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Comparative study of the interaction of fullerenol nanoparticles with eukaryotic and bacterial model membranes using solid-state NMR and FTIR spectroscopy

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Abstract Native fullerene is notoriously insoluble in water and forms aggregates toxic to cell membranes, thus limiting its use in nanomedicine. In contrast, water-soluble fullerenol is compatible with biological systems and shows low in vivo toxicity on human cell lines. The interaction mechanism between these hydrophilic nanoparticles and biological membranes is however not well understood. Therefore, in this work, the effect of fullerenol on model eukaryotic and bacterial membranes was investigated using ^{31}P- and ^{2}H solid-state NMR as well as FTIR spectroscopy. DPPC/cholesterol and DPPC/DPPG bilayers were used to mimic eukaryotic and bacterial cell membranes, respectively. Our results show low affinity of fullerenol for DPPC/cholesterol bilayers but a clear interaction with model bacterial membranes. A preferential affinity of fullerenol for the anionic phospholipids DPPG in DPPC/DPPG membranes is also observed. Our data suggest that fullerenol remains at the water/bilayer interface of eukaryote-like membranes. They also indicate that the presence of a polar group such as DPPG's hydroxyl moiety at the bilayer surface plays a key role in the interaction of fullerenol with membranes. Hydrogen bonding of fullerenol nanoparticles with DPPGs' OH groups is most likely responsible for inducing lipid segregation in the lipid bilayer. Moreover, the location of the nanoparticles in the polar region of DPPG-rich regions appears to disturb the acyl chain packing and increase the membrane fluidity. The preferential interaction of fullerenol with lipids mostly found in bacterial membranes is of great interest for the design of new antibiotics.

Keywords DPPC · DPPG · Antimicrobial activity · Membrane interaction · Lipid phase separation · Polyhydroxylated fullerene

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Introduction

Fullerene (C_{60}) is a spherical molecule containing 60 carbon atoms and 30 double conjugated bonds, which has a diameter of about 1 nm. Since its discovery (Kroto et al. 1985), fullerene's applications have encompassed several technological fields such as biosensors, cosmetics, drug delivery systems, electronics, lubricants, polymer fillers and nanomedicine (Wilson 2002; Bosi et al. 2003; Nakamura and Mashino 2009; Partha and Conyers 2009). The solubility, size, and charge of fullerene can be modulated by chemical modifications and, thus, create novel nanoparticles (NPs) with specific chemical, structural, mechanical and electronic properties (Wilson 2002; Mateo-Alonso et al. 2006; Hirsch 2010).

In water, fullerene is poorly soluble and forms aggregates that perturb human cell membranes (Sayes et al. 2004; Nielsen et al. 2008; Partha and Conyers 2009). By changing their surface chemistry (Hirsch 2010), fullerene NPs become attractive for several biological applications, and are currently used and investigated as effective anti-HIV, antitumor and antimicrobial agents, as well as enzyme inhibitors (Bosi et al. 2003; Bakry et al. 2007; Nielsen et al. 2008; Nakamura and Mashino 2009; Partha and Conyers 2009). So far, it has been shown that water-soluble fullerene derivatives—namely carboxylated and hydroxylated C_{60}—have antibiotic activity (Mashino et al. 1991; Bosi et al. 2003;

Aoshima et al. 2009; Nakamura and Mashino 2009), and induce low toxicity and fast excretion in vivo in mice and rats as compared to native fullerene (Nielsen et al. 2008 and references therein). The low toxicity of hydroxylated C_{60} was also demonstrated in vitro using different cell lines such as human dermal fibroblasts and liver carcinoma cells (Sayes et al. 2004) as reviewed by Nielsen et al. (2008). The in vitro toxicity is usually related to the formation of ROS (reactive oxygen species) causing membrane cell damage. Nevertheless, it is well recognized that fullerene and some of its derivatives are able to disrupt lipid bilayers (Braun and Hirsch 2000; Bosi et al. 2003; Spurlin and Gewirth 2007). For example, by intercalation into the microbial cell walls, fullerene and fullerene-based NPs can alter the membrane permeability and cause cell death at nanomolar concentrations (Bosi et al. 2003). Using atomistic simulations, Qiao et al. (2007) showed that ($C_{60}(OH)_{20}$) nanoparticles tend to remain on the membrane surface but can slowly passively diffuse into dipalmitoylphosphatidylcholine (DPPC) bilayers.

Size, charge and flexibility are important factors to investigate in the design of bioactive NPs because they can modulate their insertion into cell membranes (Bakry et al. 2007; Leroueil et al. 2007; Nakamura and Mashino 2009; Hirsch 2010). Only little is known of the interaction mechanism between water-soluble fullerenes and biological membranes (Spurlin and Gewirth 2007). The development of fundamental knowledge in that field is, thus, of great importance to evaluate the toxicity of these novel supramolecules for human cells and, eventually, to develop new antibiotic tools with increased efficiency and specificity (D'Rozario et al. 2009).

Our research is focused on the evaluation of the antibiotic potential of the water-soluble hydroxyfullerene derivative $C_{60}(OH)_{24}$ also known as fullerenol (FulOH). More specifically, we have studied the interaction of these NPs with model bacteria and eukaryote membranes using solid-state nuclear magnetic resonance (SS-NMR) and Fourier transform infrared (FTIR) spectroscopy. These techniques allow the assessment of different aspects of molecular interactions with a membrane such as changes in the phospholipids' dynamics and chemical environment around the polar headgroups and hydrophobic acyl chains.

The composition of natural membranes being too complex to allow direct NMR and FTIR analyses, model phospholipids membranes are generally employed (Warschawsky et al. 2011). Because *Escherichia coli* inner and outer membranes contain about 20 mol% of negatively charged phosphatidylglycerol (PG) on average (Seydel 2002; Goldfine 1984), vesicles composed of dipalmitoylphosphatidylcholine (DPPC) with 20 mol% of dipalmitoylphosphatidylglycerol (DPPG) were used in our study to mimic bacterial membranes (DPPC/DPPG). To verify any preferential affinity of the NPs to bacterial membranes, comparison was made with model eukaryote membranes. To do so, DPPC vesicles containing 20 mol% of cholesterol (DPPC/Chol) were employed. PCs and cholesterol are natural phospholipids and sterols found in most mammalian cell membranes (Warschawsky et al. 2011).

Solid-state NMR is a powerful technique that enables the study of exogenous particles in a membrane environment. By observing the appropriate nuclei, SS-NMR allows probing the effects of molecules on the membrane phospholipids. More specifically, ^{31}P SS-NMR spectra can reveal membrane perturbations at the surface of the polar headgroups (Seelig 1978), while ^{2}H SS-NMR using perdeuterated DPPC-d_{62} efficiently probes the effects on the hydrophobic region of the bilayer (Seelig 1977; Kilian et al. 1986; McConnell and Radhakrishnan 2006). FTIR spectroscopy gives complementary information by studying the hydrophobic core of the bilayer via the CH_2 or CD_2 asymmetric stretching frequencies as previously reported by Mendelsohn and Mantsch (1986) and Mantsch and McElhaney (1991).

Materials and methods

Materials

Water-soluble fullerenol (99 + %) was purchased in a powder form from M.E.R. Corp. (Tucson, AZ) as a mixture of nanoparticles with the general formula $C_{60}(OH)_{16\text{-}18}(ONa)_{6\text{-}8}$. Dipalmitoylphosphatidylcholine with perdeuterated acyl chains (DPPC-d_{62}), dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol sodium salt (DPPG) were obtained from Avanti Polar Lipids (Alabaster, AL). Deuterium oxide was purchased from Cambridge Isotope Laboratories (Andover, MA), while ^{2}H-depleted water and potassium chloride were acquired from Sigma Aldrich Inc. (St Louis, MO). All chemicals were ACS grade or higher, and were used as received without purification. Fullerenol and phospholipids were stored at −20 °C.

Sample preparation

For the NMR experiments, multilamellar vesicles (MLVs) were prepared by mixing DPPC, DPPC-d_{62} and cholesterol in a molar ratio of 2:2:1 to mimic eukaryote-like membrane, while negatively charged MLVs were prepared with DPPC, DPPC-d_{62} and DPPG in a molar ratio of 2:2:1 to model bacterial membranes. Non-labeled DPPC was used to minimize the expensive utilization of perdeuterated DPPC-d_{62}. All model membranes were prepared as follows: 20 mg of the phospholipids (or sterol) mixture were weighted and co-dissolved in chloroform (400 µl), evaporated in vacuo for 24 h then lyophilized for 3 days before use. The dry material was then suspended in 100 µl of a 50 mM KCl solution prepared in deuterium-depleted

water, giving a total proportion of 20 % w/v of phospholipids in water. The samples were submitted to three series of freeze (liquid N_2)/thaw (52 °C)/vortex shaking cycles until a homogenous gel was obtained, and were stored at −20 °C prior to analysis. The incorporation of the NPs into the MLVs was performed by rehydrating the phospholipids (± sterol) powder with the KCl solution containing 1.5 mg of dissolved FulOH. Then three cycles of freeze/thaw/vortex shaking was applied, leading to samples with a 5 mol% content of NPs and a neutral pH. About 50–55 mg of material were transferred into a 4-mm zirconium oxide rotor for NMR analysis. For FTIR study, the samples were prepared similarly, except that deuterium oxide was used instead of the 50 mM KCl buffer and the membranes were prepared using perdeuterated DPPC-d_{62} only.

Solid-state NMR experiments

Spectra were recorded with a 600 MHz Varian Inova Unity spectrometer (Agilent, Santa Clara, CA) operating at frequencies of 246.86 MHz for ^{31}P and 92.12 MHz for ^{2}H using a 4-mm magic-angle spinning (MAS) probe. All spectra were collected in duplicate from 30 to 50 °C. A 600-s delay was allowed for temperature equilibration between the experiments. Data were analyzed using the MatNMR package (van Beek 2007).

^{31}P static NMR spectra were recorded using a phase-cycled Hahn echo pulse sequence with broadband proton decoupling at a radiofrequency field of 50 kHz during the acquisition; 4,000 scans were recorded with a spectral width of 50 kHz, a 90° pulse length of 4.5-µs, a 3-s recycle delay and 28-µs echo delays. Magic angle spinning was performed at a spinning frequency of 5 kHz, and 512 scans were collected with a spectral width of 50 kHz, an acquisition time of 10 ms, a 3-s recycle delay and a 200-µs echo delay. Exponential line-broadening functions of 25 and 10 Hz were applied for static and MAS spectra, respectively, and zero filling up to 16 k was applied to MAS spectra only. Chemical shifts were referenced relative to H_3PO_4 85 %. ^{2}H static NMR experiments were performed using a quadrupolar echo pulse sequence; 8,000 scans were collected with a spectral width of 500 kHz, a 90° pulse length of 3 µs, an acquisition time of 10 ms, a 1-s recycle delay and a 45-µs echo spacing. Typically, a line broadening of 50 Hz was applied to the spectra.

FTIR experiments

Spectra were acquired on a Nicolet Nexus 670 spectrometer (Thermo-Nicolet, Madison, WI) equipped with a narrow-band mercury-cadmium-telluride (MCT) detector and a germanium-coated KBr beam splitter. Typically, 20 µl of freshly made sample were placed between CaF_2 windows separated by a 6-µm Mylar spacer. Fifty scans were collected from 4,000 to 400 cm^{-1} with a resolution of 2 cm^{-1}. Spectra were acquired at 1 °C increments from 32 to 55 °C. The spectrometer was continually purged with dry air. Spectra were corrected for water vapor and CaF_2 contributions by substraction of a reference spectrum. Data were analyzed using Grams/A1 version 7.02 (Galactic Industries Corporation, Waltham, MA).

Results

Interaction of fullerenol with model eukaryote membranes

Because phosphorus-31 nuclei have a 100 % natural abundance and a high gyromagnetic ratio, ^{31}P NMR stands as a unique tool to study the motion and average orientation of the phosphate group in model phospholipid membranes. Since the electron cloud surrounding the phosphorus nucleus is not evenly distributed, the ^{31}P resonance frequency (and resulting chemical shift) depends on the phospholipids' orientation with respect to the magnetic field. The chemical shift anisotropy (CSA)—which reflects the spread of NMR frequencies with orientation—can be measured from the powder spectrum of non-oriented static samples. Dynamical information can be extracted from the CSA measurement as increased motion of the phospholipids will lead to its partial averaging. More specifically, following the notation of Seelig the CSA ($\Delta\sigma$) can be expressed as (1978):

$$\Delta\sigma = \sigma_{//} - \sigma_{\perp} \quad (1)$$

where $\sigma_{//}$ and $\sigma\perp$ are the chemical shifts respectively obtained for parallel and perpendicular orientations with respect to the magnetic field (B_0) direction. The isotropic chemical shift can be introduced and these expressions rearranged to obtain:

$$\sigma_{iso} = 1/3\,(\sigma_{//} + 2\sigma_{\perp}) \quad (2)$$

$$\Delta\sigma = 3(\sigma_{iso} - \sigma_{\perp}) \quad (3)$$

The value of σ_{iso} can be determined from the ^{31}P NMR spectra acquired by magic-angle spinning (MAS) of the sample at an angle of 54.7° with respect to B_0, while $\sigma_{\perp}$ is obtained by measuring the chemical shift of the 90° edge of the static ^{31}P NMR spectrum at 90 % of the maximum intensity (Picard et al. 1999).

We have thus used ^{31}P SS-NMR spectroscopy to investigate the effects of 5 mol% fullerenol on model DPPC/Chol membranes. The static spectra are presented in Fig. 1, while the spectra acquired at an MAS frequency of 5 kHz are shown in Fig. 2. As can be observed in Fig. 1,

the static spectra are typical of a lipid mixture organized into MLVs, with axial symmetry of DPPC's ^{31}P chemical shift tensor as described by Seelig (1978). In the presence of 5 mol% FulOH, a minor effect is observed on the spectra (Fig. 1) below and above DPPC's gel-to-liquid crystal phase transition (melting) temperature ($T_m = 41$ °C) (Vist and Davis 1990; Guo and Hamilton 1995). The CSA values calculated from Fig. 1 and 2 are reported in Table 1 and show <1 % of variation at 35 °C only. Together, those observations suggest minimal interaction of FulOH with DPPC's headgroups.

Additional information on the interaction of fullerenol with the phospholipid headgroups in DPPC/Chol membranes can be obtained from the ^{31}P NMR MAS spectra displayed in Fig. 2. The isotropic chemical shift is characteristic of each phospholipid, but, most importantly, depends on the phosphorus nucleus electronic environment. It is thus possible to verify shielding/deshielding effects of the NPs on the lipid phosphate region by monitoring changes in the σ_{iso} value. In addition, changes in headgroup dynamics can lead to changes in the full width at half height of the isotropic resonances obtained by MAS. Similarly to the static spectra, Fig. 2 shows that the MAS spectra of DPPC/Chol system without and with 5 mol% FulOH are almost identical, with a σ_{iso} value difference of <0.01 ppm observed only at 40 °C (Table 1).

To investigate the interaction of FulOH with the hydrophobic region of DPPC/Chol bilayers, phosphatidylcholines with deuterated acyl chains (DPPC-d_{62}) were used and the ^{2}H NMR spectra recorded. The degree of organization along these chains is reflected by changes of the quadrupolar splitting ($\Delta\nu_Q$) value of a given C–D bond. The quadrupolar splitting for a C–D bond in a lipid bilayer with axial symmetry is given by:

$$\Delta\nu_Q(\delta) = \frac{3}{4}\frac{e^2qQ}{h}(3\cos^2\theta - 1)S_{CD} \quad (4)$$

where (e^2qQ/h) is the quadrupole coupling constant ($\sim$167 kHz), θ is the angle between the bilayer normal and the lipid long axis, and S_{CD} is the order parameter of a deuterium bond vector (Davis 1983; Seelig and Seelig 1980). An increase in the quadrupolar splitting is indicative of an ordering effect of the NPs on the lipid acyl chains, while a decrease reflects a disordering effect (Davis 1983).

Fig. 3 shows that with 5 mol% FulOH content, the spectra are rigorously identical to the control membranes. The line shapes are characteristic of phospholipids assembled in an ellipsoidal vesicles in a so-called "unique liquid ordered" phase below and above DPPC's T_m. At 35 °C, the spectra are broad, and the edges corresponding to the plateau region of the chains (close to the lipid headgroup) are partially flattened, suggesting a slight gel phase contribution

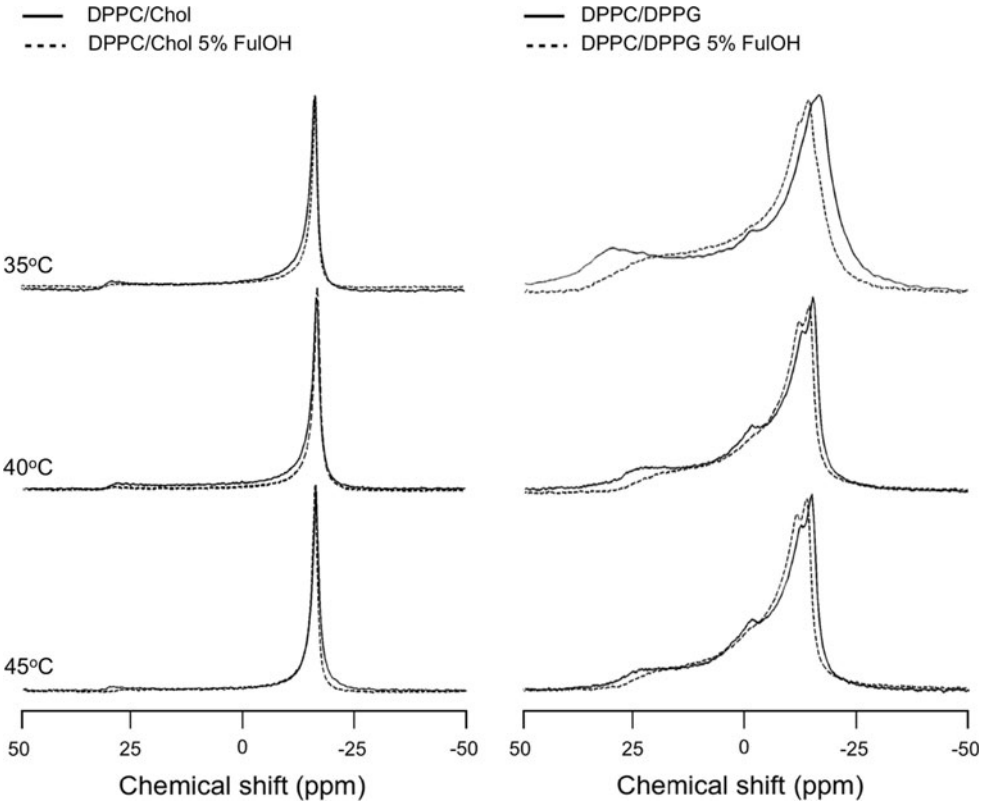

Fig. 1 Temperature dependence of the static ^{31}P NMR spectra of DPPC/Chol (*left column*) and DPPC/DPPG membranes (*right column*) at a molar ratio of 4:1, without and with 5 mol% FulOH

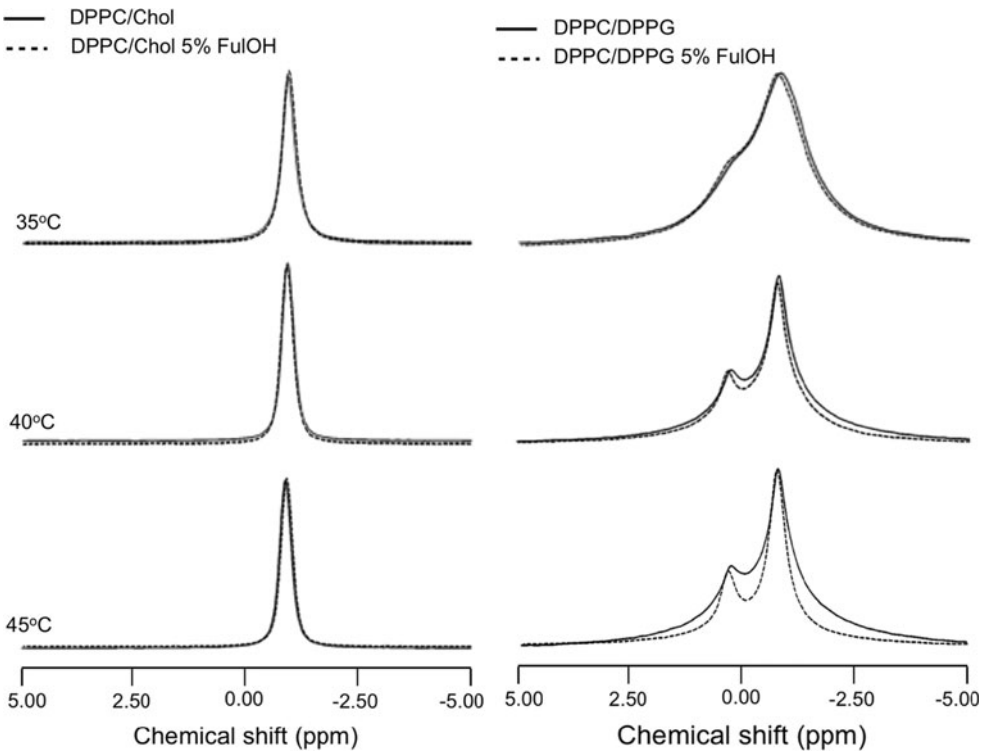

Fig. 2 Temperature dependence of the ^{31}P MAS NMR spectra of DPPC/Chol (*left column*) and DPPC/DPPG membranes (*right column*) at a molar ratio of 4:1, without and with 5 mol% FulOH obtained at an MAS frequency of 5 kHz

Table 1 ^{31}P chemical shift anisotropy ($\Delta\sigma \pm 0.2$ ppm) of DPPC in DPPC/Chol membranes calculated from static and MAS spectra

Temperature (°C)	FulOH (moI %)	σ_{iso} (ppm)	$\sigma_{\perp}$ (ppm)	$\Delta\sigma$ (ppm)
35	0	−0.91	−16.5	46.8
35	5	−0.91	−16.4	46.5
40	0	−0.90	−16.4	46.5
40	5	−0.89	−16.4	46.5
45	0	−0.87	−16.3	46.3
45	5	−0.87	−16.3	46.3

to DPPC-d_{62}/Chol spectra at 35 °C (Endress et al. 2002; McConnell and Radhakrishnan 2006).

To further assess the effect of FulOH on the hydrophobic region of the model eukaryote membranes, the thermotropic behavior of DPPC in the DPPC/Chol mixture has been studied. It can be obtained by plotting the value (in cm^{-1}) of the methylene symmetric or asymmetric ($\nu_{asym}CH_2$) stretching frequencies in the lipid acyl chains as a function of temperature (Mendelsohn and Mantsch 1986). These stretching vibrations are sensitive to changes in the trans/gauche conformer ratio and inform on structural rearrangements in lipid bilayers such as the gel-to-liquid crystal phase transition (Casal and Mantsch 1984; Mendelsohn and Mantsch 1986).

The study of the asymmetric stretching frequency displayed in Fig. 4a shows that cholesterol slightly attenuates the clear phase transition normally observed for pure DPPC, with a broader melting zone observed between 39 and 42 °C (Mannock et al. 2010). The presence of 5 mol% FulOH in the model membranes does not affect the transition temperature of the bilayer. With and without fullerenol, T_m approximatively corresponds to the theorical value of 41 °C for DPPC with 20 mol% cholesterol (Vist and Davis 1990; Guo and Hamilton 1995). A slight increase in the vibration frequency of $\sim 0.5\ cm^{-1}$ is observed above T_m in the presence of 5 mol% FulOH and can be ascribed to a minor increase of motion in the lipid chains (Mendelsohn and Mantsch 1986; Mantsch and McElhaney 1991).

Interaction of fullerenol with model bacterial membranes

SS-NMR and FTIR spectroscopy were also exploited to probe the effect of fullerenol on model bacterial membranes. First, the interaction of the NPs with the phospholipid headgroups in DPPC/DPPG bilayers was studied by ^{31}P static NMR as shown in Fig. 1. DPPC and DPPG are perfectly miscible phospholipids (Vincent et al. 1993). All spectra without and with 5 mol% FulOH are typical of a lamellar phase with axial symmetry (Seelig 1978). However, at 35 °C, i.e., below the phase transition temperature of the pure lipids ($T_m \sim 40$–41 °C) (Killian et al. 1986; Vist and Davis 1990; Guo and Hamilton 1995), the DPPC/DPPG spectrum is broader because of residual dipolar couplings, typical for lipids in the gel phase

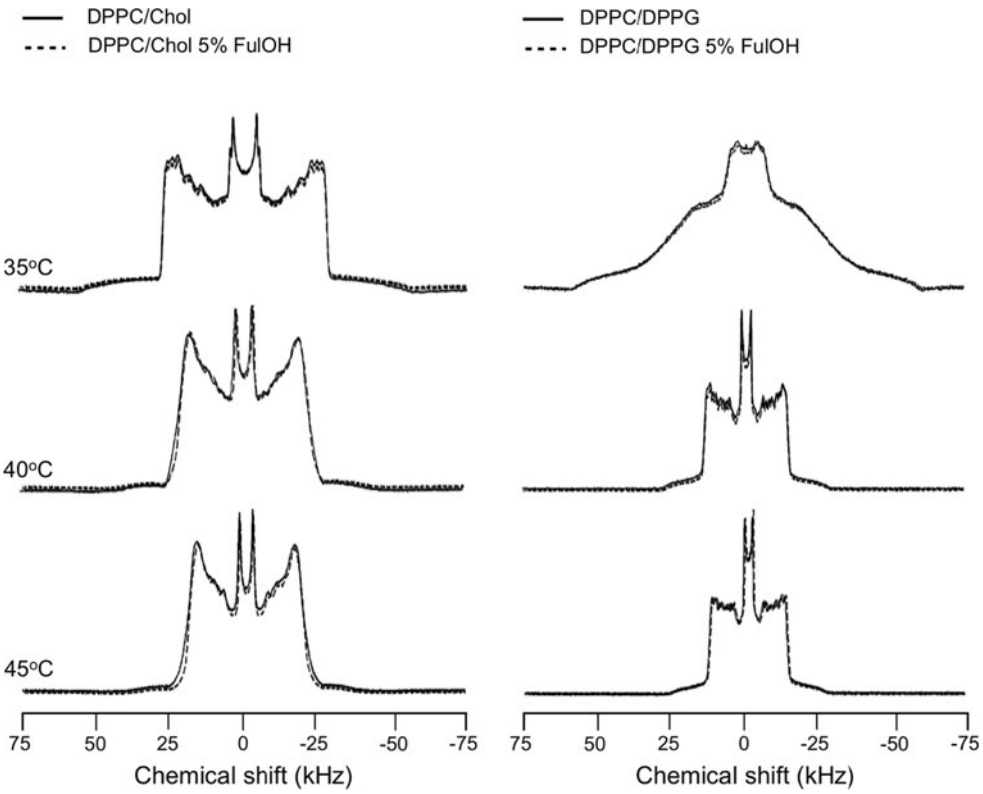

Fig. 3 Temperature dependence of the ^{2}H NMR spectra of DPPC-d_{62}/Chol (*left column*) and DPPC-d_{62}/DPPG membranes (*right column*) at a molar ratio of 4:1, without and with 5 mol% FulOH

(Killian et al. 1986). The gel phase is not observed in the presence of the NPs. The presence of 5 % FulOH would thus lower the gel-to-fluid transition temperature.

A closer analysis of the ^{31}P static SS-NMR spectra in Fig. 1 reveals two distinct spectral components. As reported by Seelig and Seelig (1980), PC and PG have similar headgroup structures and motions, and the larger CSA is attributed to DPPC (Table 1) (Marcotte et al. 2004). A small isotropic component is visible in all spectra, including the control membrane. Bensikaddour et al. (2008) reported a similar isotropic component for pure DPPG and assigned it to the presence of smaller vesicles.

In the presence of fullerenol, the spectra show a change in the orientation distribution between the 0° and 90° edges, with an increase in the 90° edge intensity for all temperatures studied. This can be ascribed to a greater contribution of membrane phospholipids oriented at 90° with respect to B_0 (Marcotte et al. 2004). This effect is accentuated with the temperature. The spectral widths are also narrowed, and calculation of the CSA (Table 2) shows an average decrease of $\sim$13 % for DPPG and 10 % for DPPC at 40 and 45 °C. This result could be attributed to increased motions of the lipid head group or the whole lipid (Smith and Ekiel 1984). Noteworthily, the CSA variation in the presence of 5 mol% FulOH is more important at 35 °C, i.e., below the mixture's melting temperature: a decrease of 16 and 13 % is observed for DPPG and DPPC respectively.

The presence of two spectral components is also visible in the ^{31}P NMR MAS spectra at a spinning frequency of 5 kHz (Fig. 2). No significant effect can be observed on the isotropic chemical shift of both phospholipids in the presence of 5 mol% FulOH at 35 °C. However, an important averaged downfield shift of 0.11 ppm is observed for DPPG at 40 and 45 °C. Also, narrowing of DPPC and DPPG's isotropic resonances is seen in the MAS spectra, and a better separation between the two spectral components of the phospholipids is observed (Fig. 2). This effect is more important in the presence of the NPs at 45 °C, i.e., above the mixture's T_m. Interestingly, the linewidth of DPPG decreases by 40 %, whereas the one of DPPC only decreases by 30 %. A greater perturbation of the PG headgroup by FulOH is thus observed.

To probe the insertion of FulOH into the bilayer, the effect of the NPs on DPPC-d_{62} acyl chains in the DPPC/DPPG model membranes was studied by ^{2}H SS-NMR. As shown in Fig. 3, the spectra obtained without and with 5 mol% FulOH show minimum perturbation of the phosphatidylcholine acyl chains in the DPPC-d_{62}/DPPG mixture. Interestingly, at 35 °C, i.e., below the lipid phase transition temperature, the spectra are superimposable and show a broad distribution associated with lipids in the gel phase. As observed by ^{31}P SS-NMR, spectra acquired above 35 °C are typical of fluid phase lineshapes and quasi identical with and without NPs.

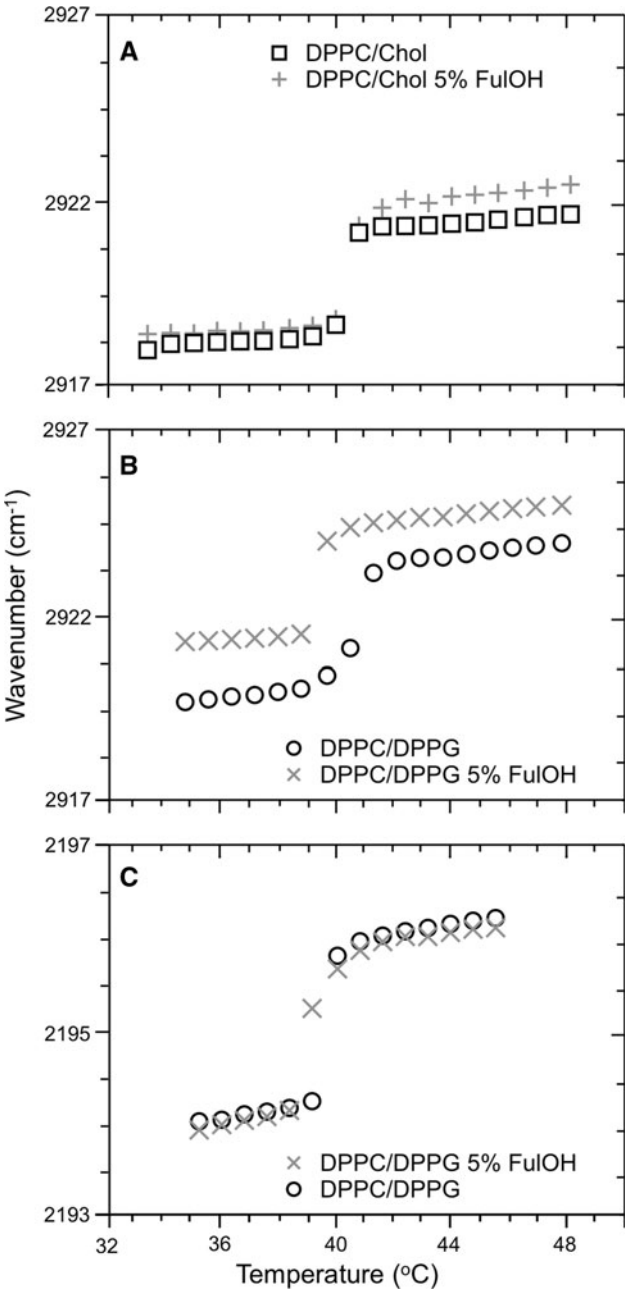

Fig. 4 Temperature dependence of the frequency of the CH_2 asymmetric stretching vibration of **a** DPPC in DPPC/Chol, **b** DPPG in DPPC/DPPG and **c** the CD_2 asymmetric stretching vibration of DPPC-d_{62} in DPPC-d_{62}/DPPG membranes at a molar ratio of 4:1, without and with 5 mol% FulOH

In order to verify a preferential interaction of fullerenol NPs with DPPG, we have studied by FTIR spectroscopy the CH_2 and CD_2 asymmetric stretching vibration frequencies of DPPG and DPPC-d_{62}, respectively, as a function of temperature in DPPC-d_{62}/DPPG model membranes. It is known that the mass variation due to isotopic substitution changes the frequency of the absorption band without affecting the force constants (Mendelsohn and Mantsch 1986). It is therefore possible to study the effect of FulOH on the two phospholipids in a single thermotropic analysis using one lipid with perdeuterated acyl chains and one protonated lipid. Such a study, thus, complements the information obtained on the hydrophobic core of the membranes by ^{2}H NMR.

As shown in Fig. 4c, DPPC-d_{62} acyl chains are not perturbed by the presence of 5 mol% FulOH. As well, the gel-to-liquid crystal phase transition temperature is unchanged, i.e., $\sim$39 °C which is close to the value of 38 °C reported for DPPC-d_{62} (Kilfoil and Morrow 1998). Only a slight broadening of the melting zone is visible in the presence of fullerenol, indicating a less cooperative melting process that could be ascribed to phase separation (Mendelsohn and Mantsch 1986; Mantsch and McElhaney 1991; Mannock et al. 2010). Indeed, analysis of $\nu_{asym}CH_2$ associated to DPPG's acyl chains in Fig. 4b reveals that DPPG's melting temperature is in the expected range of 40–41 °C (Kilian et al. 1986) in the pure model membranes. However, a 2 °C decrease of DPPG's T_m and an increase ($\sim$1 cm^{-1}) in the vibration frequencies for all temperatures studied (Fig. 4b) are observed in the presence of 5 mol% FulOH. These results indicate a decreased ordering of the chains because of a greater number of gauche conformers in DPPG's acyl chains. They also reveal a selective affinity of the NPs for the anionic DPPG in DPPC/DPPG membranes.

Discussion

Interaction of fullerenol with eukaryote model membranes

Fullerenol is a hydrosoluble spherical amphiphilic molecule containing a "waxy" carbon cage surrounded by 24 polar hydroxyl units (Mateo-Alonso et al. 2006). The NMR and FTIR investigations of the interaction between FulOH and DPPC/Chol bilayers seem to indicate no affinity of these NPs for eukaryote membranes. Indeed, the results obtained by ^{31}P static and MAS SS-NMR spectroscopy show insignificant effects of FulOH on DPPC phosphate groups. The study of DPPC acyl chains reveals a small decrease in the quadrupolar splittings of $\sim$1 kHz on the ^{2}H NMR spectra as well as a slight augmentation of the CH_2 asymmetric stretching vibration frequency in FTIR, but these changes fall within the experimental error of the measurements. Our results are in agreement with molecular dynamics simulations carried out by D'Rozario et al. (2009) on $C_{60}(OH)_{20}$ in DPPC bilayers, which suggest that the polar NPs would preferentially remain at the bilayer/water interface. Nevertheless, FulOH is a polar NP that has the ability to create H-bonds with water and cholesterol.

Table 2 ^{31}P chemical shift anisotropy ($\Delta\sigma \pm 0.2$ ppm) of DPPC and DPPG in DPPC/DPPG membranes calculated from static and MAS spectra

Temperature (°C)	FulOH (mol%)	DPPG			DPPC		
		σ_{iso} (ppm)	$\sigma_{\perp}$ (ppm)	$\Delta\sigma$ (ppm)	σ_{iso} (ppm)	$\sigma_{\perp}$ (ppm)	$\Delta\sigma$ (ppm)
35	0	0.25	−14.3	43.7	−0.79	−16.3	46.5
35	5	0.29	−11.9	36.6	−0.74	−14.1	40.1
40	0	0.27	−12.3	37.7	−0.78	−14.8	42.1
40	5	0.37	−10.8	33.5	−0.71	−12.4	35.1
45	0	0.27	−12.3	37.7	−0.78	−14.8	42.1
45	5	0.39	−10.5	32.1	−0.72	−12.2	34.4

A potential interaction with cholesterol, which would only be observable at higher concentrations, is therefore possible.

Interaction of fullerenol with model bacterial membrane

Contrary to eukaryote-like membranes, the results obtained by SS-NMR and FTIR spectroscopy confirm a clear interaction of the $C_{60}(OH)_{24}$ NPs with model bacterial membranes. Indeed, FulOH increases the phospholipids' mobility in the bilayer, as revealed by the decrease in the CSA measured from the ^{31}P SS-NMR spectra, especially in the gel phase (Table 2). Concomitantly, the narrowing of the isotropic peak width for both lipids (Fig. 2) indicates a longer transverse relaxation time (T_2) due to increased lipid motion. Moreover, an augmentation in the proportion of phospholipids oriented at 90° with respect to the magnetic field is also observed on the ^{31}P static SS-NMR spectra when FulOH is added (Fig. 1). This suggests the formation of more elongated DPPC/DPPG vesicles as was observed for met-enkephalin in DMPG vesicles (Marcotte et al. 2004). This could be explained by greater bilayer fluidity.

Although perturbing all lipids' organization and motion in the bilayer, FulOH preferentially interacts with DPPG molecules in the bilayer. ^{2}H SS-NMR results show that the NPs have no effect on the deuterated acyl chains of DPPC-d_{62} in DPPC-d_{62}/DPPG bilayers at the concentration studied (Fig. 3). This is confirmed by FTIR spectroscopy where no significant changes are seen on the CD_2 asymmetric stretching vibration frequencies of DPPC-d_{62} in the temperature range examined (Fig. 4c). However, the thermotropic behavior of DPPG's $\nu_{asym}CH_2$ (Fig. 4b) clearly reveals a 2 °C decrease of the phase transition temperature as well as an important increase in the vibration frequency at all temperatures in the presence of 5 mol% FulOH, indicative of a disordering of DPPG acyl chains both the gel and liquid crystalline phases. These results also indicate a lipid segregation in the bilayer due to the preferential interaction of $C_{60}(OH)_{24}$ NPs with the anionic phospholipids.

Considering the lack of interaction of FulOH with DPPC molecules and the propensity of fullerenol to remain at the bilayer/water interface, the presence of lipid polar groups such as the glycerol hydroxyl moiety at the membrane surface seems to play a key role in the interaction of FulOH NPs with a membrane. The OH groups surrounding the fullerene center of the NPs would most likely selectively interact with DPPGs' phosphatidylglycerol groups. This would force the phospholipids to form DPPG- and DPPC-rich domains in the bilayer. The hydroxyl group in PGs' polar head is thought to be involved in strong H-bonding to the phosphate moiety of neighboring molecules (Zhang et al. 1997). An interaction of the NPs with the OH groups would disturb the molecular packing of the phospholipids and allow greater motion of the acyl chains, in addition to more fluidity of the overall bilayer. H-bonding of FulOH with DPPG's phosphate group seems improbable since the ^{31}P CSA of DPPG is decreased in the presence of the NPs, and no significant variation in the isotropic chemical shift is observed (Epand and Vogel 1999).

Conclusion

The results obtained by FTIR, ^{31}P and ^{2}H SS-NMR spectroscopy reveal low affinity of fullerenol for DPPC in DPPC/Chol membranes. The NPs would remain at the bilayer/water interface, although H-bonds with cholesterol cannot be ruled out. Interestingly, a selective interaction was identified with negatively charged PGs in DPPC/DPPG membranes, thus causing lipid segregation in the bilayer. Our results indicate that FulOH would remain in the polar region of the bilayer because of H-bonding with the phosphatidylglycerol hydroxyl group. As a result, the DPPG packing would be perturbed as well as the bilayer fluidity. The selective interaction of fullerenol with PG and perturbing effect on bacterial model membranes are of great interest since PG is not found in eukaryotic membranes. Our study demonstrates that new fullerenol-based antibiotics could be designed to target bacterial membranes similarly to the action mechanism of antimicrobial peptides.

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