane stability and lipid dynamics.
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1. Introduction:

Cellular membranes, which contain abundant phospholipids, such as phosphatidylcholine, are major targets subjected to the damage caused by free radicals. Cellular damage due to lipid oxidation is strongly associated with ageing, carcinogenesis and other diseases (Kujoth et al, 2005). Antioxidants are reducing agents, and limit oxidative damage to biological structures by passivating free radicals. Oxidative damage caused by oxidative stress which may be defined as an imbalance between pro-oxidant and antioxidant agents, in favour of the former, this imbalance may be due to an excess of pro-oxidant agents, a deficiency of antioxidant agents or both factors simultaneously. Oxidative stress is free radical which has been characterized by an elevation in the steady state concentration of reactive oxygen species including super oxide anion, hydrogen peroxide, and hydroxyl radical. The oxidative stress state results in an excess of free radicals (which are molecules or atoms with unpaired electrons) which can react with cellular lipids, proteins, and nucleic acids leading to local injury and eventual organ dysfunction. Lipids are probably the most susceptible bio-molecule to free radical attack. There is increasing evidence connecting oxidative stress with a variety of pathological conditions including cancer, cardiovascular diseases, chronic inflammatory disease, post-ischaemic organ injury, diabetes mellitus, xenobiotic/drug toxicity, and rheumatoid arthritis.

Fortunately, our bodies have developed a number of defense protective mechanisms known as the “antioxidant defense system”. The protective mechanisms are enzymes and non enzymatic antioxidants like vitamins (classified to water soluble and lipid soluble vitamins) and minerals.

Liposomes have been used extensively as biological models for in vitro lipid oxidation studies. The resemblance between the liposomal and membrane bilayer core makes liposomes a very useful tool to investigate the significance of the antioxidant-membrane interactions for antioxidant activity. The antioxidant activity of a compound is strongly influenced by numerous factors including the nature of the lipid substrate, the hydrophilic–lipophilic balance of the antioxidant, the physical and chemical environments of the lipids, and various other interfacial interactions. In a large number of studies, liposomes have been used for the evaluation of the antioxidant properties of several lipophilic and hydrophilic antioxidants against oxidant insults. In addition, but to a lesser extent, studies have also examined the use of liposomes for the delivery of water-soluble and lipid-soluble antioxidants as well as antioxidant enzymes to different organs and tissues for the treatment of oxidative stress-induced damage. (Suntres, 2002).

The main aim of this work is to stress the influence of three structurally different antioxidants: vitamin C, vitamin E and Zinc on membrane fluidity and to establish a relationship between the interactions of the antioxidants with the membrane and their consequent antioxidant activity. The structures of vitamin C and vitamin E are presented (figure 1). The evidence from several studies have shown that administration of antioxidants did not seriously modify the injurious actions of oxidants, an

observation attributed mostly to their inability to cross cell membrane barriers and/or to their rapid clearance from cells. So, the liposomes (which are phospholipid bilayer vesicles) are used as a model membrane (due to their structural similarity to cell membranes) to study and understand the molecular basis of the pharmacological behavior of antioxidant and their effect on membrane fluidity and as the result the possible implications in their antioxidant activity (Suntres and Omri, 2006).

In this study, we use UV spectroscopy experiments to show antioxidants induce changes in the fluidity of the lipid bilayer and to estimate the subtle perturbation of the lipid bilayer structure.

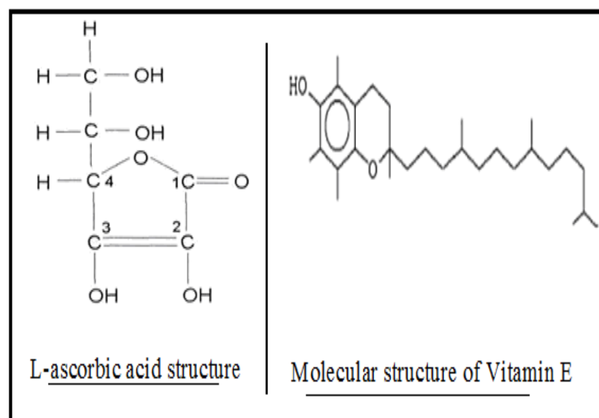


Fig. 1: Chemical structures of the antioxidants involved in the study: Ascorbic acid (vitamin C) and vitamin E.

2. Materials and Methods

High purity L-α-Dipalmitoyl phosphatidylcholine (DPPC), with a molecular weight of 734 (99% pure), Trizma buffer, with a molecular weight of 121.1, Dicetyl phosphate (DCP), with a molecular weight of 546.9 (99% pure) and, Stearyl amine (SA), with a molecular weight of 269.5 (99% pure) were purchased from Sigma (ST. Louis, Mo, USA) and were used without further purification. Vitamin C (VC), with a molecular weight of 176.13, vitamin E (VE), with a molecular weight of 430.72 and, Zinc, with a molecular weight of 65.409 were purchased from EIPICO (Egyptian International Pharmaceutical Industries Co, Egypt). All other chemicals used in this work were of research grade. Solutions were prepared in de-ionized ultra pure water.

Preparation of control liposomes

Aliquots of 10 mg of DPPC were dissolved in 10 ml ethanol (EtOH), and evaporated in a Rotavapor (Vacuum System). The film was dried under vacuum (Vacutherm, Heraeus Instrument, Hannover, Germany) overnight at 42 °C. Ten ml of buffer (10 mM Trizma adjusted to pH 7) was then added to the flask which was flashed through with nitrogen stream and immediately stoppered. The flask was mechanically shaken for 1 hr at 42 °C. The hydration step is done at a temperature above the liquid crystalline transition temperature T_c of the lipid. The suspension was then centrifuged at 8000 rpm for 20 min and the supernatant was discarded. The liposomes were prepared following classical methods (Deamer and Uster, 1983.)

Preparation of antioxidants - doped liposomes

Two alternative incorporation strategies were performed:

Preparation I for VC and VE

Antioxidant liposomes were prepared following the same classical methods as described above using 10 mg of DPPC were mixed with each of 0.68 mg of vitamin C (molar ratio of 7:2) or 1.37 mg of vitamin C (molar ratio of 7:4), 1.6766 mg of vitamin E (molar ratio of 7:2) or 3.35 mg of vitamin E (molar ratio of 7:4) in round bottom flasks. 10 ml of EtOH were added to each mixture, and then a vigorous vortex took place to assure a complete solvation for each mixture. The mixture was evaporated and dried as before. The previous preparation was for neutral preparations. A net positive or negative charge was introduced by the addition of either SA (0.506 mg) or DCP (1.027 mg) to the lipid composition.

Preparation II for Zinc

0.509 mg of zinc in a molar ratio of 7:2 or 0.76 mg molar ratio of 7:4 was added to 10 ml of buffer (10 mM Trizma adjusted to pH 7) which previously contained 0.5 ml of conc HCL and each mixture added to 10 mg of DPPC which previously dissolved in 10 ml of EtOH. The mixture was evaporated under the same conditions of control as before and the film was similarly dried.

The above preparation was suited to be the neutral preparations. A net positive or negative charge was introduced by the addition of either SA (0.506 mg) or DCP (1.027 mg) to the lipid composition.

UV spectroscopy measurements

To characterize the antioxidants stability before and after incorporation, UV spectra of the liposomal suspension were taken on a PC Scanning Spectrophotometer (Uvikon 930, Italy). From UV spectra [200-360 nm], the resonance absorption peaks of antioxidants were recorded. All measurements were performed at room temperature.

3. Results and discussion

UV spectroscopy measurements

Figure 2 shows the typical UV-absorption spectra for pure DPPC [A], vitamin C [B], vitamin E [C] and Zinc [D] respectively. The characteristic bands was found at 221 nm for pure DPPC, 214 and 268 nm for vitamin C, 240, 280 and 287 nm for vitamin E and 227 nm for Zinc.

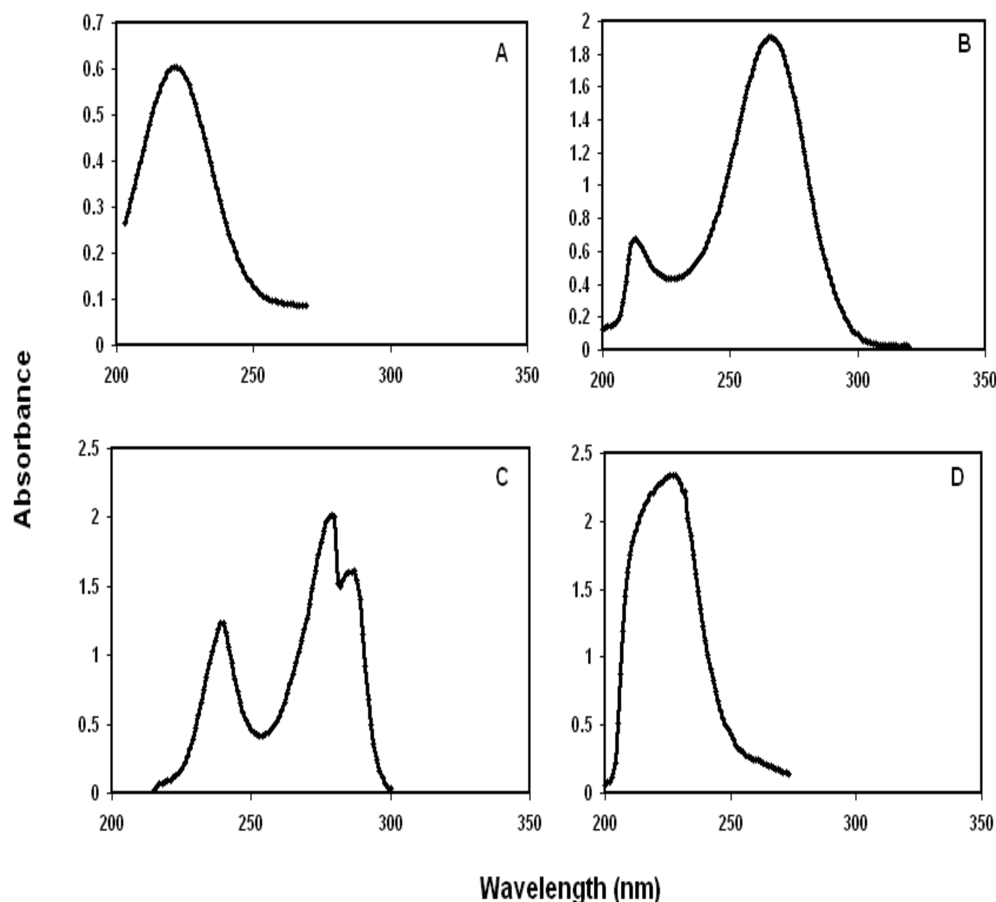
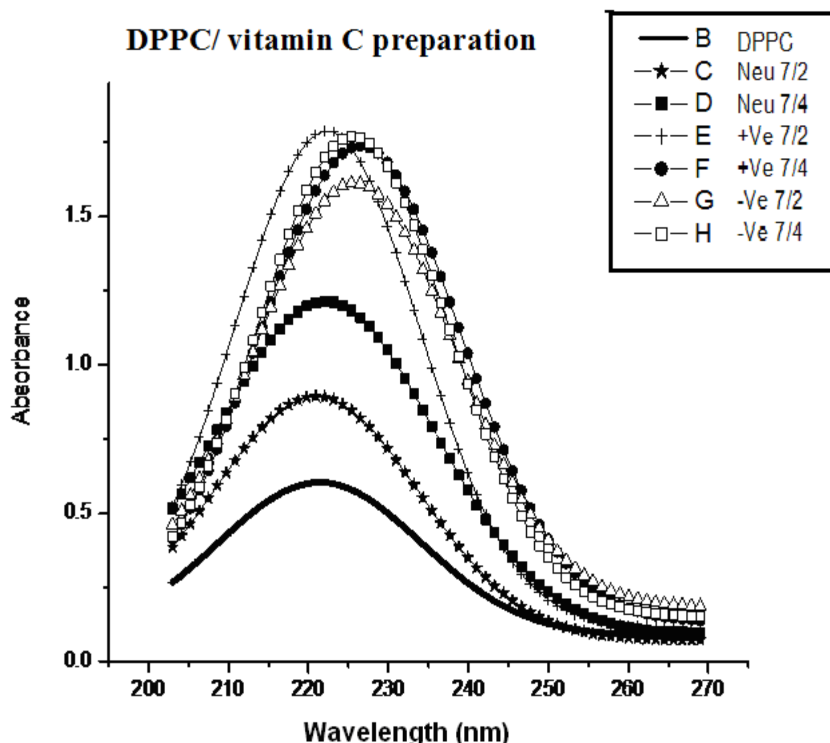


Fig. 2: Typical UV- spectra for [A] DPPC, [B] vitamin C, [C] vitamin E and [D] Zinc

The UV-absorption spectra of various formulations of vitamin C in DPPC liposomes (Figure 3), shows that positively charged liposomes doped with vitamin C at molar ratio 7:4 and both negatively charged liposomes at molar ratio 7:2 and 7:4 exhibited an average 5-6 nm bathochromic shift (which assigned for less energy) with respect to the absorption spectrum in tris buffer solution, indicating that these formulations are embedded in the lipid vesicle. Moreover, the characteristic bands of vitamin C as integral molecule at 214 and 268 nm are not any more visible. The strong electrostatic reaction between phospholipids and vitamin C should not be ignored, as they might play a vital part in the interactions of vitamin C with phospholipids. The existence of $N(CH_3)_3^+$ group in the polar head of DPPC may attract ascorbate negative ions of vitamin C which may be expected to disappear the characteristic band of vitamin C. In polar solvents aggregates would be formed between vitamin C and water molecules, and these would shift the absorption maximum to higher wavelengths. The data suggest that the hydrophobic core is poorly affected by vitamin C molecules and, consequently, they cannot insert deeply into the bilayer and the interaction is mainly localized at the polar head level.

These findings from UV spectroscopy experiments showed that vitamin C was unable to interact with the hydrophobic zone of the bilayer. (Table 1) summarizes the spectral changes monitored upon incorporation of vitamin C into different formulations of DPPC liposomes.



(Fig. 3): Typical UV-spectra of vitamin C incorporated into different formulations of DPPC liposomes.

Table 1: Spectral changes upon incorporation of Vitamin C into DPPC liposomes

VC / liposomes Samples	Wavelength	Bandwidth	Absorbance
DPPC	221±0.7	25± 2.1	0.7
Neutral (7:2)	221±0.8	26 ±2.3	1
Neutral (7:4)	*223±0.6	27 ±2	1.3
+Ve (7:2)	220±0.5	*19 ±1.2	1.7
+Ve (7:4)	*227±0.5	*18 ±1.1	1.77
-Ve (7:2)	*227±0.4	*16 ±1.3	1.8
-Ve (7:4)	*226± 0.5	*19 ± 1.2	1.9

*Statistically significant

Figure 4 represents the absorbance spectrum of various formulations of vitamin E in DPPC liposomes. The results indicate that the positively charged liposomes doped with Vitamin E at molar ratio 7:2 and neutral liposomes at molar ratio 7:2 exhibited an average 4-5 nm bathochromic shift with respect to the absorption spectrum in tris buffer solution, indicating that this formulation is significantly interacted with lipid bilayers. The bathochromic shift would occur not only in polar solvents (by the formation of aggregates with solvent molecules) but also in non-polar solvents (by the formation of intra-molecular hydrogen bonds). Moreover, the characteristic bands of vitamin E as integral molecule at 240, 280 and 287 nm are disappeared by incorporation into liposomes. The results indicate that the incorporated vitamin E can not insert deeply into the bilayer and the interaction is mainly localized at the polar head level.

It has been concluded that vitamin E induces a disordering effect in the gel phase in the phospholipids membranes (Severcan, 1997). The effect of α-tocopherol on a phospholipid model membrane has been investigated by Electron Spin Resonance and Saturation Transfer ESR by using stearic acid and perdeutero- di-t-butyl nitroxide spin probes. It is observed that in the gel phase it induces a decrease of order and increase in fluidity; while in the liquid crystalline phase an indication of a slight increase in ordering and a clear decrease in fluidity

are registered (Severcan and Cannistraro, 1990). (Table 2) summarizes the spectral changes upon incorporation of vitamin E into different formulations of DPPC liposomes.

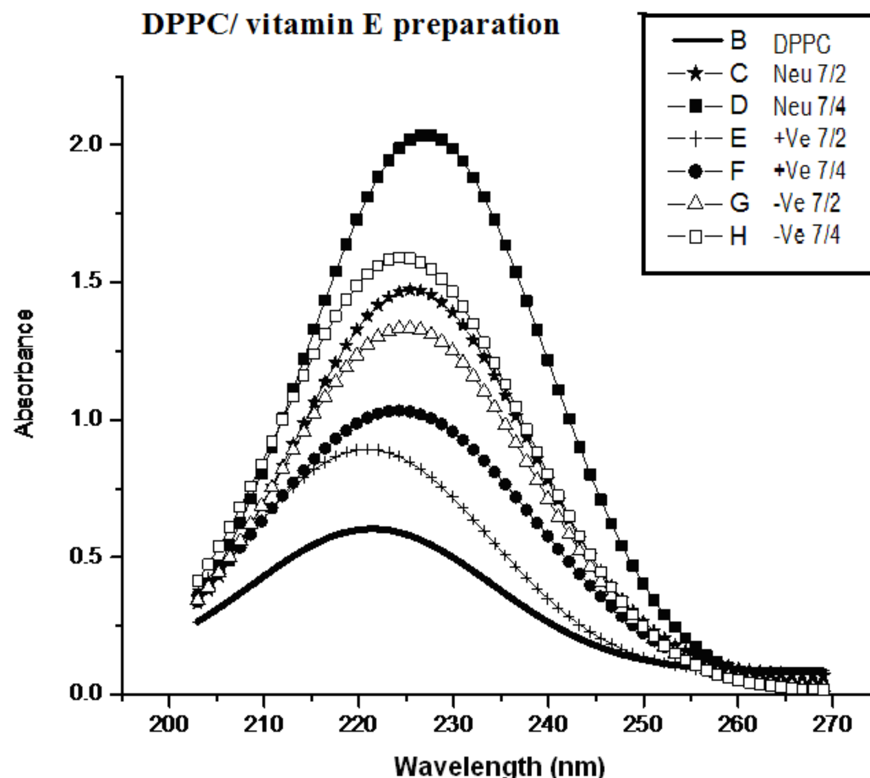


Fig. 4: Typical UV-spectra for vitamin E incorporated with different formulations of DPPC liposomes.

Table 2: Spectral changes upon incorporation of vitamin E into DPPC liposomes

VE / liposomes Samples	Wavelength	Bandwidth	Absorbance
DPPC	221± 0.7	25± 2.1	0.7
Neutral (7:2)	*223 ±0.4	22± 1.2	1.5
Neutral (7:4)	*225±0.3	24± 0.7	2
+Ve (7:2)	*226±0.6	27± 1.3	0.5
+Ve (7:4)	*223 ±0.5	26± 1.1	1.1
-Ve (7:2)	*222± 0.5	23± 0.9	1.4
-Ve (7:4)	*223± 0.4	23± 0.8	1.6

*Statistically significant

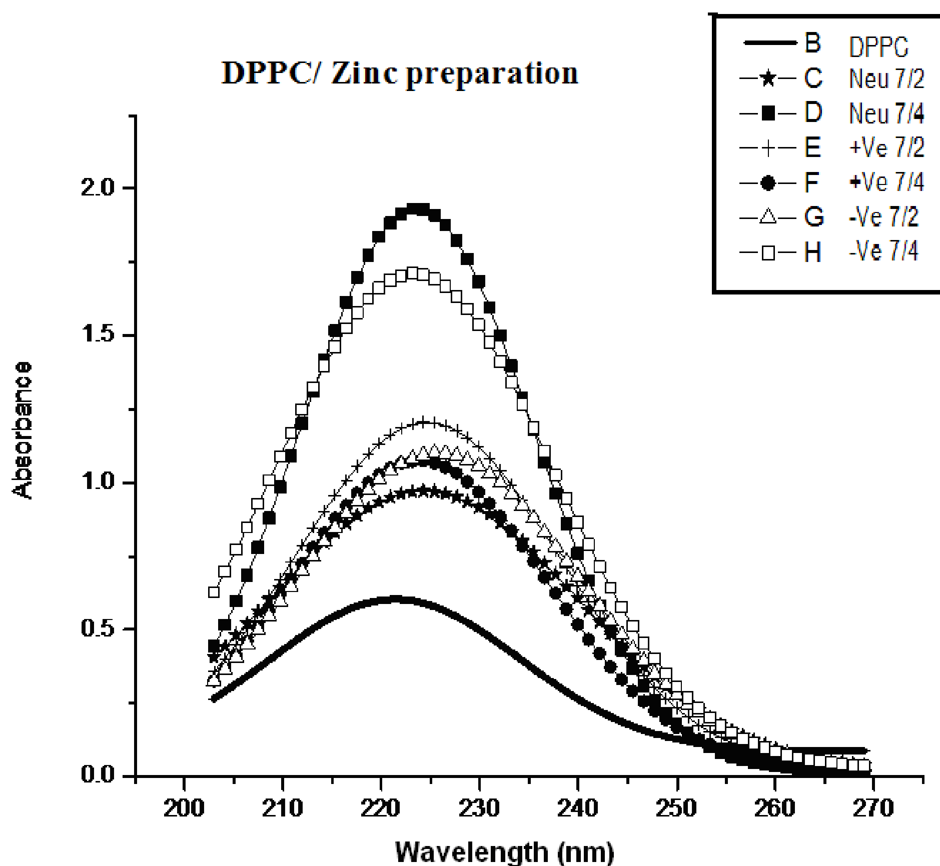
It is well known that the antioxidant action of vitamin E significantly depends upon its location in the membrane bilayer (Fukuzawa et al., 1981; Burton and Ingold, 1986; Niki, 1989). On one hand the phenolic hydroxyl group of vitamin E acts as a capture of peroxyl radicals. Recently, on the basis of numerical kinetic investigation, it was shown that the pro-oxidant activities of vitamin E mostly due to the reduction of hydroperoxides by vitamin E (Tavadyan et al., 2007). On the other hand the existence of a long hydrophobic phytyl side chain allows one to monitor the location of vitamin E in the membrane bilayer and hence to vary its antioxidant action.

It has been considered (Avellone et al., 2002) that UV absorption spectroscopy is a suitable technique to provide direct information on the location of vitamin E in the DPPC. UV absorption band of vitamin E (-tocopherol; Fig. 1) as an aromatic group-containing compound exhibits fine vibrational structure in non-polar solvents, such as heptane or cyclohexane. On the other hand, in relatively polar solvent chloroform and in ethanol the

fine structure is vanished and just broad absorption is observed. These observations provided to develop an elegant approach for studying the local microenvironments of vitamin E in liposomes and hence membranes.

The absorption spectra of Zinc into different formulations of DPPC liposomes are shown in figure 5. The results indicate interestingly that the both negatively charged liposomes at molar ratio 7:2 and 7:4 are displayed contrary effect. While the negatively charged liposomes at molar ratio 7:2 exhibits a siginifant bathochromic shift (6nm) , the negatively charged liposomes at molar ratio 7:4 exhibits hypsochromic shift (3nm). Zinc was known as from metal ion with positive charge and when these metal ions react with liposomes, it make destruction of the liposomes. It has been investigated that the efficiency of encapsulation of several divalent metal ions, mainly Zn (II) ion, into liposomes with particular attention to the stability of liposomal membrane against the transition metal (II) ions. The stronger interaction of metal ions to liposomes led to the less formation of liposomes. (Sunamoto et al, 1980).

Like all metal cations, zinc interacts electrostatically with anionic groups. In contrast to other divalent metal cations with similar ionic radius such as Mg^{+}_{2}, zinc ions possess a higher affinity to electronegative groups (Gresh and Šponer 1999). Zn^{+}_{2} obviously bind more tightly. It forms a stable complex with the zwitterionic phospholipid headgroup. The changes in its conformation, hydration and the effect on gel – liquid-crystal phase temperature of 1-palmitoyl-2-oleoyl-*sn*glycero- 3-phosphocholine (POPC) were studied by (Binder et al. 2001). Dehydration of phospholipid headgroups due to complexation with zinc cations was suggested to increase fusogenic potency of lipid membranes. (Table 3) summarizes the spectral changes monitored upon incorporation of Zinc into different formulations of DPPC liposomes.



(Fig. 5): Typical UV-spectra for Zinc incorporated with different formulations of DPPC liposomes.

Table 3: Spectral changes upon incorporation of Zinc into DPPC liposomes

Zn / liposomes Samples	Wavelength	Bandwidth	Absorbance
DPPC	221± 0.7	25± 2.1	0.6
Neutral(7:2)	*225 ± 0.6	*32± 1.4	1
Neutral(7:4)	*219± 0.4	22± 0.8	2
+Ve (7:2)	*225 ± 0.5	*23± 1.1	1.3
+Ve (7:4)	221± 0.5	22± 1.1	1.1
-Ve (7:2)	*227 ± 0.5	22± 1.0	1.2
-Ve (7:4)	*218 ± 0.3	*21± 0.8	1.8

*Statistically significant

Conclusion

On the basis of the above results, antioxidants are the effective modulators of physical properties of model and natural membranes as a scavenger of what called free radical. The results revealed complex interactions between antioxidants and polar head group, which strongly influence membrane stability and lipid dynamics.

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