Interaction of Fullerene Nanoparticles With Biomembranes: From the Partition in Lipid Membranes to Effects on Mitochondrial Bioenergetics

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Fullerenes, also called buckyballs, discovered in 1985 by Robert Curl (Kroto et al., 1985), are hollow spherical molecules exclusively made of carbon atoms. They are characterized by small size (diameter of approximately 0.7 nm), large surface area, high reactivity (Aschberger et al., 2010), and lipophilicity (Fang et al., 2007). A wide variety of fullerenes is obtained by addition of functional groups resulting in nanoparticles with different physicochemical properties. Fullerene chemical modification by adding hydroxyl groups leads to a wide variety of new nanoparticles, called fullerolns or fullerols, which are less hydrophobic than the original molecules. Due to their unique characteristics, fullerenes have been intensively investigated in order to develop formulations with specific biological activities to be used in different biomedical fields, from diagnosis methods to therapeutic applications (Bakry et al., 2007; Bosi et al., 2003; Markovic and Trajkovic, 2008; Partha and Conyers, 2009). The intensive production and use of fullerenes result in their release and, for the most stable, accumulation in the environment, increasing the risk of biological systems to be exposed to high concentrations of these nanoparticles (Benn et al., 2011; Gottschalk et al., 2009). Studies using atomistic molecular dynamics simulations suggested that fullerenes deeply incorporate into cellular membranes, whereas fullerolns can barely penetrate the lipid bilayer (Bedrov et al., 2008; Qiao et al., 2007). Consistently, fullerenes perturb lipid order (Jeng et al., 2005) and induce membrane damage by lipid peroxidation, as studied in diverse types of cells (Sayes et al., 2004, 2005) and organisms, such as juvenile largemouth bass (Oberdörster, 2004). On the other hand, accumulation of fullerenes in lysosomes of oyster hepatopancreas cells (Ringwood et al., 2009), damage of DNA in mammalian cells (Matsuda et al., 2011) and human lymphocytes (Dhawan et al., 2006) and

Partition and localization of C_{60} and its derivative C_{60}(OH)_{18–22} in lipid membranes and their impact on mitochondrial activity were studied, attempting to correlate those events with fullerene characteristics (size, surface chemistry, and surface charge). Fluorescence quenching studies suggested that C_{60}(OH)_{18–22} preferentially populated the outer regions of the bilayer, whereas C_{60} preferred to localize in deeper regions of the bilayer. Partition coefficient values indicated that C_{60} exhibited higher affinity for dipalmitylophosphatidylcholine and mitochondrial membranes than C_{60}(OH)_{18–22}. Both fullerenes affected the mitochondrial function, but the inhibitory effects promoted by C_{60} were more pronounced than those induced by C_{60}(OH)_{18–22} (up to 20 nmol/mg of mitochondrial protein). State 3 and p-trifluoromethoxyphenylhydrazone-uncoupled respirations are inhibited by both fullerenes when glutamate/malate or succinate was used as substrate. Phosphorylation system and electron transport chain of mitochondria are affected by both fullerenes, but only C_{60} increased the inner mitochondrial membrane permeability to protons, suggesting perturbations in the structure and dynamics of that membrane. At concentrations of C_{60}(OH)_{18–22} above 20 nmol/mg of mitochondrial protein, the activity of F_{1}F_{0}-ATP synthase was also decreased. The evaluation of transmembrane potential showed that the mitochondria phosphorylation cycle decreased upon adenosine diphosphate addition with increasing fullerenes concentration and the time of the repolarization phase increased as a function of C_{60}(OH)_{18–22} concentration. Our results suggest that the balance between hydrophilicity and hydrophobicity resulting from the surface chemistry of fullerene nanoparticles, rather than the cluster size or the surface charge acquired by fullerenes in water, influences their membrane interactions and consequently their effects on mitochondrial bioenergetics.

Key Words: carbon nanoparticles; fullerenes; mitochondria; membrane partition.
increase of malformations and mortality in zebrafish embryos (Usenko et al., 2007) have been reported.

The size, shape, surface chemistry, and surface charge were identified as the physicochemical properties of water-colloidal fullerenes that can influence biological interactions and consequently their toxicity (Li et al., 2008; Sayes et al., 2004). However, fundamental cause-effect relationships have not been clarified yet. The disposition of carbon nanoparticles in biological systems (from bacteria to man) necessarily involves the passage across cell membranes, whatever the site of first contact between nanoparticles and organisms. Thus, it is important to unravel the relationship between physicochemical properties of nanoparticles and their impact on cell membrane structure and dynamics, in order to better predict the potential risks arising from the exposure to those compounds for health and for the environment. Several biological processes have been shown to be affected by membrane physical properties. Mitochondrial bioenergetics is an example of a crucial cell function that strictly depends on membrane integrity and is highly influenced by membrane mechanical properties. Not surprisingly, many lipophilic pollutants have been shown to exert toxic effects by affecting mitochondrial function (Moreno et al., 1995; Palmeira et al., 2009; Videira et al., 2001).

In the present study, we investigate how the physicochemical characteristics (size, surface chemistry, and surface charge) of 2 fullerenes (C60 and its derivative C60(OH)18–22) modulate their partition and localization across the bilayer thickness as well as their effects on the activities of inner mitochondrial membrane protein complexes involved in mitochondria bioenergetics.

**MATERIALS AND METHODS**

**Chemicals.** Fullerene C60 (99.5%) was obtained from Sigma Chemical Company (St Louis, Missouri) and C60(OH)18–22 (99.5%) was purchased from Buckypsa (Houston, Texas). The spectrophotometric probes, 16-(9-anthroylthio)palmitic acid (16-AP) and 2-(9-anthroyloxy)-stearic acid (2-AS), were obtained from Molecular Probes, Inc (Plano, Texas). Dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids, Inc (Murcia, Spain). All other chemicals used were obtained from Sigma Chemical Company and are of the highest grade of purity commercially available.

**Preparation of fullerene aqueous suspensions.** C60 aqueous suspensions were prepared transferring fullerene nanoparticles fromtoluene solution into the aqueous phase using ultrasonic treatment, as described elsewhere (Andrievsky et al., 2002) with some modifications. Briefly, 4 mg of solid C60 was added to 2 ml of toluene and stirred for several minutes to yield a purple solution. This solution was then added to 100 ml of deionized ultrapure water and stirred vigorously to promote formation of an emulsion of fullerene in water. The emulsion was sonicated (50 W, 40 kHz) for 24 h in a water bath to remove toluene by evaporation and to promote the transfer of the C60 nanoparticles into the aqueous phase. The mixture was filtered under reduced pressure through a cellulose nitrate filter with a pore size of 450 nm to remove the solid material and suspended particle agglomerates with average size above. The filtered colloidal suspension was transferred to a round-bottom flask and approximately half the volume of solvent was evaporated in a rotatory evaporator, at 50°C, to concentrate the sample and remove any traces of toluene. The absence of toluene was confirmed by gas chromatography/mass spectrometry, after headspace solid-phase microextraction. The UV-visible absorption spectra of C60 aqueous suspensions were recorded in the range of 200–700 nm with 1 nm of resolution, using a Lambda 45 UV/visible spectrophotometer (Perkin Elmer). The fullerene C60 concentration was determined considering 68 000 dm3 mol-1 cm-1 as molar absorption coefficient (ε) at 343 nm (Andrievsky et al., 2002). The polyhydroxy-C60 aqueous solution was prepared adding 68.0 mg of C60(OH)18–22 to 100 ml deionized ultrapure water and sonicating the mixture for 4 h in a water bath to obtain a stable fullerene aqueous solution. Both fullerene preparations were stored in the dark, at room temperature.

**Measurement of the size and zeta potential of fullerene nanoparticles in aqueous suspensions.** The size and surface charge, in terms of zeta potential, of C60 and C60(OH)18–22 nanoparticles in aqueous suspensions were assessed with a Zeta Plus analyzer (Brookhaven). The average diameters of both fullerene nanoparticles in water and in the buffer solutions used in partition and bioenergetics studies were determined by dynamic light scattering (DLS) at 25°C, using a helium-neon laser wavelength of 635 nm and a detector angle of 90°. Individual run times were 60 s (5 runs per each measurement). Nanoparticles size distributions were evaluated by number-weighted diameters. The zeta potentials acquired by each type of fullerene nanoparticle in water and in the buffer solutions used in partition and bioenergetics studies were determined, at 25°C, by measuring electrophoretic mobility using phase analysis light scattering, as described by Bratt et al. (2005). Data were recorded with at least 6 runs with a relative residual value (measure of data fit quality) of 0.03. Additionally, particles were morphologically characterized by transmission electron microscopy (TEM). For this purpose, a drop of fullerene aqueous suspension was placed on a 200-mesh Formvar-precoated grid. After air drying at room temperature, samples were observed using a JEOL JEM-100 SX electron microscope at 80 kV.

**Preparation of DPPC large unilamellar vesicles for fluorescence studies.** Large unilamellar vesicles (LUV) of DPPC were prepared from multimammalian vesicles by standard extrusion techniques as described elsewhere (Videira et al., 2001). Briefly, DPPC was dissolved in chloroform in a round-bottom flask and the solvent was evaporated to dryness in a rotatory evaporator. The resulting dry thin film on the wall of the round-bottom flask was hydrated with an appropriate volume of buffer solution (50 mM KCl, 10 mM Tris maleate pH 7.0) and dispersed under nitrogen atmosphere by handshaking in a water bath set at 50°C (9°C above the transition temperature of DPPC) to obtain DPPC multilamellar vesicles. These vesicles were then subjected to 13 repeated extrusions through 2 stacked polycarbonate membranes of 200-nm pore size to obtain a homogeneous population of LUV with an average diameter of 200 ± 10 nm, as determined by DLS. Phospholipid concentration in the final (stock) vesicle suspension (discarding the lipids retained by filters during the extrusion procedure) was determined by measuring the inorganic phosphate (Bartlett, 1959) released after hydrolysis of dried lipids, at 180°C, in 70% HClO4 (Böttcher et al., 1961). Then, DPPC vesicle suspensions with the required lipid concentrations were prepared by dilution of the stock preparation.

**Incorporation of probes and fullerenes into DPPC and mitochondrial membranes.** Fluorescent probes (16-AP and 2-AS) were added in aliquots (1.25 µl) from concentrated dimethylformamide solutions (2 mM) to LUV suspensions (2.5 µl) with lipid concentrations ranging from 100 to 600 mM, to give a bulk probe concentration of 1 µM and a progressive decrease of the probe/lipid molar ratio. In the range of probe/lipid molar ratios used (from 1/100 to 1/600), the probes exhibited high fluorescence intensities and did not induce significant changes in the biophysical properties of DPPC bilayers (Cadenhead et al., 1977; Videira et al., 1999). The mixtures were allowed to equilibrate in the dark, to protect probes from light, under magnetic stirring, at 50°C for a period of 2 h, to ensure the random incorporation of the fluorescent probe into lipid bilayers. Then, fullerene nanoparticles were added from concentrated aqueous suspensions to give molar concentrations ranging from 1.4 to 37.6 mM and the mixtures were incubated in the same conditions, for another half an hour, before recording the fluorescence spectra. A similar procedure was applied to mitochondria. In this case, the mitochondrial lipid concentration, previously determined in the mitochondrial lipid extract, varied from 50 to 200 µM and the incubation temperature was set at 37°C.
**Fluorescence measurements: fullerene membrane localization and partition coefficients.** Fluorescence quenching was used to assess fullerene localization and partition into synthetic and native membranes. The preferential localization of \(C_{\alpha}\) and \(C_{\alpha}(OH)_{18-22}\) across the thickness of lipid bilayers was deduced from their differential quenching effects on the fluorescence of the probes 16-AP, located in the hydrophobic core of the membrane, and 2-AS, located close to the polar regions of the bilayer. The most sensitive fluorescent probe to fullerene-induced quenching was used to determine the partition coefficient of the corresponding fullerene molecules (quencher).

Fluorescence emission spectra of 16-AP and 2-AS incorporated in DPPC or in mitochondrial membranes were recorded, in the absence or in the presence of fullerenes, between 390 and 550 nm, using a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies) provided with a thermostated cell holder. The excitation wavelength was set at 365 nm and the excitation and emission bandpass (slits) were both 5 nm. The fluorescence studies were performed in DPPC membranes in the fluid phase (at 50 ± 0.1°C) and in mitochondrial membranes at 37 ± 0.1°C, i.e., close to the animal physiological temperature. All the fluorescence measurements were corrected for the contribution of light scattering, recording the emission spectra of blanks (without added fluorescent probes) prepared from membranes containing each quencher (fullerene) at each concentration used.

The lipid-water partition coefficients were determined from the Stern-Volmer modified equation, as previously reported (Fato et al., 1986; Santos et al., 2003):

\[
\frac{1}{K_{pp}} = \frac{1}{K_q} + \frac{1}{K_p} \alpha_{pp} + \frac{1}{K_p}
\]

where \(K_{pp}\) is the apparent bimolecular quenching constant experimentally determined by Stern-Volmer plots, \(K_q\) is the bimolecular quenching constant for the fullerene in the membrane, \(K_q\) is the partition coefficient, and \(\alpha_{pp}\) is the volume fraction of the membrane phase. Thus, a plot of \(1/K_{pp}\) versus \(\alpha_{pp}\) yields a linear function with an intercept on the ordinate equal to \(1/K_q\) and a slope corresponding to \((1/K_q - 1/K_p)\), thus allowing the determination of fullerene partition coefficient \((K_p)\) into the membrane. The membrane volume fraction \((\alpha_{pp})\) was determined assuming a lipid-specific volume of 0.984 μl/mg of lipid (White et al., 1987). Mitochondrial membrane lipid concentration was determined, as previously described, after lipid extraction by the method of Bligh and Dyer (1959) and the mean molecular weight of phospholipids (775 g/mol) was used to estimate the mitochondrial membrane volume.

**Isolation of rat liver mitochondria.** Rat liver mitochondria were isolated from 8-week-old male Wistar rats, by differential centrifugation according to conventional methods (Gazotti et al., 1979) with minor modifications. The medium used for liver homogenization and mitochondria isolation contained 250 mM sucrose, 0.5 mM ethylene glycol tetraacetic acid (EGTA), 5.5 mM EDTA, 10 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), pH 7.4, and 0.1% (wt/ vol) fatty acid-free bovine serum albumin (BSA). EGTA, EDTA, and BSA were omitted from the final washing medium, which was adjusted to pH 7.2. The final concentration of the mitochondrial protein was determined by the biuret method (Gornall et al., 1949), using BSA as standard.

**Measurement of mitochondrial oxygen consumption.** Mitochondrial oxygen consumption (respiration rate) was measured polarographically using a Clark-type oxygen electrode (Estabrook, 1967) connected with a suitable recorder, in a 1 ml thermostated water-jacketed closed glass chamber with magnetic stirring, at 30°C. A volume of the mitochondrial fraction corresponding to 1 mg of protein was added to 1 ml of standard respiratory medium (250 mM sucrose, 10 mM HEPES pH 7.2, 20 mM KCl, 5 mM KHP04, and 2 mM MgCl2). Nanoparticles of fullerene \((C_{\alpha}\) or \(C_{\alpha}(OH)_{18-22}\)) were added from concentrated aqueous suspensions to give molar concentrations from 0 to 94 μM (ie, 5–94 nmol/mg of protein). The mixtures were incubated 5 min before the addition of the respiratory substrate, i.e., before the beginning of the respiratory activity. A mixture of 10 mM glutamate plus 5 mM malate was used to activate the mitochondrial respiratory chain from the complex I, whereas 10 mM succinate was used to activate the complex II. The standard respiratory medium was supplemented with 2 μM rotenone when succinate was used as respiratory substrate. The state 3 respiration was evaluated upon addition of 150 nM of adenosine diphosphate (ADP). Uncoupled respiration was initiated by the addition of 1 μM p-trifluoromethoxyphenylhydrazone carbonylanide p-trifluoromethoxyphenylhydrazone (FCCP). Oxygen consumption was calculated considering a saturation oxygen concentration of 232 mM O2 per milliliter of reaction medium, at 30°C. The respiratory control rate respiratory control ratio (RCR) was calculated according to Chance and Williams (1956).

**Measurement of mitochondrial transmembrane potential (ΔΨ).** The mitochondrial transmembrane potential (ΔΨ) was measured indirectly by measuring the transmembrane distribution of the lipophilic cation tetraphenylphosphonium (TPP+) using a TPP+-selective electrode in combination with a Ag/AgCl-saturated reference electrode, as previously described (Kamo et al., 1979). The potential difference between the selective electrode and the reference electrode resulting from mitochondrial TPP+ uptake was measured with an electrometer and recorded continuously in a Linear 1200 recorder. A linear voltage response of the TPP+ electrode as a function of log[TPP+] was obtained, with a slope of 59 ± 1, at 25°C, in good agreement with Nerst equation.

Reactions were carried out in an open thermostated water-jacketed glass chamber with 1 ml of standard respiratory medium supplemented with 3 μM TPP+ and 1 mg of mitochondrial protein, with magnetic stirring, at 30°C. Before the energization of mitochondria with respiratory substrates (glutamate/malate or succinate), fullerene nanoparticles \((C_{\alpha}\) or \(C_{\alpha}(OH)_{18-22}\)) were added from concentrated aqueous suspensions and the mixture was incubated for 5 min. The standard respiratory medium was supplemented with 2 μM of rotenone when succinate was used as substrate. No correction was made for the ‘passive’ binding of TPP+ to mitochondrial membranes, because the purpose of the experiments was to study the changes induced by fullerenes in the mitochondrial transmembrane potential as comparison to a control (in the absence of fullerenes) rather than to determine absolute values. Electrode calibration was performed in the presence of \(C_{\alpha}\) and \(C_{\alpha}(OH)_{18-22}\) to exclude any direct interference of fullerenes on the electrode signal.

**F.F1-ATPase.** The activity of the F.F1-ATPase of liver mitochondria was determined by an electrometric technique (pH electrode), as previously described (Madeira et al., 1974). The kinetics of ATP hydrolysis, which results in transmembrane proton translocation, was followed by recording the continuous pH changes in the reaction medium with a pH electrode. The reaction occurred at 30°C, in an open reaction thermostated chamber with permanent magnetic stirring, in 2 ml of reaction medium (130 mM sucrose, 60 mM KCl, 0.5 mM HEPES, and 2.5 mM MgCl2, pH 7.0), supplemented with 3 μM rotenone and 1 mg of mitochondrial protein. The reaction was initiated by the addition of 2 mM ATP-Mg. The release of protons was followed continuously with a Crison Titrino pH electrode resulting from mitochondrial TPP+ uptake was measured with an electrometer and recorded continuously in a Linear 1200 recorder. A linear voltage response of the TPP+ electrode as a function of log[TPP+] was obtained, with a slope of 59 ± 1, at 25°C, in good agreement with Nerst equation.

**Statistical analysis.** All the experiments were performed using 3 or more independent experiments with different preparations. The values were expressed as mean ± SD. The values were statistically compared by 1-way ANOVA with the Student-Newman-Keuls as posttest. Statistical significance was set at \(p < .05\).

**RESULTS**

**Characterization of \(C_{\alpha}\) and \(C_{\alpha}(OH)_{18-22}\) Aqueous Suspensions.** Fullerene nanoparticles dispersed in water or in the buffer solutions used in partition and bioenergetic studies were analyzed by DLS (Figs. 1A and 1B) and TEM (Figs. 1C and 1D). DLS data indicated that nanoparticles with an average
diameter around 34.5 ± 4.5 nm predominated in C_{60} aqueous suspensions, suggesting the presence of hydrated clusters formed by several fullerene molecules, whereas C_{60}(OH)_{18–22} water-colloidal systems exhibited nanoparticles with sizes up to 3.0 nm, which may represent essentially single fullerene molecules in hydrated state. TEM analysis (Figs. 1C and 1D) showed that C_{60} nanoparticles exhibit spherical shape and confirmed the hydrodynamic size distribution determined by DLS. Additionally, TEM micrographs showed a quasi-continuous network of C_{60}(OH)_{18–22} nanoparticles upon sample dehydration, which should have resulted from the interaction of individual molecules by hydrogen bonds. Zeta potential measurements, evaluated by electrophoretic mobility of colloidal nanoparticles (Fig. 1, insert), indicated that hydrated nanoparticles of both fullerenes acquired negative surface charge (−25.8 ± 3.9 and −37.8 ± 4.7 mV for C_{60} and C_{60}(OH)_{18–22} in water, respectively). The negative values of zeta potential suggest that fullerene nanoparticles were surrounded by a well-organized layer of hydrogen-bonded water molecules (a stable hydrophilic shell), which promoted a negative surface charge and prevented the interaction with similar neighboring molecules or clusters. The physical characteristics (particle size and zeta potential) of these 2 colloidal systems were preserved when nanoparticles were diluted in the buffer solutions used in partition and bioenergetics studies (Fig. 1, insert). Curiously, C_{60} nanoparticles dilution in Dulbecco’s modified Eagle medium (DMEM), a more physiological medium used to grow human cell lines, promote a significant reduction in particle size (22.2 ± 1.8 nm) without affecting zeta potential (−24.5 ± 2.3 mV). Additionally, size and zeta potential of C_{60}(OH)_{18–22} nanoparticles upon dilution in DMEM (2.0 ± 0.2 nm; −33.1 ± 3.7 mV) were not significantly different from those observed in water.

**Interactions of C_{60} and C_{60}(OH)_{18–22} With Lipid Membranes**

The characterization of fullerene membrane interactions is an essential issue to understand how different parameters, including size, surface chemistry, and surface charge, modulate
Fullerene Membrane Interactions

nanoparticle biological activity, selectivity, and toxicity. Thus, partition coefficients of $C_{60}$ and $C_{60}(OH)_{18-22}$ nanoparticles between water and membranes as well as their preferential localization across the bilayer thickness were investigated by fluorescence quenching methods. Two fluorescent probes were used: 16-AP, which probes the bilayer core; or 2-AS that monitors the hydrophobic region of the bilayer closer to the lipid-water interface (Videira et al., 1999). Figure 2 shows that the fluorescence intensity of 16-AP (Fig. 2A) and 2-AS (Fig. 2B) incorporated in large unilamellar DPPC bilayers decreased with increasing $C_{60}$ nanoparticle concentrations up to 14 µM. Although for both probes fluorescence quenching was described by Stern-Volmer equation, the quenching efficiency (proportional to apparent Stern-Volmer constant) was greater for 16-AP probe than for 2-AS probe (Fig. 3A), suggesting that $C_{60}$ molecules were deeply buried in the membrane, interacting with phospholipid hydrophobic chains. On the other hand, $C_{60}(OH)_{18-22}$ (0–37.6 µM) exerted limited effects on fluorescence intensity of 16-AP probe, but significantly decreased the fluorescence intensity of 2-AS probe (Figs. 2C and 2D and 3B), suggesting a preferential localization close to the polar region of the lipid bilayer. Thus, the partition studies with $C_{60}$ and 2-AS with $C_{60}(OH)_{18-22}$ were performed using the probes 16-AP and 2-AS, respectively.

FIG. 2. Fluorescence emission spectra of 16-AP probe (A, C) and 2-AS probe (B, D) incorporated in large unilamellar dipalmitoylphosphatidylcholine vesicles (200 µM) in the absence and in the presence of increasing concentrations of $C_{60}$ (0–14 µM) and $C_{60}(OH)_{18-22}$ (0–37.6 µM) nanoparticles. For both probes, the excitation wavelength was set at 365 nm.
Figures 4A and 4B show the Stern-Volmer plots for quenching of 16-AP and 2-AS incorporated in mitochondrial membranes as function of C$_{60}$ and C$_{60}$(OH)$_{18-22}$ nanoparticle concentration, respectively. For both fullerenes, the apparent Stern-Volmer constant decreased as the amount of mitochondrial membranes was increased (from 50 to 200 µM in lipid). Additionally, a linear relationship between the reciprocal of apparent Stern-Volmer constant (1/K$_{app}$) and the lipid membrane volume fraction ($\alpha_m$) was obtained with C$_{60}$ (Fig. 4B) and C$_{60}$(OH)$_{18-22}$ (Fig. 4D), thus allowing to determine the partition coefficients (K$_P$) between water and mitochondrial membranes for both fullerene nanoparticles (Table 1). The partition coefficients of both fullerene nanoparticles between DPPC membranes and water were also determined from Stern-Volmer plots at different lipid concentrations (100–600 µM).

In both membranes (DPPC and mitochondrial membranes), C$_{60}$ showed a higher partition coefficient than C$_{60}$(OH)$_{18-22}$ (Table 1). Additionally, both fullerene nanoparticles incorporated in higher extent into mitochondrial membranes than in DPPC membranes in the fluid phase. Thus, the partition coefficients in mitochondria (36 003 for C$_{60}$ and 6315 for C$_{60}$(OH)$_{18-22}$) were an order of magnitude higher than those found in synthetic lipid bilayers (1247 for C$_{60}$ and 212 for C$_{60}$(OH)$_{18-22}$). These results indicate that the lipid heterogeneity and protein content of mitochondrial membranes may have favored the partitioning of fullerene nanoparticles into these biological membranes.

**Effects of C$_{60}$ and C$_{60}$(OH)$_{18-22}$ on Mitochondrial Respiration**

The effects of C$_{60}$ and C$_{60}$(OH)$_{18-22}$ on mitochondrial respiration were evaluated by measuring the respiratory rates (state 3, state 4, and FCCP-stimulated respiration) supported by glutamate/malate (reducing complex I) or succinate (reducing complex II), as shown in Figure 5.

In order to perform an accurate analysis of data, 2 fullerene concentration ranges will be considered: from 0 to 20 nmol/mg of mitochondrial protein, which will allow to establish a comparison between the effects exerted by the 2 fullerenes, and a range covering concentrations above 20 nmol/mg of mitochondrial protein, at which only C$_{60}$(OH)$_{18-22}$ could be used. This resulted from the fact that C$_{60}$(OH)$_{18-22}$ presented a much higher solubility in water than C$_{60}$, allowing to obtain much more concentrated colloidal suspensions, from which small aliquots could be taken to be added to the 1 ml respiration medium contained in the reaction chamber. Therefore, comparing the effects of both fullerenes in the same bulk concentration range, it is noticeable that whereas C$_{60}$ stimulated state 4 respiration, promoting an increase of 15% and 23% in the state 4 rate, with glutamate/malate and succinate as substrates, respectively (Figs. 5A and 5B), C$_{60}$(OH)$_{18-22}$ had a negligible effect on this respiration state, with an increase of about 4% of its rate with both respiratory substrates (Figs. 5C and 5D). On the other hand, increasing concentrations of both fullerene nanoparticles exerted a progressively higher inhibition of state 3 respiration induced by ADP addition. In the same concentration range, both fullerenes also inhibited FCCP-induced (uncoupled) respiration at an extent comparable to that observed for state 3 respiration, with both respiratory substrates. These effects had, as expected, impact on the respiratory control ratio (RCR, given by the ratio of state 3 to state 4 respiration rates), which reflects the coupling of the mitochondrial oxidative phosphorylation. This important parameter of mitochondrial functioning showed high values in control experiments (4.35 ± 0.18 and 5.42 ± 0.21) when glutamate/malate or succinate was used as respiratory
In the presence of increasing concentrations of both fullerenes, up to 20 nmol/mg of mitochondrial protein, RCR showed a progressive and significant decrease with both respiratory substrates.

Effect of C₆₀(OH)$_{18-22}$ on mitochondrial respiration were also studied, as previously mentioned, at concentrations above 20 nmol/mg of mitochondrial protein. The interest in this study is based on the fact that C₆₀(OH)$_{18-22}$ presented a partition coefficient in mitochondrial membranes 6-fold lower than C₆₀, its effective concentration in membrane being hence more reduced than that of C₆₀. For example, when the aqueous bulk fullerene concentration is 20 nmol/mg protein (20 µM), the predicted mitochondrial membrane concentration is 63mM for C₆₀ and 40.7mM for C₆₀(OH)$_{18-22}$.

Regarding the state 4 respiration rate, it did not show significant alterations with the increase of C₆₀(OH)$_{18-22}$ up to 94 nmol/mg of mitochondrial protein, for complex I– and for complex II–activating substrates. An interesting effect was observed with respect to state 3 and FCCP-induced respiration, whose rates diverged above or at the concentration of 76 nmol/mg protein, when complex I or complex II was activated, respectively. Thus, considering the highest concentration of C₆₀(OH)$_{18-22}$ used (94 nmol/mg protein), a decrease of 31% and 30% was induced in state 3 respiration rate relative to the control (without nanoparticles added), using glutamate/malate and succinate as respiratory substrates, respectively. In the same conditions, the FCCP-stimulated respiration rate underwent a decrease of 16% and 10.5%, with the substrates glutamate/malate and succinate, respectively. RCR continues to decrease at concentrations of C₆₀(OH)$_{18-22}$ above 20 nmol/mg of mitochondrial protein, although in a less pronounced way than in a lower concentration range.

Effects of C₆₀ and C₆₀(OH)$_{18-22}$ on Mitochondrial Membrane Potential

The effects of fullerenes on mitochondrial phosphorylation efficiency were evaluated by following the fluctuations of the transmembrane potential (ΔΨ) associated with the oxidative phosphorylation in mitochondria energized by glutamate/malate or succinate. The addition of the respiratory substrates led to the development of a ΔΨ around 210 mV (negative inside), which was not affected significantly by the incubation of the mitochondrial preparation with increasing concentrations of fullerenes, as illustrated in Figure 6 for mitochondria energized with succinate. However, both fullerenes decreased the depolarization amplitude induced by ADP in mitochondria whose respiration was supported by succinate (Fig. 6) as well amounts of mitochondria membranes (50–200µM in lipid) as a function of C₆₀(A) and C₆₀(OH)$_{18-22}$(B) nanoparticles at 37°C. 1/K$_{app}$ determined from the slopes of the lines corresponding to the best fits to the experimental points in A and B, as a function of the volume fraction of membrane phase (α$_{m}$) is represented in plot C, in order to calculate the partition coefficients of C₆₀ and C₆₀(OH)$_{18-22}$.
as by glutamate/malate (data not shown). For the same concentration, the effects exerted by C$_{60}$ were more severe than those exerted by C$_{60}$ (OH)$_{18-22}$. Thus at 20 nmol/mg of mitochondrial protein, C$_{60}$ promoted a decrease in the depolarization amplitude of 31% and 35% with glutamate/malate and succinate as respiratory substrates, respectively, whereas C$_{60}$ (OH)$_{18-22}$ induced a decrease of 3.7% and 7.2%, in identical conditions. At the highest concentrations of C$_{60}$ (OH)$_{18-22}$ used (76 and 94 nmol/mg of mitochondrial protein), the time for repolarization (lag phase) also increased with both substrates. Nevertheless, the membrane potential achieved after repolarization for both substrates and fullerenes was not significantly affected.

**Effects of C$_{60}$ and C$_{60}$ (OH)$_{18-22}$ on F$_{o}$F$_{-}$ATPase Activity**

The action of C$_{60}$ and C$_{60}$ (OH)$_{18-22}$ on the enzymatic activity of the mitochondrial complex V (F$_{o}$F$_{-}$ATPase) was also evaluated, although considering the reverse reaction, i.e., ATP hydrolysis instead of ATP synthesis, as described in Materials and Methods section. Figure 7 shows that C$_{60}$ in the concentration range assayed did not affect F$_{o}$F$_{-}$-ATPase activity, whereas C$_{60}$ (OH)$_{18-22}$ at 20 nmol/mg protein induced a slight but significant inhibition, and, at concentrations above 74 nmol/mg protein, promoted 30% decrease in the enzymatic activity.

**DISCUSSION**

The release and accumulation of fullerenes in ecosystems should lead to the exposure of a great number of organisms to those nanoparticles. Cellular membranes are often the first site of contact (in the absence of cell wall) and the most universal barrier to foreign molecules, although it could also be one of the most vulnerable targets for lipophilic molecules that strongly incorporate into the lipid bilayer. Therefore, the study of fullerene interactions with membranes emerges as an important step to predict the damage that these nanoparticles can inflict on organisms. In this context, the investigation of the impact of fullerenes on mitochondrial bioenergetics, which depends largely on reactions that occur at the membrane level, may contribute to evaluate the capacity of fullerenes to cause adverse effects on organisms. In fact, mitochondrial systems have been used as excellent models to assess the toxicity of a variety of pollutants (Moreno et al., 2007; Palmeira et al., 1995; Pereira et al., 2009; Videira et al., 2001) and rat liver mitochondria have been often chosen for this type of studies because the liver is the main organ where detoxification processes take place and pollutants can accumulate. On the other hand, because mitochondria bear architectural and functional similarities to their prokaryotic ancestors, and mitochondrial proteins have undergone a very conservative evolution, data obtained with mitochondria from a certain animal species (eg, rat) can be predictably extrapolated to other animal species (eg, human).

In the present work, respiration of rat liver mitochondria showed to be affected by both fullerenes (stimulation of state 4 and inhibition of state 3 and FCCP-uncoupled respiration), in the same bulk concentration range (up to 20 µg/mg of mitochondrial protein). The main difference between the effects exerted on mitochondrial respiration by the 2 fullerenes concerned state 4 respiration, which was much more stimulated by C$_{60}$ than by C$_{60}$ (OH)$_{18-22}$ nanoparticles. This effect is generally interpreted as resulting from an increase of the inner mitochondrial membrane permeability to protons (proton leak), afforded by perturbations in lipid packing and organization.

### TABLE 1

Values of the Apparent Stern-Volmer Constants (K$_{app}$) for C$_{60}$ and C$_{60}$ (OH)$_{18-22}$ Nanoparticles Using 16-AP and 2-AS Probes, Respectively, Incorporated in Dipalmitylophosphatidylcholine (DPPC) Unilamellar Liposomes (LUV) in the Fluid Phase (50°C) and in Membranes of Mitochondria (37°C), at Different Lipid Concentrations

<table>
<thead>
<tr>
<th>[DPPC] µM</th>
<th>C$_{60}$ Fluorescence Quenching of 16-AP</th>
<th>C$<em>{60}$ (OH)$</em>{18-22}$ Fluorescence Quenching of 2-AS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K$_{app}$ (mol$^{-1}$ dm$^{-3}$)</td>
<td>K$_{app}$ (mol$^{-1}$ dm$^{-3}$)</td>
</tr>
<tr>
<td>100</td>
<td>129 ± 161±3104</td>
<td>1247</td>
</tr>
<tr>
<td>200</td>
<td>118 ± 372±3600</td>
<td>13719 ± 400</td>
</tr>
<tr>
<td>300</td>
<td>102 ± 305±2904</td>
<td>13444 ± 345</td>
</tr>
<tr>
<td>400</td>
<td>100 ± 177±3130</td>
<td>13278 ± 325</td>
</tr>
<tr>
<td>600</td>
<td>88 ± 512±3097</td>
<td>12056 ± 297</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>[Lipid of Mitochondria] µM</th>
<th>K$_{app}$ (mol$^{-1}$ dm$^{-3}$)</th>
<th>K$_{app}$ (mol$^{-1}$ dm$^{-3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>20 4368 ± 6547</td>
<td>36 003</td>
</tr>
<tr>
<td>100</td>
<td>128 207± 4395</td>
<td>17 761 ± 494</td>
</tr>
<tr>
<td>150</td>
<td>95 105± 3702</td>
<td>15 684 ± 398</td>
</tr>
<tr>
<td>200</td>
<td>74 389 ± 3204</td>
<td>13 813 ± 399</td>
</tr>
</tbody>
</table>

Nanoparticle partition coefficients (K$_{p}$) in both membranes are also indicated.
Fluorescence quenching studies showed that both fullerenes partitioned strongly in synthetic lipid bilayers as well as in native membranes, although the affinity of \( \text{C}_{60} \) for DPPC or mitochondrial membranes was about 6 times higher than that of \( \text{C}_{60}(\text{OH})_{18-22} \) (Table 1). On the other hand, these studies also demonstrated that \( \text{C}_{60}(\text{OH})_{18-22} \) preferentially populated the outer regions of the bilayer, extending their interactions to the polar headgroups of lipid molecules, whereas \( \text{C}_{60} \) preferred to localize in deeper regions of the bilayer. Therefore, taking into consideration the differential effects of fullerenes on mitochondria state 4 respiration, we can infer that the physical properties of mitochondrial membranes should have been more affected by \( \text{C}_{60} \) than by \( \text{C}_{60}(\text{OH})_{18-22} \) nanoparticles, which could be explained by their different effective membrane concentrations and/or their distribution across the bilayer thickness (\( \text{C}_{60} \) deeply incorporated in the membrane and \( \text{C}_{60}(\text{OH})_{18-22} \) interacting at the level of the outer regions of the bilayer). On the other hand, both fullerenes induced a decrease in state 3 and FCCP-uncoupled respiration. However, whereas in the presence of increasing concentrations of \( \text{C}_{60} \), state 3 and FCCP
respiration decreased in parallel, concentrations of C₆₀(OH)₁₈–₂₂ above 20 nmol/mg of mitochondrial protein induced an inhibition of state 3, which was more pronounced than that observed in FCCP-uncoupled respiration, thus indicating that these particles, besides inhibiting the electron transport chain, also exerted a perturbation of the phosphorylation system. This finding is in agreement with the decrease in FₒF₁-ATPase activity at concentrations of C₆₀(OH)₁₈–₂₂ above 20 µg/mg of mitochondrial protein and with the increase in length of the repolarization phase (lag phase) induced by these C₆₀(OH)₁₈–₂₂ concentrations, which indicates an uncoupling between the oxidation of reduced substrates at the electron transport chain level and the phosphorylation of ADP by the ATP synthase. These effects associated with a decrease of the depolarization amplitude, as denoted by measuring the fluctuations of transmembrane potential (ΔΨ), clearly demonstrate that ADP phosphorylation was seriously affected by the polar fullerene.

Interestingly, most of the effects induced by both fullerenes on mitochondria bioenergetics were exerted regardless of the respiratory substrates used. This fact suggests a relatively nonspecific action on mitochondrial complexes, which could be mediated by changes in the lipid environment. Taking into account the decrease in FoF₁-ATPase activity and the increase in the length of the repolarization phase, it seems likely that the polar fullerene induces a perturbation of the lipid bilayer of the inner mitochondrial membrane, which could affect the function of the ATP synthase. This hypothesis is supported by the results of the experiments shown in Fig. 7, which demonstrate that the perturbation of the lipid environment is not specific to the respiratory substrates used, and that it has a significant effect on the function of the mitochondrial FoF₁-ATPase.
account that nanoparticles of $C_{60}$ and its polar derivative displayed both negative values of zeta potential and that, despite the different size of nanoparticles in aqueous suspensions, both fullerenes strongly partitioned in mitochondrial membranes, differences in mitochondrial effects between the 2 types of nanoparticles might mainly result from their preferential localization within the membrane (deeper for $C_{60}$ than for $C_{60}(OH)_{18-22}$), which is modulated by nanoparticle surface chemistry. Regarding $C_{60}$ fullerene, a dynamic equilibrium should occur between large clusters of $C_{60}$ molecules (34.5 nm) in aqueous suspension and free molecules. Consistently, molecular dynamics simulation studies (Li et al., 2007) indicate that the partitioning of $C_{60}$ molecules into the anisotropic membrane is favored compared with that of enlarged aggregates, whose accommodation would require high energy to drive the consequent distortion of packed lipid bilayers. Because functional mitochondria display a transmembrane electric potential across the inner mitochondrial membrane, generated by the translocation of protons from the matrix to the intermembrane space, it is expectable that proton abundance in this mitochondrial compartment attracts the negative fullerene nanoparticles, favoring the partition of fullerene individual molecules into mitochondrial membranes. Additionally, the incorporation of individual $C_{60}$ molecules within the 5-nm-thick bilayers of biological membranes could promote changes in membrane physical properties, which, although not compromising the bilayer structure and mitochondrial membrane integrity, could impact on mitochondria bioenergetics. A diagram highlighting the main aspects of our proposal regarding fullerene mitochondria interactions is shown in Figure 8.

Another relevant question that should be taken into consideration in order to guarantee relevant translational data from the in vitro assays to human health assessment concerns the compound concentrations used in those assays. This is a transversal drawback in toxicology, including in lethality assays, where the concentrations required to achieve this endpoint are often much higher than those able to promote adverse effects in the organism. In this context, basic studies of biochemical nature, such as those we performed with fullerenes, can offer more significant data, but ideally connections should be established between the molecular events and the biological responses in vivo. However, 2 aspects should be taken into account: firstly, drug concentrations used in laboratory are conditioned by technical constraints. In the present work, the concentration range used for each fullerene in mitochondria assays was defined considering the maximal concentrations of stable stock aqueous

![Figure 8](image-url)

**FIG. 8.** Schematic representation of a mitochondrium (A) highlighting some aspects of fullerene mitochondria interactions. In water phase, large clusters of hydrated $C_{60}$ molecules with a negative surface charge (a) are in equilibrium with free molecules (b), whereas $C_{60}(OH)_{18-22}$ nanoparticles (c) occur mainly as single hydrated molecules, also with a negative surface charge. Functional mitochondria display a transmembrane electric potential across the inner mitochondrial membrane due to proton translocation from the matrix to the intermembrane space. Proton abundance in this mitochondrial compartment attracts the negative fullerene nanoparticles, favoring the partition of fullerene individual molecules into mitochondrial membranes. In B, the preferential localization of $C_{60}$ and $C_{60}(OH)_{18-22}$ molecules within membrane bilayers, according to fluorescence quenching studies, is shown. In C, the mitochondrial respiratory system is represented, emphasizing the interactions of fullerenes with the complexes I–V and the promotion of proton leak (higher in the presence of $C_{60}$ than in the presence of $C_{60}(OH)_{18-22}$ molecules).
suspensions prepared \((C_{60}(OH))_{18-22}\) being much more soluble than \(C_{60}\) allows stock aqueous suspensions with higher concentrations). In these assays, the volume (1 ml) of reaction medium precluded the use of very dilute fullerene aqueous suspensions. The other aspect regards a very important toxicological concept, the dose threshold. This is relevant not only in laboratory work (it depends on the technique and equipment sensitivity) but also in vivo because organisms may deal differently with different drugs (drug disposition). Disposition data of fullerenes are scarce in literature. However, an interesting review of Aschberger et al. (2010) reports 0.5% as the estimated concentration of fullerene in creams used as cosmetic (the main source of human exposure to fullerenes), which is of the same order of magnitude of the concentrations we used in our and in other in vitro assays. In summary, we are confident that the biochemical approach addressed in the present work offers a mechanistic basis for future toxicity studies with fullerenes, although we are aware that more data should be gathered to predict the toxic activity of these compounds in vivo.

CONCLUSIONS

Overall, the results of the present study indicate that the surface chemistry of fullerene nanoparticles, rather than nanoparticle cluster size or surface charge, influenced fullerene membrane interactions and, consequently, their effects on mitochondrial bioenergetics. Thus, studies on nanoparticle partitioning and distribution in lipid membranes, in parallel with assays that unravel their impact on cellular functioning, such as those regarding mitochondrial bioenergetics (which supplies more than 95% of the cell’s total energy requirement), constitute a promising strategy to evaluate the toxicological potential of fullerenes and their derivatives. On the other hand, studies on mitochondrial function may provide novel insights to unveil the molecular mechanisms underlying fullerene biological activity.

FUNDING


REFERENCES


