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Hydrophobic double walled carbon nanotubes interaction with phospholipidic model membranes: \(^1\text{H}, \, ^2\text{H}, \, ^3\text{P}\) NMR and ESR study

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A B S T R A C T

The interactions of carbon nanotubes synthesized by catalytic chemical vapour deposition with phospholipidic bilayers, mimicking biological membranes, have been investigated using solid state \(^3\text{P}\)- and \(^2\text{H}\) NMR, \(^1\text{H}\)- and \(^3\text{P}\) NMR in liquids and ESR studies. It was found that carbon nanotubes can integrate the bilayer, depending on the overall cohesion of the membrane used. Whereas no direct interaction can be observed in small unilamellar vesicles or directly in the presence of short-chained phospholipids, carbon nanotubes incorporate into the membrane of multibilayers. The result is a significant 2–3 K lowering of the transition temperature in multibilayers of dimyristoyl lecithins, which is more markedly associated with increased fluidity in the most superficial part of the membrane below the transition temperature (292–300 K range). However, no ionophoric property was found on large unilamellar vesicles.

1. Introduction

Carbon nanotubes (CNTs) that belong to the class of nanoparticles, consist of carbon atoms arranged in hexagonal sp\(^2\) structures, themselves organized in tubular superstructures (part of the family of fullerenes, besides other allotropic forms of carbon, such as diamond and graphite). Following the preparation method, the dimensions and number of CNT walls dramatically differ, from single-wall systems (diameters ranging from 0.4 to 2 nm with a length of several \(\mu\)m), while multi-walled CNTs range from 2 to 50 nm (up to 100 nm) in diameter for 1–50 \(\mu\)m in length (Lin et al., 2004). This important diversity directly results from the synthesis method used: catalytic chemical vapour deposition (Cassell et al., 1999; Flahaut et al., 2003), electric arc discharge (Journet et al., 1997), or laser ablation (Bronikowski et al., 2001), as well as high pressure carbon monoxide (Thess et al., 1996) and surface mediated growth of vertically aligned tubes. Each method produces CNTs of different quality, mainly differing by the chemical purity, the presence of residual catalyst and the presence of other forms of carbon species, such as amorphous or graphitic. Finally, specific physico-chemical, mechanical, thermal or electrical properties can be obtained and various applications are currently available, not only in industrial (construction, car assembly, chemistry, surface treatments, and thermal isolation) and domestic uses, in foodstuffs, sun creams, and fabrics (Stern and McNeil, 2008). More recently, biomedical and therapeutic applications have been proposed (Ning et al., 2007). However, a prerequisite for any medical use is the absence of any toxicity, which depends on the degree of systemic bioavailability of such compounds. Interestingly, the evidence for asbestos related occupational disease pointed out the link between toxicity and nanostructures. In the case of CNTs, interactions with biological systems were almost unknown up to the end of the 20th century and remained to be studied and understood. The study of such interactions rests currently with two basic properties of carbon nanotubes. First, their small size and high specific surface area, as well as secondly, possible defects or free carbon residues in these structures which potentially confer functional ability to penetrate living organisms by crossing lipid barriers, possibly leading to irreversible cell damage perhaps by inflammation and/or oxidative stress (Engler, 2004). Besides, most of the CNT preparation methods use heavy metals such as Ni, Co, Mo and Fe as catalysts, which also exert specific toxic effects. As part of the possible biological or medical uses of CNTs, for instance as drug (Mehra et al., 2008), DNA (Lee and Mijovic, 2009) or MRI contrast agent (Faraj et al., 2008) carriers, harmful effects should be studied and cytotoxicity assessed. Alongside different studies performed on lungs after respiratory exposure to CNTs including inflammation, granuloma formation or ROS related damages (Donaldson et al., 2006), various cell studies (e.g. keratinocytes (Monteiro-Riviere et al., 2005), fibroblasts (Tian et al., 2006), and embryo kidney cells (Cui et al., 2005)) have addressed more or less specific mechanisms including membrane permeation for hydrosoluble derivatives, inflammation, cell

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apoptosis or immune perturbations (Monteiro-Riviere et al., 2005). Conversely other studies found neither alteration in cell viability or metabolism in human umbilical vein endothelial cells (Flahaut et al., 2006). By way of contrast there are only few reports of more general aspecific interactions of CNTs with membranes (Aartyukhin et al., 2005; Thauvin et al., 2008; Wallace and Sansom, 2009).

This paper presents such a study, using double wall nanotubes (DWNTs) as CNTs and various phospholipid membrane models to mimic unfunctionalised membranes, which employs NMR and ESR methods.

2. Materials and methods

2.1. Synthesis and characterisation of CNTs

DWNTs were produced by CCVD decomposition of CH₄ over Mg₁₋₁₋₂₋₃₋₄₋₅₋₆₋₇₋₀₋₉₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀₋₀

2.2. Membranes preparation

l-α-Phosphatidyl choline (EPC) and phosphatidic acid (EPA) from egg yolk and synthetic dimyristylphosphatidyl choline (DMPC) were obtained from Sigma, La Verpillière, France. DMPC deuterated on both chains (DMPDF-d₅₄) was purchased from Interchim, Montluçon, France.

2.2.1. Small unilamellar vesicles (SUV)

EPC in its chloroformic solution (100 mg/ml) was evaporated to a film and resuspended in pure D₂O to a final lipid concentration of 10 mM in 500 µl SUV. LUV were formed by 1 h bath sonication. For CNTs (0.2 mg)/SUV systems, CNTs was either added to SUV (7 mM) and vortexed for 5 min to form the echo. The tube was placed in a 3 mm diameter quartz holder, and insert into the cavity of the ESR spectrometer.

2.2.2. Large unilamellar vesicles (LUV)

LUV were prepared following the method of Szoka and Papahadjopoulos. Briefly, EPC and EPA were mixed in their chloroformic solution (respectively 100 and 10 mg/ml, 9:1: M/M); the solvent was removed by an evaporation under reduced pressure, followed by freeze drying. The pellet was then suspended in 5 ml ether-external medium (NaH₂PO₄, pH 6.5, 0.4 M), 1/4 (v/v) then sonicated twice (2 × 2 min).

The phase transition was obtained by slow evaporation under low pressure (N₂, atmosphere). The external medium was then added (NaH₂PO₄, pH 6.5, 0.4 M), and polycarbonate filters with decreasing porosities were finally used to calibrate the vesicles to a final diameter of 0.4 µm and a total lipid concentration of 20 mM.

2.2.3. Multilayers vesicles (MLV)

DMPC in its chloroformic solution was evaporated to a film and resuspended in pure D₂O. The liposomes were formed by fast freezing and thawing cycles. The liposomes were removed by treatment of the sample with a concentrated aqueous HCl solution. High-resolution transmission electron microscopy showed that a typical sample consists of ca. 80% DWNTs, 20% Single walled nanotubes, and a few triple-walled carbon nanotubes.

The diameter distribution of the DWNTs ranged from 0.5 to 2.5 nm for inner tubes and from 1.2 to 3.2 nm for outer tubes. The length of individual DWNTs usually ranges between 1 and 10 µm, although bundles may be much longer (up to 100 µm at least). Due to the synthesis and catalyst-elimination process, the walls of the DWNTs are not expected to be functionalized and certainly not by oxygen-containing functional groups.

2.3. NMR experiments

2.3.1. High-resolution NMR

1H NMR experiments were recorded at 300 K on a Bruker AM-400 spectrometer using a 4000 Hz spectral width, 32 K digitization points, a recycling delay of 2 s and a presaturation of the solvent resonance was used for all the experiments in aqueous medium.

1P NMR experiments in LUV were recorded at 300 K on a Bruker AM-400 spectrometer using a 20 ppm spectral width, 16K digitization points, a recycling delay of 1.5 s, 50 µs pulses of 5 µs and a composite pulse proton decoupling.

2.3.2. Solid state NMR

the membrane curvature (radius about 10–20 nm), and the cohesion forces could be strong enough to preclude strong interactions with the relatively big size CNT systems.

This led to use other models of higher size, large unilamellar vesicles, LUV (EPC/phosphatidic acid 9/1 M/M, 20 mM), to observe membrane ionic permeability in the conditions of cation/proton exchange conditions, as classically described by Gary-Bobo and coworkers (Cybulskia et al., 1986). Briefly, these membranes enclose a significant aqueous volume (20% of the total sample volume), thus separating two compartments, intra- and extravesicular. If the aqueous medium consists of NaH₂PO₄, ³¹P NMR is a suitable tool to separate and identify on a spectrum the internal and extravesicular contributions by simply creating a pH gradient: hence, ³¹P chemical shift directly depends on the pH, as classically described (Gorenstein, 1984). The result is shown on the insert of Fig. 1, and, plotted truncated on the bottom trace. Under these circumstances, pH equilibration induces a progressive shift of the internal resonance (high-field resonance) to the chemical shift corresponding to the external pH (the most intense peak). By adding 8 µM MnCl₂ 0.1 M, the resonance arising from the extravesicular contribution vanishes by paramagnetic effect, while the resonance of enclosed (not accessible to paramagnetic ion) phosphate remains unaffected (middle trace of Fig. 1). A slight line width broadening was simply observed (from 30 to 35 Hz), as a result of the paramagnetic effect on external deuterated water thus resulting in a loss of homogeneity. Successive spectra were then acquired up to 3 h (top trace). As no significant shift of the resonance occurred, it could be deduced that no membrane permeabilization had occurred (i.e. no ionophoric property). Furthermore, total signal intensity (the integral of the peak) was unchanged, indicating that no destruction of the LUV had occurred. However, a significant line broadening (to more than 55 Hz) strongly suggested that CNT–membrane interaction, at least a binding of CNTs at the surface had occurred: in the absence of any chemical shift variation, the line broadening could only be attributed to increased correlation time.

In order to study the dynamics and structural consequences of CNT presence, multibilayers of DMPC (dimyristoyl phosphatidyl choline) were finally used: MLV are big sized (up to 100 µm) multibilayers with very small intermembrane medium and relatively weak intramembrane cohesion forces. Such systems are well adapted to study dynamic and structural modifications, and the use of these systems and ³¹P and ²H solid state NMR, and ESR allowed to observe such parameters at the different levels of the membrane and to study of collective motions in the presence of highly concentrated models (80 mM) (Dufourc et al., 1992; Seelig, 1977).

3.2. ³¹P NMR of DMPC dispersions (MLV)

The left trace presented on Fig. 2 is representative of axially symmetric powder phosphorus spectrum of standard bilayer structures of DMPC below phase transition (Debouzy et al., 1989). The temperature dependence of ³¹P Chemical Shift Anisotropy (CSA, figured by an horizontal bar) was measured on the phosphorus-NMR spectra of multilayers (MLV) (Pennequin et al., 2000) of pure DMPC or in the presence of CNTs (see legend of Fig. 2). As classically described (Duchene et al., 1992), CSA value can be considered as representative of the fluidity of the superficial part of a membrane, where phosphorus nuclei are located. By comparison with DMPC, no significant modification of the line shape was detected on the spectra of MLV in the presence of CNTs (under the same experimental conditions) indicating that the main bilayer structure was not significantly modified. Hence, no resolved resonance indicative of detergent effect or membrane destruction was observed at the isotropic position. Also, below 292 K, both line shape and the CSA values of pure DMPC and of CNT–DMPC systems were quite similar. By the way of contrast, a significant reduction of the CSA measured in the presence of CNTs occurred in the temperature domain, 294–298 K (295 K, see Fig. 2, right the top trace of

![Fig. 1. ³¹P NMR of LUV. Left: spectrum of LUV (EPC/EPA 9/1 M/M, 20 mM in NaH₂PO₄ 0.4 M); extravesicular (out) and internal (in) contributions are separated by using a 1 u pH gradient. Right: (C) same as left spectrum with magnification for a better identification of intravesicular contribution; (B) same sample after addition of paramagnetic ions that broaden under detection the extravesicular contribution; (A) same sample in the presence of 1 mg CNTs after 3 h evolution.](image-url)

![Fig. 2. ³¹P NMR (left trace) of DMPC dispersion over transition temperature. The arrow represents CSA range (ppm), measured between the left shoulder (σ₁) and the high-field edge (σ₂) of the spectrum. Right: temperature dependence of the CSA, measured on pure DMPC (●), or on CNT–DMPC systems (1/50 W/W) (○).](image-url)
CNTs/lipid 1/50 W/W). Besides, the phase transition temperature was found to be 2 K lower than that of pure DMPC, whereas the measures performed on traces at higher temperatures (298–313 K) also exhibited a significant reduction, revealing a higher fluidity at the polar head level where phosphorus is located.

3.3. 2H NMR of chain perdeuterated DMPC liposomes

3.3.1. 2H NMR lineshape

The bottom spectrum of DMPC-d54 dispersions (DMPC with perdeuterated chain) presented on the spectrum of Fig. 3 is typical of phospholipid bilayers below phase transition (temperature of 296 K) (Douliez et al., 1996; Fauvelle et al., 1997). Such a spectrum appears as a superimposition of symmetrical doublets, each doublet corresponding to a methylenic CD2 group or to the terminal methyle CD3 group of the acyl chain. For a given doublet, the splitting (quadrupolar splitting, $\Delta Q$) is directly related to the local order following the relation:

$$\Delta Q = A \times (3 \times \cos^2 \theta - 1)$$

where $A$ 170 kHz (for CD2 bound in DMPC) and $\theta$ is the averaged value of the solid angle of reorientation. This splitting can be used in a first approximation as an order parameter. As the acyl chain fluidity decreases from the terminal methyl group (CD3) to the methylenic groups close to the polar head of the lipids (the so-called “plateau region”, from C2 to C8 of the chain), the resulting spectrum consists of (i) an inner doublet with a quadrupolar splitting (in the 2–4 kHz range depending on temperature) attributed to the CD3 methyl group, (ii) doublets with increasing quadrupolar splittings assigned to successive CD2 groups from C14 to C9, (iii) the external edge doublet, attributed to the deuterium of the C2–C8 plateau region where quadrupolar splitting is measured between 24 and 30 kHz.

The spectra recorded in the same conditions in the presence CNTs (R = 1/50 W/W) exhibited significantly reduced values of quadrupolar splitting on all the doublets resonances (see CD2, C10 and plateau curves on Fig. 3, left) in the low temperature range 293–298 K, indicating increased mobility of these groups of the layer. Besides, the traces are consistent with a 2 K lowering of the transition phase, whereas the part of the traces plotted at high temperature (i.e. over normal phase transition temperature for DMPC, 297 K) are quite similar to those of pure DMPC systems except for the plateau region, i.e. the most superficial groups of the chains. This feature is also supported by the corresponding lineshapes and linewidths: as shown on Fig. 3 (left), pure DMPC trace is typical of multilayers in gel phase below transition, 296 K (linewidth around 750 Hz for the CD3 group) while reduced linewidths (linewidth of 600 Hz for CD3 resonance) and splittings on CNTs containing systems reveal the presence of liquid crystal phase over transition temperature. It is also clear from the plots Fig. 3 (right), that the mobility of the different groups were inequally influenced by the presence of CNS at high temperature. While this fluidization is markedly observed for the superficial groups (the plateau region), this dynamic effect is less important in the middle of the chain (that vanishes at 313 K), and completely absent at the terminal methyl group level.

3.4. ESR experiments

ESR Spin label experiments were performed to assess the membrane fluidity in different temperature conditions. Two probes were separately used, 5 NS give information about superficial membrane fluidity, while 16 NS concerned the inner membrane region.

The overall result (Fig. 4) shows an increase in the mobility of the two probes contribution in the two groups with the temperature increase. As previously described in MLV model (Dufourc et al., 1992; Follot et al., 2009), the phase transition in control groups occurs near 297 K. The 5 NS results are drawn in Fig. 4A. A shift of the phase transition, 3° lower, could be observed in the nanotube group. In the inner compartment, 16 NS results (Fig. 4C) show a major effect of the carbon nanotubes with a total disappearance of phase transition, while it occurs at the same temperature as 5 NS experiments in the control group.

Thus, from all the results observed in multibilayer systems, it is apparent that a dramatic fluidization of the membrane is observed...
cisely to observe the contribution of the phospholipid matrix and structure in such interactions. The present study shows that CNTs truly interact with model membranes, by inducing an overall fluidization of the layer more markedly observed at the superficial level, accompanied by a significant lowering of the transition temperature of DMPC dispersions. Besides, no significant modification of the main bilayer structure is present and no detergent effect of ionophoric property is found. The biological relevance of these results is the next step to investigate, using natural models (ghosts or erythrocytes) of living cells grown in labelled media to allow 2H NMR studies, and finally to use a metabolomic approach of cell metabolism in the presence of CNTs, by HRMAS NMR methods. These investigations are now in progress.

Conflict of interest

The authors declare that there are no conflicts of interest.

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