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A Biophysical Research on the Determination of Morphological and Structural Properties of Coumarin-Loaded Liposomes

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Keywords
Coumarin; Liposomes; Phospholipids; ATR-FTIR; FE-SEM

Abstract

The major goal of this research was to examine how coumarin affects lipid model membranes. For this reason, liposome membranes were formed using dimyristoyl phosphatidylcholine (DMPC) as zwitterionic lipid. The influence of coumarin on the morphology, packing order, fluidity, and hydration state of lipid membranes was specifically investigated by means of microscopic (field emission scanning electron microscopy (FE-SEM)) and spectroscopic (attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy) techniques. Taken into account the results obtained with FE-SEM images and analysis, liposomes without and with coumarin have uniform structures and spherical shapes in appearance. However, coumarin-loaded liposomes are observed with an increase in size when compared to a mean diameter of unloaded-liposomes. Considering ATR-FTIR analysis, the investigation of the vibrational bands which belong to the hydrophobic and hydrophilic parts of DMPC lipid reveals that coumarin alters the physical features of the DMPC liposomes by decreasing the order and increasing the fluidity of the system and making hydrogen bonding with the interfacial and headgroup regions of zwitterionic lipid DMPC. Finally, performing more biophysical studies on the interactions of biologically active compounds with model membranes plays an important role in determining the molecular action mechanisms of these compounds in drug discovery and formulations.

Kumarin Yüklü Lipozomların Morfolojik ve Yapısal Özelliklerinin Belirlenmesi Üzerine Biyofiziksel Araştırma

Anahtar kelimeler
Kumarin; Lipozomlar; Fosfolipitler; ATR-FTIR; FE-SEM

Öz

Bu araştırmanın esas amacı kumarinin lipit model membranları nasıl etkilediğini incelemektir. Bu nedenle, zwitteriyonik lipit olarak dimiristoil fosfatidilkolin (DMPC) kullanılarak lipozom membranlar oluşturulmuştur. Kumarinin lipit membranların paketleme düzeni, akışkanlığı, hidrasyon durumu ve morfolojisi üzerine etkisi, özellikle mikroskobik (alan emisyonlu taramalı elektron mikroskobu (FE-SEM)) ve spektroskopik (zayıflatılmış toplam yansıma Fourier dönüşüm infrared (ATR-FTIR) spektroskopisi) teknikleri ile incelenmiştir. FE-SEM görüntüleri ve analizleri ile elde edilen sonuçlar dikkate alındığında, kumarinsiz ve kumarinli lipozomlar görünüm olarak düzgün yapılara ve küresel şekillere sahiptir. Bununla birlikte, kumarin yüklü lipozomların, yüklenmemiş lipozomların ortalama çapı ile karşılaştırıldığında boyutunda bir artış gözlenmiştir. ATR-FTIR analizleri göz önüne alındığında, DMPC lipitinin hidrofobik ve hidrofilik kısımlarına ait titreşim bantlarının incelenmesi, kumarinin sistemin düzenini azaltarak ve akışkanlığını artırarak ve zwitteriyonik lipit DMPC'nin arayüzey ve baş grup bölgeleri ile hidrojen bağı yaparak DMPC lipozomlarının fiziksel özelliklerini değiştirdiğini ortaya koymaktadır. Sonuç olarak, biyolojik olarak aktif bileşiklerin model membranlar ile etkileşimleri üzerine daha fazla biyofiziksel çalışmaların gerçekleştirilmesi, ilaç keşfi ve formülasyonlarında bu bileşiklerin moleküler etki mekanizmalarının belirlenmesinde önemli rol oynamaktadır.

1. Introduction

Liposomes are microstructures made up of a bilayer of natural or synthetic lipids that produce an amphipathic environment with a polar headgroup and a long hydrophobic tail (e.g., phospholipid or lecithin) (Briuglia *et al.* 2015). Liposomes are also spherical vesicles with a mimetic cell membrane consisting of an aqueous inner core and a lecithin bilayer shell. They are used for encapsulating antibiotics, peptides, polyphenols, as well as several chemicals owing to their excellent biocompatibility, low toxicity, and ability to shield the active compounds from surroundings (Zhang *et al.* 2020). Therefore, liposomes are effective drug delivery systems for both hydrophobic and hydrophilic agents, and also powerful models for biological membrane research in biophysics, biochemistry, and structure–function relationships (Zhao and Feng 2006). Liposomes are utilized to examine how drugs interact with membranes, which can reveal important details about various pharmacokinetic characteristics of drugs (Mahajan and Mahajan 2013, Sreekanth and Bajaj 2013). Furthermore, by using liposomes as model systems, it is possible to investigate the membrane properties such as membrane trafficking, membrane fusion, cell adhesion, molecular recognition, and others (Pereira-Leite *et al.* 2013). Gene delivery systems, vaccines, signal enhancers in medical diagnostics, and solubilizers for various chemicals are also among the biomedical uses for which they are beneficial (Sorkin *et al.* 2013).

It is critical to ensure long-term stability of liposomes with regards to lipid bilayer rigidity by using lipids with a high phase transition temperature, which is influenced by polar headgroups, fatty acid side chains, chain length, and degree of unsaturation (Briuglia *et al.* 2015). For this reason, dimyristoyl phosphatidylcholine (DMPC) (Figure 1) used in this study is a well-known zwitterionic phospholipid, which is a significant ingredient of pulmonary surfactant compounds located on alveoli surfaces and it serves as a good model for the structural phosphatidylcholines (PCs) present in eukaryotic cell membranes (Wood *et al.* 2021). It contains a neutrally charged headgroup and two aliphatic chains of 14 carbon atoms (Miskowiec *et al.* 2017, Scheibe and Hauser 2018). Two endothermic processes are seen in DMPC aqueous dispersions. A less energetic process is a pretransition about at 14 °C while a more energetic one is a main phase transition around 24 °C (Prenner *et al.* 1999). DMPC is widely utilized in the preparation of biomimetic membranes. It was preferred as a model system because the structure of DMPC lipid bilayers has been extensively studied by neutron reflectivity (NR) (Burgess *et al.* 2004), atomic force microscopy (AFM) (Li *et al.* 2008), and polarization modulation infrared reflection absorption spectroscopy (PM-IRRAS) (Zawisza *et al.* 2004, Bin *et al.* 2005). In addition, NMR (Weisz *et al.* 1992, Hong *et al.* 1996, Marsan *et al.* 1999), X-ray diffraction (Mavromoustakos *et al.* 1990, Petrache *et al.* 1998), and computer simulation (Damodaran and Merz 1994, Kothekar 1996, Duong *et al.* 1999, Pasenkiewicz-Gierula *et al.* 1999, Zubrzycki *et al.* 2000) studies were performed for structural and dynamical features of DMPC.

In our previous study, we have systematically examined the molecular interactions between citrus flavonoids, hesperidin and naringin, and model lipid membranes composed of DMPC by using both experimental and theoretical techniques, differential scanning calorimetry (DSC), attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy, field emission scanning electron microscopy (FE-SEM), atomic force microscopy (AFM), and density functional theory (DFT). Methodologies demonstrated that even little structural alterations can have a significant impact on membrane bilayers and influence the distribution of these flavonoids throughout the membrane. As a result of that research, changes in the structural and dynamical features of lipid membranes can be crucial for activities concerning the inhibition of lipid peroxidation and the antioxidant mechanism (Altunayar-Unsalan *et al.* 2022a).

In our recent research, we focused on a better understanding of the molecular mechanism of action of hesperidin as an antioxidant agent. Therefore, firstly, by using spectroscopic, calorimetric and microscopic techniques (ATR-FTIR spectroscopy, DSC and AFM), we studied the influence of hesperidin incorporation in model membranes consisting of DMPC and cholesterol (CHOL) to mimic mammalian cell membranes. Secondly, molecular docking studies were carried out to predict hesperidin's inhibition potential of the human lanosterol synthase (LS), an enzyme present in the last stage of cholesterol synthesis. Then, the hesperidin's ADME/Tox (absorption, distribution, metabolism, excretion, and toxicity) profile was computed to determine its possible effect on living system. Taken into account all these investigations, it is concluded that binding properties and orientation of hesperidin in DMPC/CHOL lipid bilayer would provide valuable information on its antioxidant and lipid peroxidation inhibitory activity. Because of its antioxidant properties, hesperidin may be a high potential for use as a therapeutic drug for hypercholesterolemia (Altunayar-Unsalan *et al.* 2022b).

As polyphenols are known to have strong antioxidant properties both *in vitro* and *in vivo* (Oteiza *et al.* 2005), coumarin (coum) (1,2-benzopyrone) (Figure 1) used in this study is the simplest constituent in a broad family of naturally occurring phenolic compounds composed of fused benzene and α-pyrone rings (Mirunalini and Krishnaveni 2011). It is the primary component of coumarin derivatives and has aromatic and fragrant properties that are present throughout the plant family. Coumarin exists in a variety of plant origins, including fruits, medicinal herbs, vegetables, and spices as well as all sections of the plant-fruits, leaves, stems, and roots. It is observed in significant proportions in several cinnamon varieties (Lončar *et al.* 2020). Coumarin is also a well-known fluorescent component and it belongs to the benzopyrone family (Kalyanram *et al.* 2020). Coumarin and its derivatives have a variety of biological characteristics that are essentially determined by their chemical composition (Matos *et al.* 2017). As a result, they have been used in a variety of pharmaceutical purposes, including antioxidants (Witaicenis *et al.* 2014, Pérez-Cruz *et al.* 2018), antiviral (Mishra *et al.* 2020), antimicrobial (Al-Majedy *et al.* 2017), anti-inflammatories (Chen *et al.* 2017, Liu *et al.* 2020), anticancer (Emami and Dadashpour 2015), anti-HIV (Liu *et al.* 2020), anticoagulants (Akoudad *et al.* 2014), and antituberculosis (Keri *et al.* 2015) agents. Coumarin and its derivatives have attracted significant scientific attention as potential therapeutic candidates since these substances have antioxidant properties (Zhang and Wang 2004, Beillerot *et al.* 2008, Lin *et al.* 2008; Ćavar *et al.* 2012). Antioxidant agents like hydroxycoumarins have been examined because of their capacity to prevent neurodegenerative diseases. The possible mechanisms are associated to free radical scavenging and postponing or preventing biomolecule oxidation (Matos *et al.* 2013).

To further understand the antioxidant action mechanism of biological active compounds, knowledge of the molecular interactions of these compounds with lipids is clearly required. Changes in some physical features of the bilayer can influence the rate of protein and lipid oxidation in membranes. The rate of membrane oxidation is greatly influenced by membrane fluidity and lateral phase separation (Oteiza *et al.* 2005). To better reveal the mechanism involved in antioxidant activity, we aimed to examine the interaction of coumarin with model membranes - liposomes composed of DMPC lipid bilayers, and thus identify how coumarin is distributed in DMPC lipid bilayers. To achieve this purpose, firstly, FE-SEM (Field Emission Scanning Electron Microscopy) technique was performed to examine the microscopic appearance of coumarin-loaded and coumarin-unloaded liposomes. Secondly, attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy was utilized to determine the characteristic structures of these liposomes.

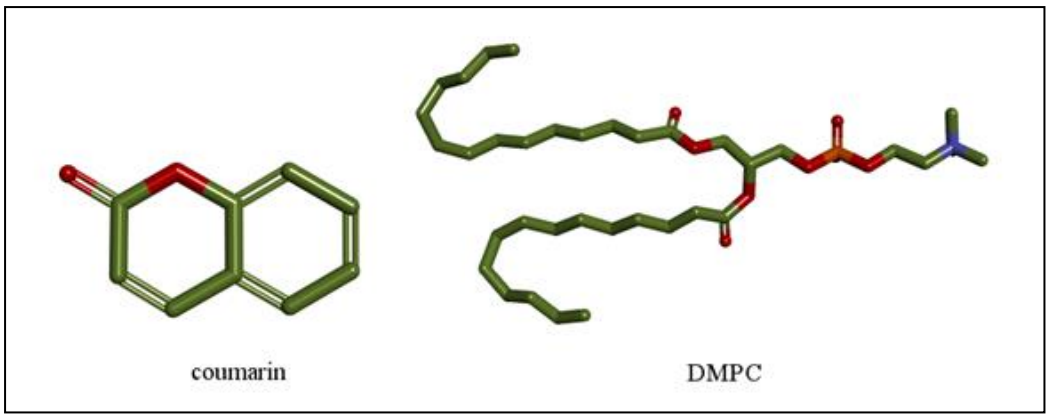


Figure 1. Chemical structures of coumarin and DMPC. Molecules were constructed by BIOVIA Discovery Studio Visualizer v20.1.0.19295 (BIOVIA, Dassault Systèmes, Discovery Studio Visualizer, v20.1.0.19295, San Diego: Dassault Systèmes, 2019).

Then, the infrared spectra indicating coumarin's capacity to bind to the DMPC lipid bilayer were analyzed in detail. Finally, these data would have a significant impact on coumarin conformation, orientation, and partitioning within DMPC lipid membranes, and also the physicochemical characteristics and functioning of the cell membranes. Thus, this type of study would also give significant and valuable insight into the comprehension of the antioxidant action mechanisms of coumarin.

2. Materials and Methods

2.1 Chemicals

1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Coumarin, chloroform, and PBS (phosphate buffered saline) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Liposomes' preparation

Bangham method (Bangham *et al.* 1965, Bangham 1978) that was previously described was used for the preparation of liposomes. DMPC and coumarin were dissolved in chloroform. For spectroscopic and microscopic investigations, the quantities of DMPC were used according to the values determined by Altunayar-Unsalan *et al.* (2022a). For coumarin-loaded liposomes, 1 mol% (low) and 40 mol% (high) concentrations of coumarin were used. These concentrations are consistent with previous studies about polyphenols in lipid bilayers (Saija *et al.* 1995, Demetzos *et al.* 2001, Fadel *et al.* 2011, Koukoulitsa *et al.* 2011, Longo *et al.* 2016, Khattari *et al.* 2017). Under a steam of nitrogen, the mixtures were evaporated. In order to form thin homogeneous, solvent-free films, the mixtures were waited in high vacuum for approximately 2 hours. 10 mM PBS buffer in deionized H_2O (pH 7.4) was used to hydrate the dried films and then, for 20 minutes these mixtures were vortexed. To check the experimental reproducibility, the mixtures were prepared in triplicate for every measurement.

2.3 FE-SEM (Field emission scanning electron microscopy)

Thermo Scientific Apreo S LoVac SEM (ThermoFisher Scientific, USA) with Schottky FEG (Field Emission Gun) was used for imaging the liposomes in high vacuum mode. Before imaging, 6 nm gold-palladium coating were applied on the samples using Leica EM ACE600 sputter coater under vacuum with argon gas (Leica Microsystems, Germany) to make the surface conductive. Imaging resolution and acceleration voltage were 0.9 nm and 1 kV, respectively. For the experiments, maximum beam of current of 50 nA and 30 kV accelerating potential were used. Micrographs were analyzed by ImageJ 1.53t software (Schneider *et al.* 2012).

2.4 ATR-FTIR (Attenuated total reflection Fourier transform infrared) spectroscopy

IR spectroscopic measurements of liposomes in the presence and absence of coumarin were made utilizing Fourier transform infrared spectrometer. The IR spectrometer used here includes an ATR set-up. A PerkinElmer Spectrum Two FTIR spectrometer (PerkinElmer Inc., Waltham, MA, USA) that uses diamond crystal as an internal reflection element and a deuterated triglycine sulfate (DTGS) detector was used to record ATR-FTIR spectra. Spectral range that was covered was 4000–1000 cm^{-1}. 64 scans were used with the spectral resolution of 2 cm^{-1}. ATR crystal was carefully cleaned with ultra-pure organic solvents and used for collecting background spectra before every measurement at ambient temperature. The spectra were evaluated by PerkinElmer Spectrum v10.5.4 software (PerkinElmer Inc., Waltham, MA, USA) and water vapor and CO_2 contributions were removed. In the 3400–3200 and 1800–1500 cm^{-1} regions, OH stretching bands due to the PBS buffer are detected and they overlap with the relevant lipid bands. Therefore, PBS spectrum was first recorded and then subtracted from the liposomes' spectra. PerkinElmer Spectrum v10.5.4 software (PerkinElmer Inc., Waltham, MA, USA) was used for manual subtraction to reach a suitable baseline in the bulk water area. In order to make detailed analysis, the subtracted original spectra were used. The positions of the peaks were determined with regard to the weight center whereas the widths of the peaks were measured from the 80% of the highest positions of the peaks. Furthermore, normalization was done for visual representation according to the CH_2 antisymmetric stretching band in the spectra.

2.5 Statistical data analysis

The results of quantitative experiments were expressed as mean ± standard deviation (SD). The statistical analysis was performed with the Mann-Whitney U test by SPSS Statistics Version 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). *p-value < 0.05 was considered as a statistically significant. All experiments were repeated three times using freshly prepared samples and microscopic images are representative images from three independent experiments.

3. Results and Discussion

3.1 Morphological analysis

Electron microscopy (EM) is a technique used to visualize liposomes at high magnifications. It is widely used for studies of liposomes consisting of lipids since the wavelength of the electron, thus diffraction-limited resolution is several orders of magnitude smaller than that of optical microscopy, allowing for high-quality imaging of liposomes. In EM, electron beam is focused on the sample's surface using different electromagnetic lenses. Then, these particles are dispersed by the specimen before being refocused and enlarged by a second set of electromagnetic lenses in the imaging section to form a projected picture (Robson *et al.* 2018).

Scanning electron microscopy (SEM) used in this study can get information on the morphology and size of a sample as well as relevant details on the concentric organization of the distinct lipid layers (Robson *et al.* 2018). Figure 2 shows the FE-SEM micrographs of DMPC liposomes without and with coumarin (1 mol% and 40 mol%). From the FE-SEM image in Figure 2a, it is obvious that liposomes without coumarin are smooth, round and they bear spherical structures. The distribution of particles seems to be quite uniform, and no aggregation is observed. Moreover, size analyses performed on FE-SEM image in Figure 2a indicate that unloaded-liposomes have a mean diameter of 130.98 ± 28.77 nm (Table 1). Figures 2b and 2c present the FE-SEM images of the coumarin-loaded liposomes. These micrographs show that liposomes with coumarin have uniform structures, smooth surfaces and free from crystalline nature of coumarin. Additionally, particles seem to have no tendency to aggregate. The mean diameters of coumarin-loaded liposomes are determined to be 139.35 ± 27.19 nm for 1 mol% coumarin and 195.95 ± 77.14 nm for 40 mol% coumarin, and the particle diameters seem to increase with increasing coumarin content (Figures 2b and 2c, Table 1). This result indicates that

coumarin has a particle size-increasing effect when compared to unloaded liposomes.

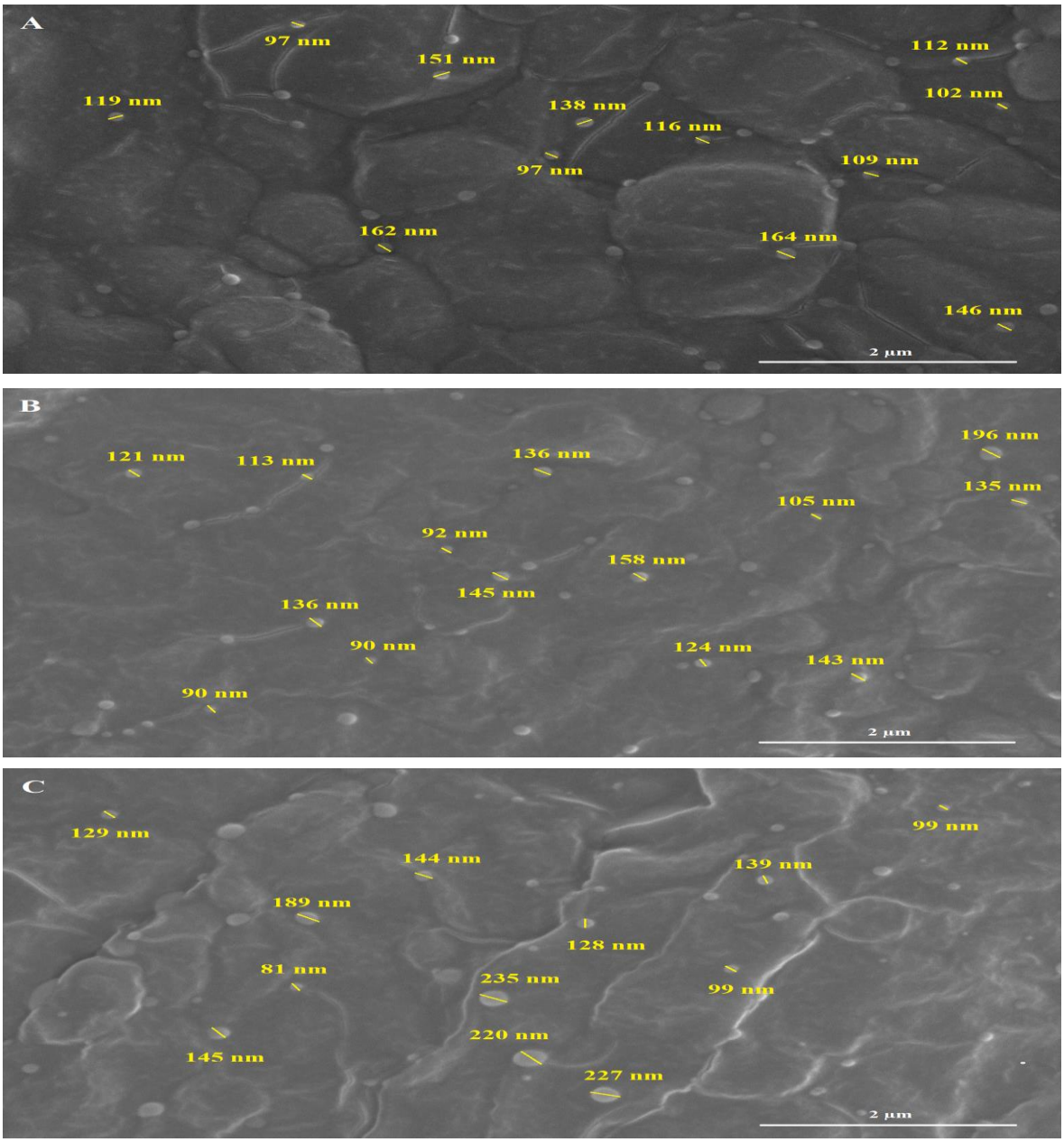


Figure 2. FE-SEM micrographs of DMPC liposomes without and with coumarin. a) pure DMPC, b) DMPC + 1 mol% coumarin, c) DMPC + 40 mol% coumarin.

Table 1. Particle sizes of DMPC liposomes without and with coumarin.

Lipid	Particle size (nm)
DMPC	130.98 ± 28.77
DMPC + 1 mol% coum	139.35 ± 27.19*
DMPC + 40 mol% coum	195.95 ± 77.14*

Asterisk (*) denotes p-value < 0.05 that was considered as a statistically significant.

In a study conducted by Niaz *et al.* (2018), SEM was applied to image the morphological characteristics of chitosomes and liposomes in the absence and presence of nisin. Their results showed that the mean diameter of liposomes is between 50–100 nm, which is in line with the literature (Trucillo *et al.* 2018). It is also stated that nisin-loaded liposomes have larger particle size (80–108 nm) when compared to the size of empty liposomes (54–60 nm), and this is due to the encapsulation of nisin in the core and bilayers of these nanovesicular system. These findings are consistent with our results, which

indicate the increase in the particle size of coumarin-loaded liposomes (139.35 ± 27.19 nm for 1 mol% coumarin and 195.95 ± 77.14 nm for 40 mol% coumarin) in comparison to that of unloaded one (130.98 ± 28.77 nm). Thus, it is obvious that coumarin is encapsulated in the liposome systems in our study. In another research, Trucillo and his colleagues (2018) examined the eugenol-loaded liposomes by using SEM, and they determined that these liposomes are spherical, and their surfaces are smooth. When they performed the size analysis on the SEM images, they revealed that these liposomes have a mean diameter of 100–200 nm, which is also in line with our findings.

3.2 Structural analysis

ATR-FTIR technique is effective to achieve relevant data concerning the lipid hydrocarbon chain orientation/conformation (νCH_2, νCH_3), lipid headgroup conformation (νPO_2^-, νCN^+C), and hydrogen bonding/hydration between and around lipids (νPO_2^-, νCO). It also provides essential data regarding the orientation and partition of these compounds in the bilayer system, and the intermolecular interactions that occur during integration (Hull *et al.* 2005).

The FTIR spectrum of membrane lipids gives numerous important data on both structure of lipid (acyl chain unsaturation/length, and headgroup) and membrane physical condition (chain ordering, phase transition) (Derenne *et al.* 2013). The spectrum is divided into two separate parts. The C-H stretching vibrations ν(C-H) contribute to the high wavenumber region of the FTIR spectrum (3100–2800 cm^{-1}). As a result, it is mostly derived from lipid acyl chains. In addition, this region primarily consists of C-H stretching bands from various vibrational modes such as $\nu_{as}(CH{=}CH)$ at 3010 cm^{-1}, $\nu_{as}(CH_3)$ at 2960 cm^{-1}, $\nu_{as}(CH_2)$ at 2920 cm^{-1}, $\nu_s(CH_3)$ at 2870 cm^{-1}, and $\nu_s(CH_2)$ at 2850 cm^{-1}. However, the polar headgroups of the lipids are mostly associated with the lower wavenumber part of the FTIR spectrum (<1800 cm^{-1}). In this region, the ester ν(C=O) band is typically dominant, and the contributions of phosphate at 1090 cm^{-1} ($\nu_s(PO_2^-)$) and 1240 cm^{-1} ($\nu_{as}(PO_2^-)$) come in turn. Besides, acyl chains contribute at 1465 cm^{-1} whereas *all-trans* conformations absorb at 1470 cm^{-1} (Derenne *et al.* 2013).

The spectral characteristics of the $\nu_{as,s}(CH_2)$ vibrational bands are related to the acyl chains' conformational status in various lipid phases such as the gel or fluid phases. They provide monitoring of the chain-melting transition, and this transition is assigned to rapid variations in the proportion of *trans* to *gauche* rotamer of CH_2 groups of lipids. As the number of *gauche* conformers in the hydrophobic region of the lipid bilayers increases, the $\nu_{as,s}(CH_2)$ vibrational bands shift to a higher wavenumber with reduced intensity and increased half-width. However, when compared to the *gauche*-rich states, the $\nu_{as,s}(CH_2)$ vibrational bands are shifted to lower wavenumber with the enhanced intensity and reduced half-width for *trans*-rich situations of lipid bilayers (Cieślik-Boczula 2018).

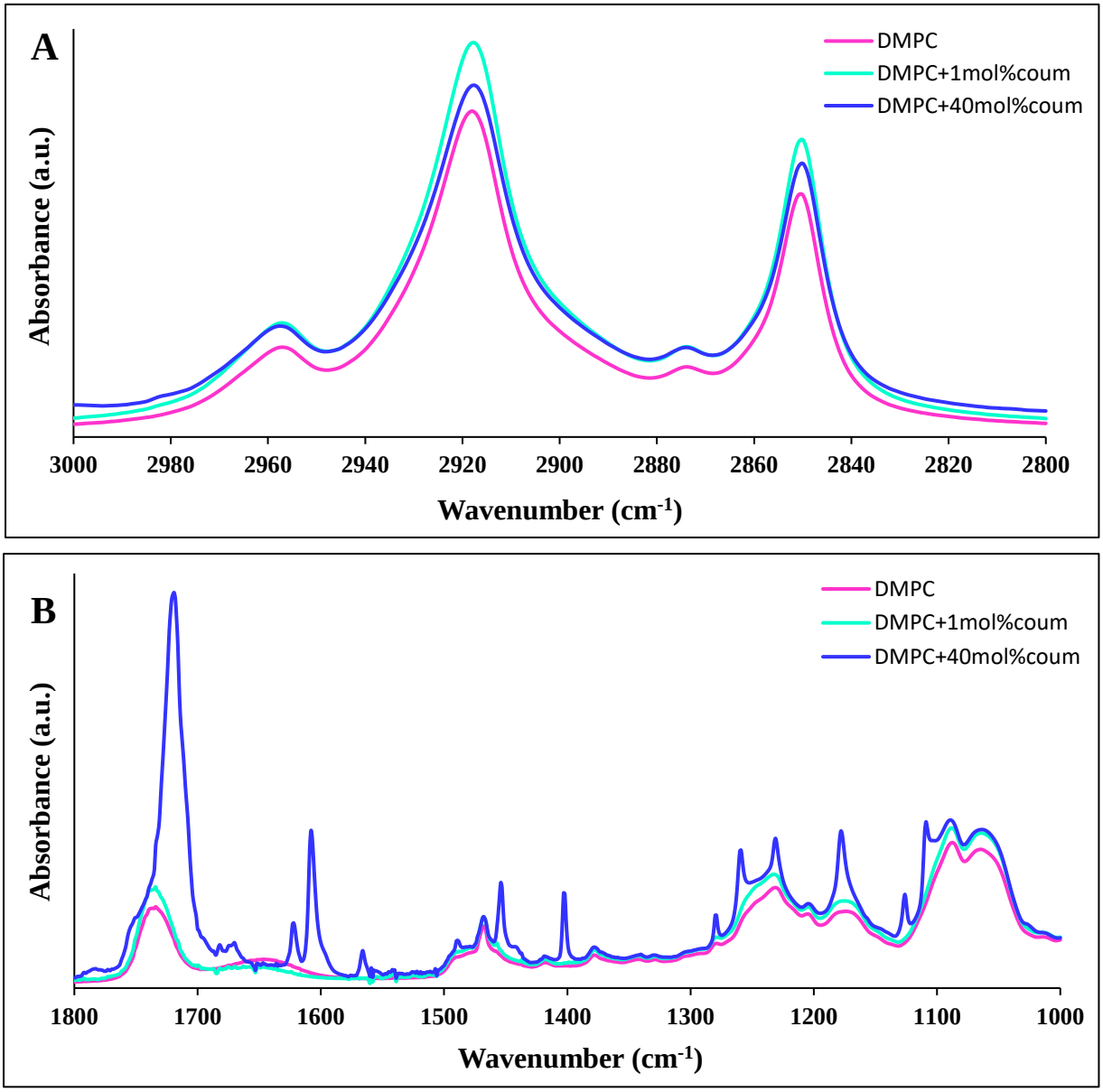


Figure 3. ATR-FTIR spectra of DMPC liposomes without and with coumarin. a) 3000-2800 cm^{-1}, b) 1800-1000 cm^{-1}.

The vibrational peaks originating from the PO_2^- and C=O groups (hydrogen bond acceptors) are observed to change while intermolecular hydrogen bonding occurs (Hull *et al.* 2005). It is known that a red shift in the wavenumber of ν(C=O) is attributed to the hydrogen bonding (Wong and Mantsch 1988, Hübner *et al.* 1994, Ueda *et al.* 1994). Besides, the hydration status of polar headgroups of lipids can be monitored using the wavenumber data of $\nu_{as}(PO_2^-)$ (López-García *et al.* 1993). Higher wavenumbers are associated with a dehydrated PO_2^- group and oppositely, lower wavenumbers correspond to a hydrated PO_2^- group (Casal *et al.* 1989).

In this work, the interactions between coumarin and the lipid model membranes were investigated to detect the physical alterations in the membranes by utilizing ATR-FTIR spectroscopy. In the ATR-FTIR spectra of DMPC liposomes both pure and containing coumarin in 1 mol% (low) and 40 mol% (high) concentrations, the methylene antisymmetric stretching mode ($\nu_{as}(CH_2)$), the carbonyl stretching mode (ν(C=O)), and the phosphate antisymmetric stretching mode ($\nu_{as}(PO_2^-)$) were taken into account. Figure 3 shows the ATR-FTIR spectra of DMPC liposomes without and with coumarin. The inclusion of coumarin induces some alterations in the intensity, width, and position of the relevant spectral bands (Figures 3a and 3b). These variations were analyzed in detail, and the corresponding values are given in Figures 4 and 5.

Figure 4a indicates the results for the coumarin concentration dependence of the wavenumber of $\nu_{as}(CH_2)$ of DMPC liposomes. 0 mol% implies pure DMPC liposomes without coumarin. When low (1 mol%) and high (40 mol%) concentrations of coumarin are added to pure DMPC liposomes, slight changes in wavenumber values of $\nu_{as}(CH_2)$ occur, and it is seen that this band slightly shifts towards higher wavenumber values. This situation shows that DMPC model membranes containing both low and high concentrations of coumarin are disordered and have more *gauche* rotamers in the acyl chains (Casal *et al.* 1980, Mantsch 1984). Changes in acyl chain conformation are linked to distinctive wavenumber alterations in methylene vibrations. These changes are thought to be caused by a Fermi-resonance interaction between the stretching and bending vibrations (Gericke and Hühnerfuss 1993, Zhao and Feng 2006).

Figure 4b shows the data acquired for coumarin concentration dependence of the bandwidth of $\nu_{as}(CH_2)$ of DMPC liposomes. The incorporation of low (1 mol%) and high (40 mol%) concentrations of coumarin into pure DMPC liposomes causes a shift of the bandwidth to higher values, reflecting an enhancement in the fluidity of membrane (Casal *et al.* 1980, Lee and Chapman 1986, Zhao and Feng 2006). Membrane fluidity is an essential physical property of biological membranes because it is associated to cellular functions including permeability, lateral motion of membrane components, transport of nutrients, regulation of enzyme activity and osmotic stability of cells. Changes in membrane fluidity have an impact on membrane protein activity by altering its lipid microenvironment and interactions (de la Haba *et al.* 2013). A reduction in fluidity, for instance, results in the loss of its functioning as a barrier (Sadžak *et al.* 2020). Several studies have found that lipid peroxidation causes a reduction in membrane fluidity in various cell membranes (Kaplán *et al.* 2000, Solans *et al.* 2000, Benderitter.

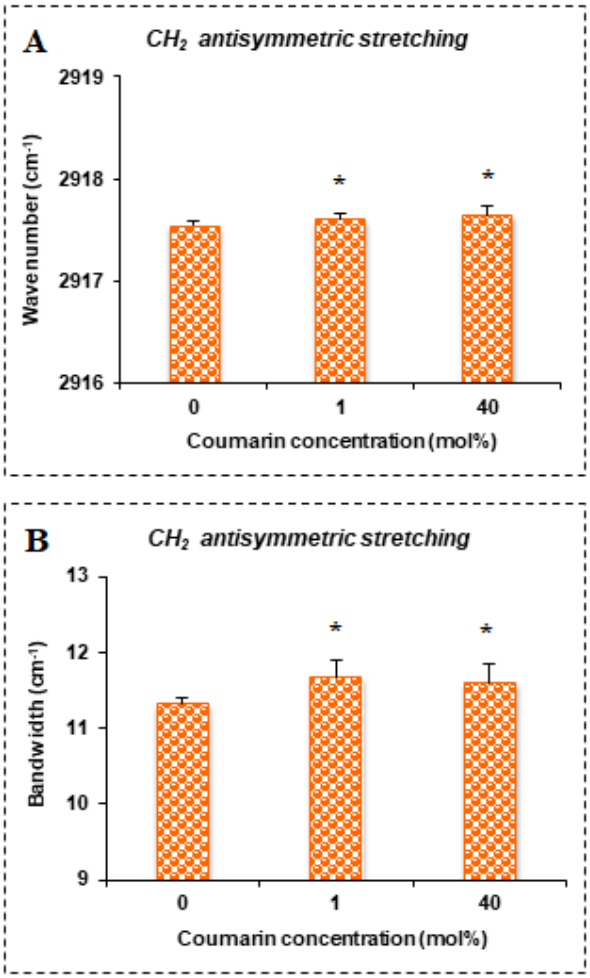
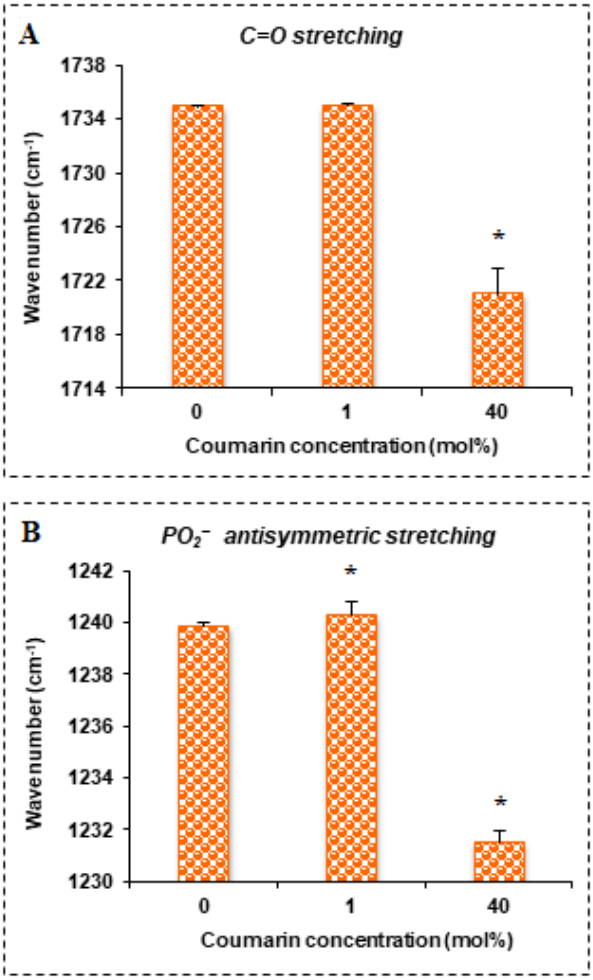


Figure 4. Coumarin concentration dependence of the wavenumber of the CH_2 antisymmetric stretching mode of DMPC liposomes (a). Coumarin concentration dependence of the bandwidth of the CH_2 antisymmetric stretching mode of DMPC liposomes (b). Asterisk (*) denotes p-value < 0.05 that was considered as a statistically significant.

et al. 2003). However, one of the mechanisms in the protection against peroxidation could be the interaction of polyphenolic components with lipid membranes. There are two probable interactions; i) if polyphenols partition in the non-polar part of the bilayer, which is related with their hydrophobic nature, they can alter membrane fluidity, ii) when the more hydrophilic flavonols interacts with the polar head groups of lipids at the membrane's lipid-water interface, they are able to form hydrogen bonds with these head groups, and thus provide some protection to the bilayer (Oteiza *et al.* 2005, Sadžak *et al.* 2020).

Coumarin concentration dependence of the wavenumber of ν(C=O) of DMPC liposomes is demonstrated in Figure 5a. As seen from graph, the addition of 40 mol% (high) concentration of coumarin into pure DMPC liposomes induces an apparent shift of the wavenumber to lower values, which is an indication of hydrogen bonding (Wong and Mantsch 1988, Hübner *et al.* 1994, Ueda *et al.* 1994). However, this shift is not observed in 1 mol% coumarin when compared to pure DMPC liposomes. This indicates that 1 mol% coumarin has no significant effect on the interfacial region of DMPC liposomes for the hydration/dehydration status. When coumarin concentration dependence of the wavenumber of $\nu_{as}(PO_2^-)$ of DMPC liposomes is examined (Figure 5b), 1 mol% coumarin causes a small shift of the wavenumber to higher values, referring to a dehydrated PO_2^- group (Casal *et al.* 1989). However, the inclusion of 40 mol% coumarin significantly decreases the wavenumber values, indicating to a hydrated PO_2^- group (Casal *et al.* 1989), and thus this coumarin concentration has a significant effect on the polar head group of DMPC liposomes for the hydration/dehydration status.

Kalyanram *et al.* (2020) studied the effects of concentration and alkyl tail length of amphiphilic amino methyl coumarin on dipalmitoyl phosphatidylcholine/dipalmitoylphosphatidylserine (DPPC/DPPS) lipid bilayer by using several experimental techniques and molecular dynamics (MD) simulations. From their fluorescence spectroscopy results, it was observed that the inclusion of lower concentration (5 mM) of short-tailed (C_5) coumarin into DPPC/DPPS liposomes shifts the spectrum towards higher wavelength (red shift), indicating the electrostatic binding and insertion of these molecules into the DPPC/DPPS lipid bilayer. This red shift was also observed for the other coumarin molecules (C_9 and C_{12}), but the long-tailed (C_{12}) coumarin has a higher shift in fluorescence when compared to C_5 and C_9 due to the intermolecular hydrogen bonds between the longer alkyl tail of coumarin and the hydrocarbon chains of



lipids. However, at 25 mM coumarin concentration, fluorescence intensity was reduced due to fluorescence quenching caused by aggregation. In addition to the experimental results, MD simulations were performed using short-tailed (C_5) and long-tailed (C_{12}) coumarin at different concentrations in the 85:15 DPPC/DPPS lipid bilayer at 323 K. When 42 C_5 coumarin (C_5-42) molecules added into the lipid system, these short-tailed molecules penetrated in the DPPC/DPPS lipid bilayer. However, their head groups interacted firstly with the head groups of these lipids. Thus, C_5 coumarin changed the physical properties of the

Figure 5. Coumarin concentration dependence of the wavenumber of the C=O stretching mode of DMPC liposomes (a). Coumarin concentration dependence of the wavenumber of the PO_2^- antisymmetric stretching mode of DMPC liposomes (b). Asterisk (*) denotes p-value < 0.05 that was considered as a statistically significant.

lipid system by increasing the area per lipid (A_L) and decreasing the bilayer thickness (D_M). For C_5-166 and C_5-209 systems at high concentrations of C_5 coumarin, these alterations were also occurred. When long-tailed (C_{12}) coumarin systems were taken into account, it was observed that coumarins at low (C_{12}-42) and high (C_{12}-166) concentrations interact with the hydrocarbon chains of lipids without alterations in A_L and D_M as a result of stable interactions. However, C_{12}-184 and C_{12}-209 coumarins disrupted the lipid bilayers as a consequence of which an aggregation is formed. In addition, their MD simulations were indicated that both C_5 and C_{12} coumarins have flip-flop behavior in the DPPC/DPPS lipid bilayer and C_5 systems have a lower flip-flop percentage than C_{12} systems at the same concentration. These findings are in agreement with our ATR-FTIR spectroscopy study indicating that the simple coumarin used in our study changes the physical properties of DMPC lipid bilayer by decreasing the order and increasing the fluidity of the system depending on the concentration.

Sarpietro *et al.* (2011) investigated the interactions of three coumarins (esculin, esculetin, and scopoletin) with DMPC multilamellar vesicles (MLVs) or monolayers as biomembrane models by applying Langmuir–Blodgett and DSC (differential scanning calorimetry) methods. Their findings showed that the addition of increasing molar fraction of these three coumarins into DMPC MLVs induces a decrease in T_m (the gel to liquid-crystalline phase transition temperature), suggesting a membrane destabilization resulting from the participating of coumarins between the phospholipids. However, they also reported that scopoletin and esculetin have a more destabilization effect than esculin. For this situation, they suggested that esculin remains near the polar headgroup of phospholipids while the other two coumarins stay close to the polar headgroup and span toward the acyl tails of them. In our study, it was observed that 40 mol% concentration of coumarin makes strong hydrogen bonding with the C=O and PO_2^- groups of DMPC. However, coumarin possess six hydrogen atoms and it is quite possible that it can construct hydrogen bonds and interact with its environment with its carboxyl oxygen and hydrogens. The skeletal coumarin in our study has not any short or long-tailed alkyl chain. Thus, the complete penetration into the hydrophobic core might not be expected based on the results discussed by both Kalyanram *et al.* (2020) and Sarpietro *et al.* (2011). Our results clearly indicate that skeletal coumarin interacted with both polar headgroup and interfacial region of the DMPC strongly depending on the concentration. In conclusion, the molecular structures of coumarins are quite important for their localization and orientation in the phospholipid bilayers. The type and location of the substituent connected to the aromatic ring of coumarin compounds have a significant impact on their antioxidant activity (Bubols *et al.* 2013).

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