

Interaction of Coenzyme Q₁₀ with Dipalmitoylphosphatidylcholine Liposomes

Medhat W.Shafaa¹, Hossam S.Kareem², Amany H. Elshazly³, Amira Z.Dakrory⁴ and Maha R. Elsyed⁴

¹Physics department, Faculty of Science, Helwan University, Cairo, Egypt,

²Physiological optics department, Research Institute of Ophthalmology, Giza,

³Departments of Biochemistry and Pharmacology, Research Institute of Ophthalmology, Giza

⁴Physics department, Faculty of women for arts, science and education Ain Shams University, Egypt.

Abstract:

The goal of this study to investigate the influence of antioxidant as coenzyme Q₁₀ on structural changes of model lipid membranes as DPPC by using Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC). The results obtained indicate that the greater effect of CoQ₁₀ upon the incorporation into negatively charged liposomes in comparison with the other liposome formulations. The incorporated CoQ₁₀ is probably associated with the lipid bilayers, interacted to a large extent with them, and perturbed them which results in the strong broadening and shift to lower temperature 94 °C of the major characteristic endothermic peak of pure DPPC that exists at 105 °C. The FT-IR spectra of samples showed that Incorporation of CoQ₁₀ into neutrally, positively and negatively charged DPPC liposomes showed significant change in the frequency of the antisymmetric CH₂ stretching bands in the acyl chain implying that CoQ₁₀ create a conformational disorder within the acyl chains of phospholipids. In other words, it had significant effect on the order of the membrane.

Key word: Liposomes – Coenzyme Q₁₀ – DSC – Encapsulation – FTIR

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.

Corresponding Author: Maha R. Elsyed³, Physics department, Faculty of women for arts, science and education, Ain Shams University
E-mail: mahareda83@yahoo.com

There is increasing evidence connecting oxidative stress with a variety of pathological conditions including cancer, cardiovascular diseases, chronic inflammatory disease, post-ischemic 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 (Suntres and Omri, 2006).

Coenzyme Q₁₀ (CoQ₁₀) is an only endogenous synthesized antioxidant existing in all cell membranes of our body. It is essential for production of adenosine triphosphate (ATP), which is the energy source for all living cells. CoQ₁₀ has been proposed to help treat or possibly even prevent many cardiovascular and neurodegenerative disorders, therefore it has become one of the most popular nutritional supplements. It efficiently protects membrane phospholipids from peroxidation and also mitochondria DNA and membrane proteins from free-radical-induced oxidative damage.

However, because of its higher molecular weight and poor water solubility, CoQ₁₀ has very low oral bioavailability from the gastrointestinal tract. Several formulation approaches have been adopted to improve in vitro dissolution and absorbability of CoQ₁₀, such as liposomes. The structure of CoQ₁₀ is presented in (Fig.1).

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. Liposomes are considered as an acceptable and superior drug delivery system because they are biocompatible, biodegradable and nontoxic. With respect to treating oxidant induced tissue injuries, it has been demonstrated that encapsulation of antioxidants in liposomes promotes their therapeutic potential against oxidant-induced tissue injuries, presumably by liposomes facilitating the intracellular uptake and extending the half-lives of the encapsulated antioxidants (Ajda Ota et al, 2015).

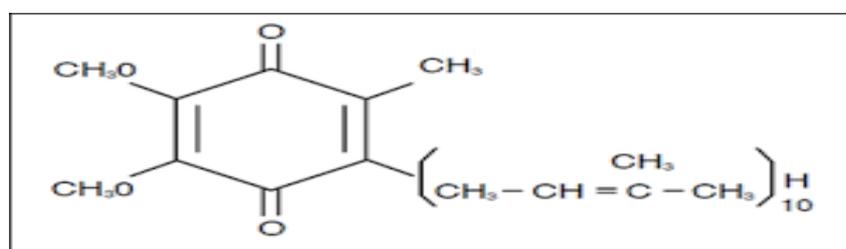


Fig (1): Chemical structure of the Coenzyme Q₁₀

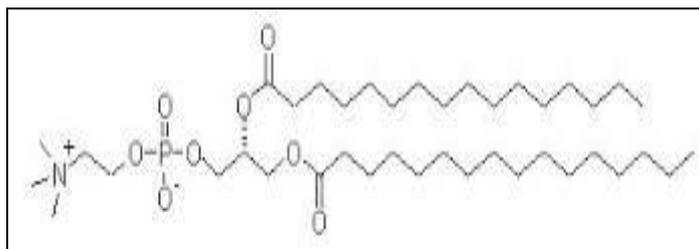


Fig (2): Chemical structure of DPPC

2- Materials and Methods

High purity L- α -Dipalmitoyl phosphatidylcholine (DPPC) is presented in (Fig.2) with a molecular weight of 734 (99% pure), Trizma buffer, with a molecular weight of 121.1, Dicaptyl 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. Coenzyme Q₁₀ (CoQ₁₀), with a molecular weight of 863.358 was 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.

(a) Preparation of control liposomes

Aliquots of 10 mg of DPPC were dissolved in 10 ml ethanol (C₂H₅OH), 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).

(b) Preparation of Coenzyme Q₁₀ - doped liposomes

CoQ₁₀ liposomes were prepared following the same classical methods as described above using 20 mg of DPPC were mixed with 3.4 mg CoQ₁₀ (molar ratio of 7:2) in round bottom flasks. 10 ml of C₂H₅OH 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.

(c) Encapsulation efficiency measurements

The encapsulation efficiency of the samples was measured using a spectrophotometer (Uvikon 930, Italy). The wavelengths were adjusted at 275 nm (the resonance absorption peaks of CoQ₁₀). The absorption of the supernatant of each sample (centrifuged at 8000 rpm for 20 min) was compared with the standard curve relating absorption to the CoQ₁₀ concentration. Mixing CoQ₁₀ with the lipid powder before dissolving it in ethanol was found to increase the encapsulation efficiency up to 90%. If CoQ₁₀ was dissolved in buffer and

added to the dry film of lipid upon hydration, the encapsulation efficiency is significantly reduced.

(d)DSC measurements

Differential scanning calorimetry (DSC) experiments were performed with differential scanning calorimeter (model TA-50 WSI, Shimadzu) calibrated with indium. Samples of empty and CoQ₁₀-loaded multilamellar liposomes were submitted to DSC analysis. The analyses were performed on 5-mg samples sealed in standard aluminum pans. Thermograms were obtained at a scanning rate of 5°C/min. Isotonic PBS buffer (pH 7.4) was employed as reference. Each sample was scanned between 25 and 200°C. The temperature of maximal excess heat capacity was defined as the phase transition temperature.

(f)FTIR Spectroscopy

FTIR spectra of lyophilized samples of DPPC liposomes and DPPC liposomes encapsulated CoQ₁₀ deposited in KBr disks were recorded on a NICOLET 6700 FTIR spectrometer (Thermo Scientific, Cambridge, England). Scanning was carried out at room temperature, in the range 400–4,000 cm⁻¹ at a speed of 2 mm/s and a resolution of 4 cm⁻¹. A final lipid concentration of 2 mg/ml was used.

3- Results and Discussion

DSC is generally used to measure a number of characteristic properties of a sample. It is possible to observe fusion, crystallization and even oxidation and other chemical reactions. The fluidity of lipid bilayers depends on the lipid or combination of lipids used and their fluid gel transition temperature. The melting point (T_m) represents the peak temperature of the endotherm for the lipid gel-to fluid phase transition recorded during the heating scan.

The pre and main transitions are often observed in PC lipids. The pre-transition, which corresponds to the conversion of a lamellar gel phase to a rippled gel phase, is mainly related to the polar region of phospholipids, whereas the main transition reflects the change from a gel phase to a liquid crystal phase. It has also been suggested that the main transition process is closely related to the acyl chains of phospholipids bilayers, which can probe the interaction between the acyl chains of phospholipids and exogenous substances.

The DPPC vesicles were used as model membranes since this phospholipid is able to mimic many aspects of biological membranes, being one of their most abundant constituents. When submitted to DSC analysis, pure DPPC vesicles upon dehydration showed a major endothermic peak at 105 °C (**Figure 3**), in accordance with **Koynova and Caffrey [1998]**. The pre-transition temperature was around 80°C for pure DPPC liposomes. A sharp endothermic peak at about 75 °C was observed for pure CoQ₁₀.

The presence of **a compound in** the DPPC membranes could interact and influence the thermotropic parameters of the vesicle transition as a function of its own physicochemical properties.

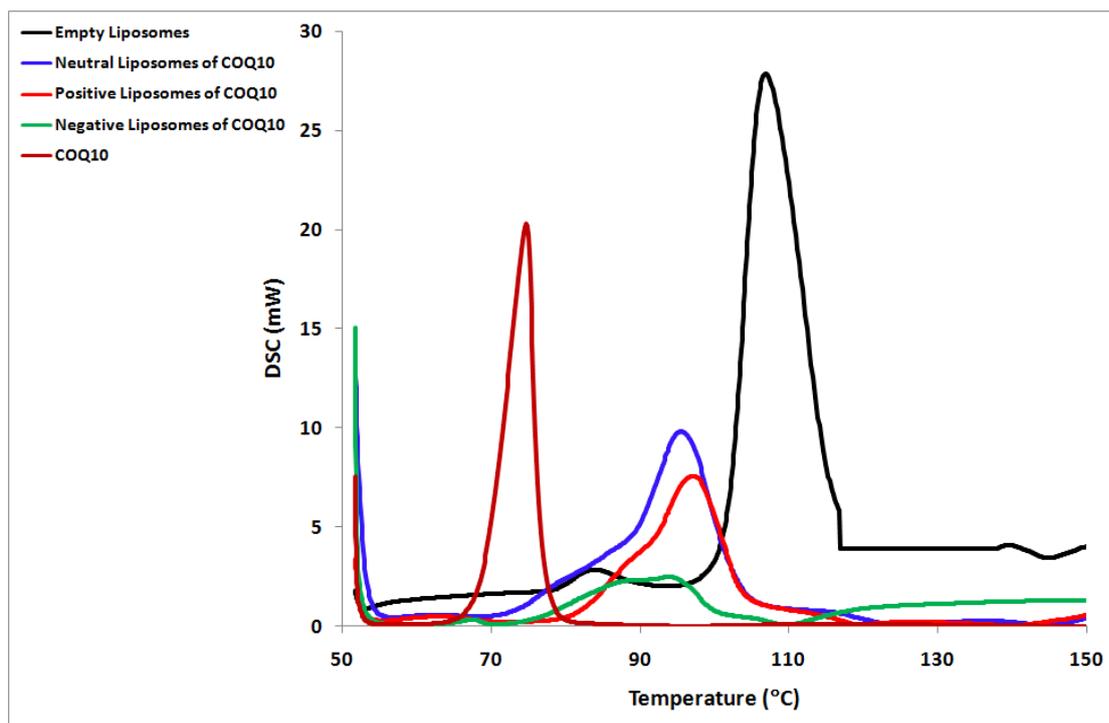


Figure 3: DSC diagrams of liposomes made of pure DPPC, liposomes doped with CoQ₁₀ and pure CoQ₁₀.

The incorporation of CoQ₁₀ into neutrally or positively charged liposomes exhibited noticeable broadening and shift to lower temperatures 96 °C and 98 °C respectively in a comparison to the major characteristic endothermic peak of pure DPPC that exists at 105 °C which suggested that CoQ₁₀ had a significant effect on the acyl chains of DPPC bilayers, and that its presence decreased the cooperative transition of the lipid acyl chains (**Rathod and Deshpande, 2010**). The lowered temperature of the main DPPC transition process indicated that the incorporation of CoQ₁₀ is more favorable to the formation of acyl chains in a disordered and loose state. The pre-transition temperature peak for neutral and positive DPPC/ CoQ₁₀ liposomes disappeared, which revealed that CoQ₁₀ interacted with the polar head group of phospholipids. The disappearance of the pre-transition is a sensitive criterion for the incorporation of substances into lipid bilayers.

Here one can observe the greater effect of CoQ₁₀ upon the incorporation into negatively charged liposomes in comparison with the other liposome formulations. The incorporated CoQ₁₀ is probably associated with the lipid bilayers, interacted to a large extent with them, and perturbed them which results in the strong broadening and shift to lower temperature 94 °C of the major characteristic endothermic peak of pure DPPC that exists at 105 °C. The change in phase transition temperature suggests that the incorporated CoQ₁₀ could be resident near the interface region and within the hydrophobic core thus giving rise to CoQ₁₀ enriched microdomains. The incorporation of CoQ₁₀ resulted in a very high miscibility of CoQ₁₀ with phospholipid bilayer which results in the disappearance of its endothermic peak at about 75 °C.

Protein and DNA structure, hydration, and binding of biomolecules have been studied using vibrational spectroscopy, as a combined theoretical and experimental approach. FTIR spectroscopy is a non-destructive technique and can provide quantitative chemical

composition and identify tissue constituents. FTIR spectroscopy was used to monitor subtle changes in the structure and function of the lipid assemblies by analyzing the frequency and bandwidth changes of different vibrational modes representing the acyl chains, interfacial, and head group region of lipid molecules.

A stack plot of the FTIR spectra of pure lyophilized DPPC liposomes compared with CoQ₁₀/DPPC liposomal samples in the region 4000–400 cm⁻¹ are presented in **Figure (4)**. The spectrum of the DPPC liposomes displayed the main characteristic bands, especially those that were because of the following: The symmetric and antisymmetric stretching vibrations of the CH₂ in the acyl chain (2,850 and 2,920 cm⁻¹, respectively), the OH stretching and bending vibrations (3,470 and 1,640 cm⁻¹, respectively), the carbonyl stretching vibration C=O (1,734 cm⁻¹), the CH₂ bending vibration CH₂ (1,470 cm⁻¹) and the symmetric and antisymmetric PO₂⁻ stretching vibrations (1,090 and 1,220 cm⁻¹, respectively).

The detailed spectral analyses were performed in three distinct wave number regions, namely 3500–2800 cm⁻¹ **Figure (5)**, 1800–1500 cm⁻¹ **Figure (6)** and 1800–800 cm⁻¹ **Figure (7)**, since identifiable Raman bands were observed mainly in these regions only. The assignment of the Raman bands of proteins, lipids and nuclei acids is based on the available literature values.

Lipids give rise to number of absorptions in Raman spectra. The most intense of these absorptions are found in the 3500–2800 cm⁻¹ region, attributed to asymmetric and symmetric stretching vibrations of CH₃ and CH₂ groups of acyl chains. The most intense band observed at 3500 cm⁻¹ in the control and treated has been assigned as presence of hydroxyl compound.

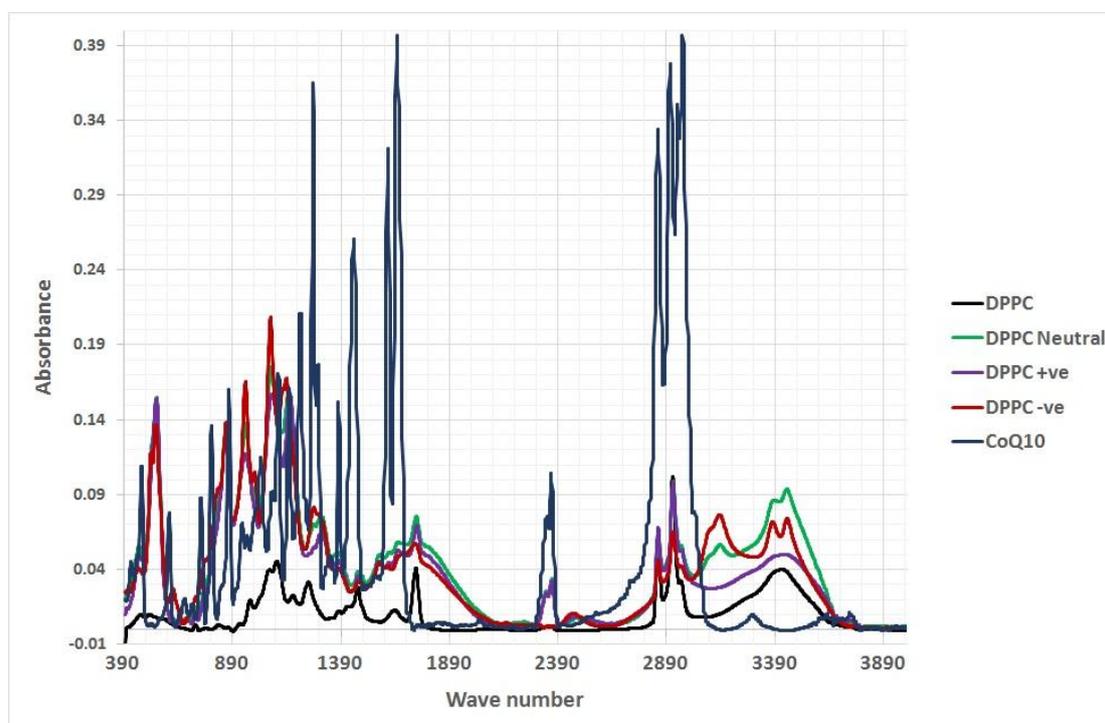


Figure 4: The full FTIR spectra of DPPC and DPPC/ CoQ₁₀ liposomal samples

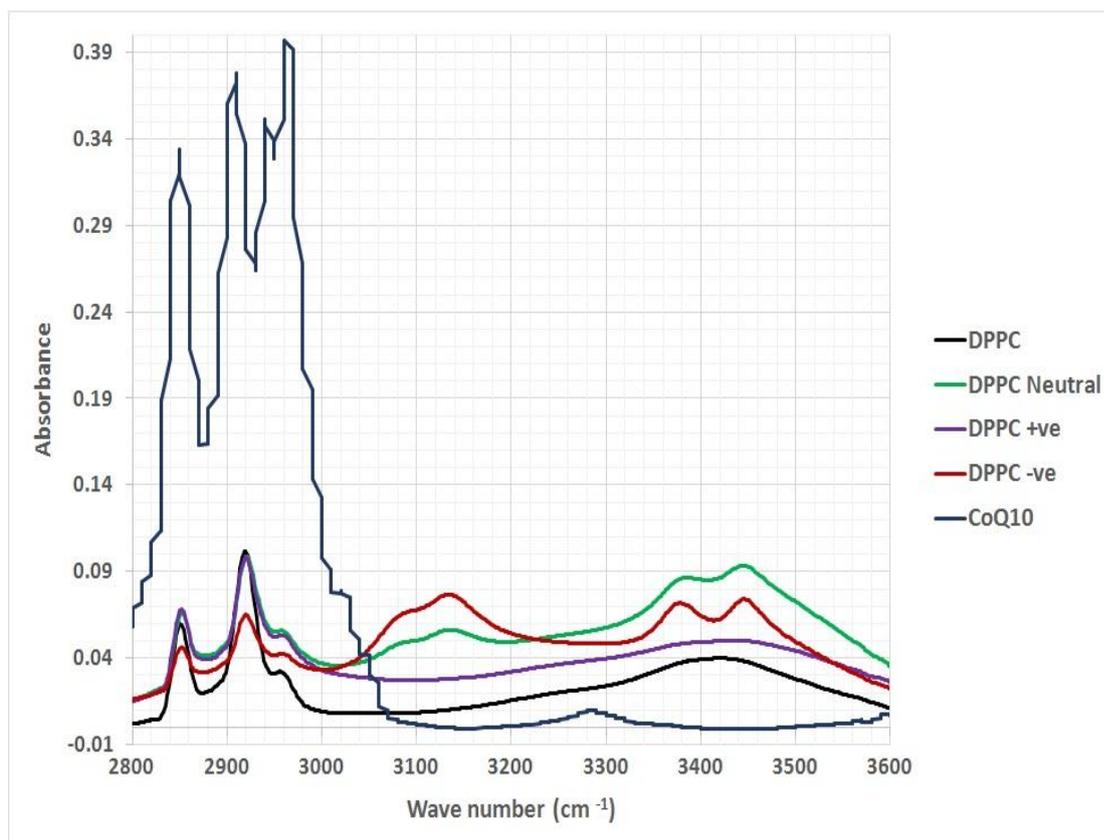


Figure 5: The magnified part (3500-2800 cm^{-1}) of FTIR spectra of DPPC and DPPC/CoQ₁₀ liposomal samples

Incorporation of CoQ₁₀ into neutrally, positively and negatively charged DPPC liposomes showed significant change in the frequency of the antisymmetric CH₂ stretching bands in the acyl chain observed in **figure (5)** implying that CoQ₁₀ create a conformational disorder within the acyl chains of phospholipids. In other words, it had significant effect on the order of the membrane.

In order to examine the interaction of CoQ₁₀ with glycerol backbone near the head group of phospholipids in interfacial region, the C=O stretching band was analyzed. The wavenumber variation of this band is shown in **Figure (6)**. As seen from the **figure(6)**, the wavenumber value of C=O group was shifted to higher degrees (from 1737 cm^{-1} to 1739.5 and 1737.5 cm^{-1}) for the neutral and negatively charged liposomes sample containing CoQ₁₀ respectively, without any evidence of hydrogen bonding formation. The degree of hydrogen-bond formation was monitored in the glycerol backbone region of the DPPC molecule by changes in the contours of the ester C=O stretching. The wavenumber value of C=O group exhibited shift towards higher value (from 1737 cm^{-1} to 1741.4 cm^{-1}) for the positive liposomes sample containing CoQ₁₀, implying dehydration about these functional groups in the interfacial region of the lipid membranes. This can be accounted for by the presence of free carbonyl groups in the system. The carbonyl absorption band around at 1730 cm^{-1} caused by the stretching vibrations of ester carbonyl groups of phospholipids is structurally sensitive to the level of hydration at the membrane interface and influenced by hydrogen bonding. Therefore, any effects in the spectra of this region can be attributed to an interaction between CoQ₁₀ and the polar/apolar interfacial region of the membrane. CoQ₁₀ tends to reduce the

forming of hydrogen bonding in the interfacial region of DPPC liposome, implying the existing of the free carbonyl groups in the system. CoQ₁₀ is probable to replace some H₂O molecules from the interfacial region and lead to an increase in the number of free carbonyl groups.

The interesting finding that can be concluded from **figure (7)** is that the CH₂ bending vibration mode which is located at 1468 cm⁻¹ is affected by the incorporation of CoQ₁₀ into DPPC liposomal preparation. As can be seen from the **figure (7)**, the wavenumber was shifted to higher values (1469.515 cm⁻¹) after the encapsulation of CoQ₁₀ into all formulations of DPPC liposomes. This implied the presence of disordering effect in acyl chain packing in the gel phases of phospholipids, in accordance with DSC studies. This might assume that the molecules of CoQ₁₀ act as small spacers of the polar head group, leading to a slight disorder in the hydrocarbon chains.

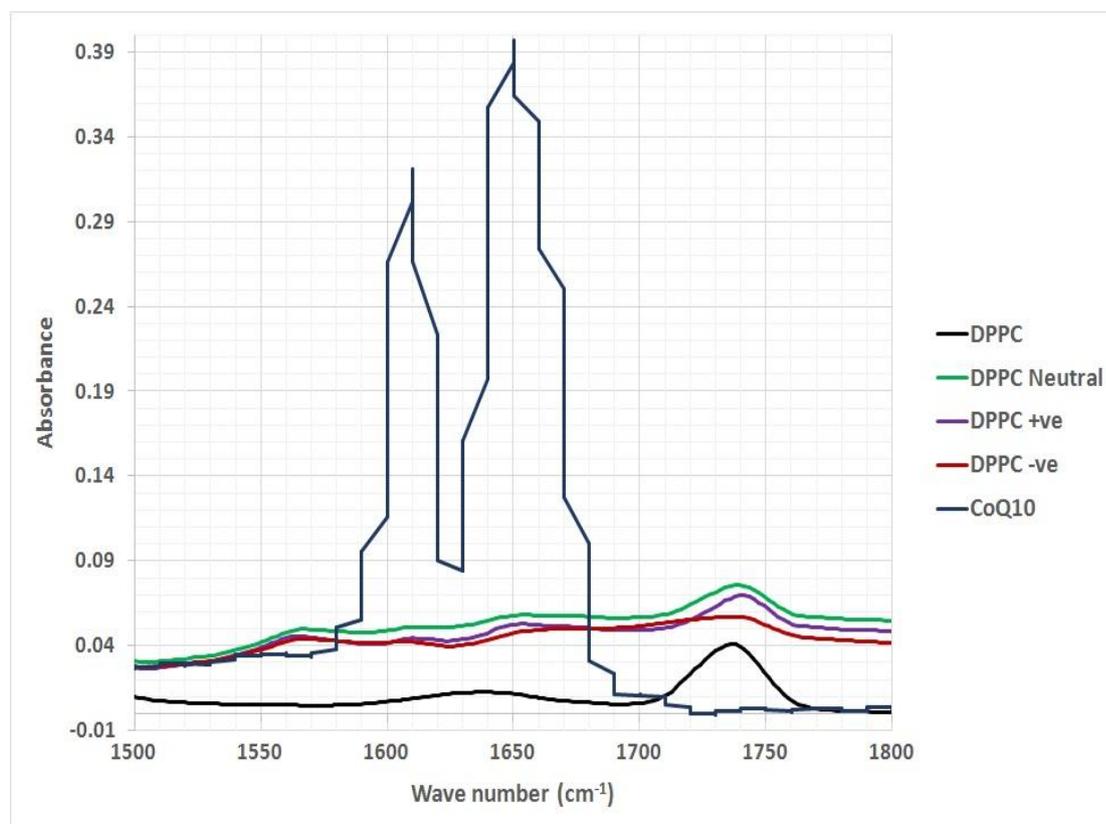


Figure 6: The magnified part (1800-1500 cm⁻¹) of FTIR spectra of DPPC and DPPC/CoQ₁₀ liposomal samples

The interaction between CoQ₁₀ and the head group of DPPC liposomes was monitored by means of the PO₂⁻ antisymmetric stretching band, which is located at 1242 cm⁻¹. **Figure (7)** shows the PO₂⁻ antisymmetric stretching band for DPPC liposomes in the absence and presence of CoQ₁₀. As can be seen from the **figure (7)**, the wavenumber was shifted to higher values 1265 cm⁻¹ after the encapsulation of CoQ₁₀ into neutrally, positively and negatively charged DPPC liposomes, respectively. This implied the reduction of hydrogen bonding between the liposome head group and CoQ₁₀ indicating an increase in dehydration of the phosphate group. In accordance with the empirical rules, decreasing frequency values displays an increase in the strengthening of existing hydrogen bonding or in the formation of new hydrogen bonding between the components ([Severcan et al., 2005](#)).

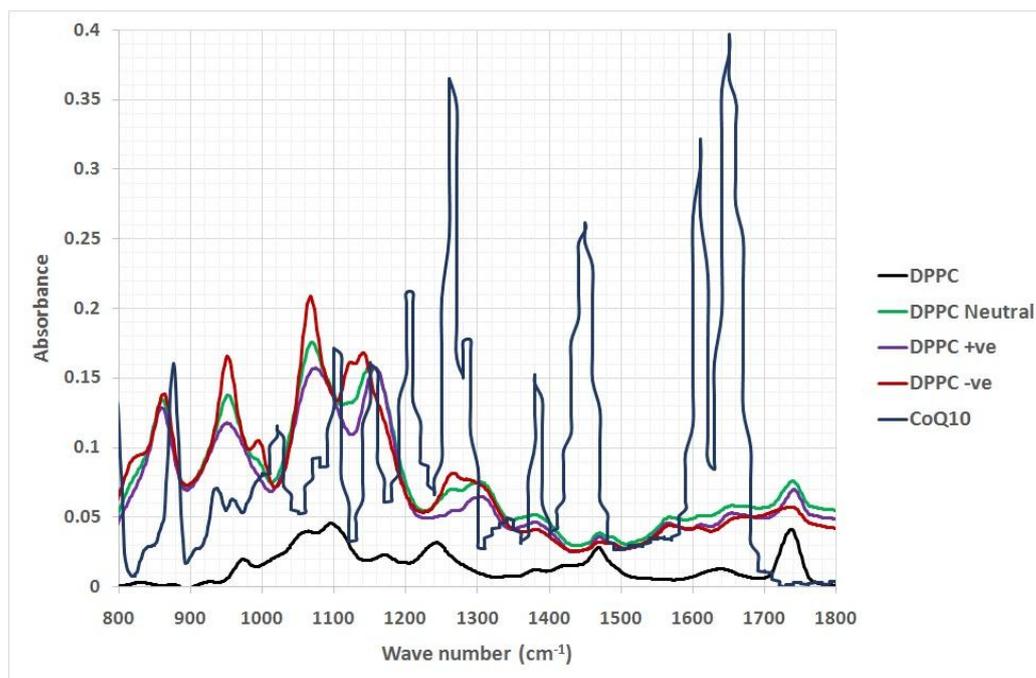


Figure 7: The magnified part (1800-800 cm^{-1}) of FTIR spectra of DPPC and DPPC/CoQ₁₀ liposomal samples

An interesting peak is appeared which corresponds to the aliphatic phosphate stretch at 995 cm^{-1} upon the encapsulation of CoQ₁₀ into negatively and positively charged DPPC liposomes, respectively possibly due to the immobilizing effect of CoQ₁₀ to the phosphate group moiety **figure (7)**. Table 1 summarizes the chemical shift observed for CoQ₁₀ after the incorporation into different formulations' of DPPC liposomes.

Table 1: The chemical shift observed for CoQ₁₀ after the incorporation into different formulations' of DPPC liposomes.

Peak assignment	Wave number	DPPC	DPPC neutral	DPPC +ve	DPPC -ve
Symmetric stretching vibrations of the CH_2 in the acyl chain	2800-2855	2850	2852.247	2852.247	2852.247
Anti-Symmetric stretching vibrations of the CH_2 in the acyl chain	2920-3000	2920	2921.673	2919.745	2919.745
OH stretching vibrations	3400-3470	3419	3444.296	3438.51	3446.224
OH bending vibrations	1640-1645	1640	1652.722	1652.722	1652.722
Carbonyl stretching vibrations C=O	1730-1740	1737	1739.504	1741.433	1737.576
CH_2 bending vibrations	1456-1470	1468	1469.515	1469.515	1469.515
Aliphatic phosphates (P-O-C stretch)	990-1050	—	—	995.1046	995.1046
Anti-Symmetric PO_2 stretching vibrations	1215-1260	1242	1265	1265	1265

5- Conclusion

We found CoQ₁₀, when incorporated in the lipid bilayers, to interact actively with the lipids and to induce changes in their physico-chemical properties. In addition, a possible location of CoQ₁₀ in the interfacial region of the membrane has been proposed. The data presented here clarify, to a certain extent, the molecular interactions of CoQ₁₀ with membrane systems and may additionally contribute to a better understanding of CoQ₁₀ physiologic properties and the development of therapeutic advanced systems.

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الملخص باللغة العربية

تفاعل الانزيم المساعد CoQ₁₀ مع الليبوسومات DPPC

- مدحت وهبه إسماعيل¹ - حسام الدين سيد عبد الكريم² - أماني حسن الشاذلي³ - اميرة زكي دكروري⁴ - مها رضا السيد⁴
- ¹ قسم الفزياء - كلية العلوم - جامعة حلوان.
- ² قسم البصريات الفسيولوجية - معهد بحوث امراض العيون - الجيزة.
- ³ قسم الكيمياء الحيوية والفارماكولوجي - معهد بحوث امراض العيون - الجيزة.
- ⁴ قسم الفيزياء - كلية النبات للآداب والعلوم والتربية - جامعة عين شمس.

هذا البحث يهدف الى دراسة الخواص البنائية لليبوسومات مثل DPPC والتي تعتبر نموذج للعشاء الخلوى وتأثيرها على مضادات الاكسدة باستخدام جهاز مسعر المسح التبياني (DSC) و مطياف الأشعة تحت الحمراء (FTIR)، وتشير النتائج الى وجود تغييرات كبيرة بعينة الليبوسومات المحملة بمضادات الاكسدة التي تحمل شحنة سالبة بالمقارنة بباقي العينات باستخدام جهاز DSC حيث حدث انخفاض كبير بالمنحنى الخاص بالعينة عند درجة حرارة 94س° اما منحنى الليبوسومات عند 105س°. وتوضح الدراسة ايضا حدوث اندماج كبير بين الانزيم المساعد (COQ₁₀) وطبقات الدهون المزدوجة الموجودة فى الليبوسومات وهذا التفاعل يستمر لوقت طويل ويزيد من فتره نصف العمر لمضادات الأوكسدة. ونتائج جهاز مطياف الأشعة تحت الحمراء أوضحت وجود إندماج كامل بين الليبوسومات وجميع عينات مضادات الأوكسدة بكل حالتها.