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Fullerene up-take alters bilayer structure and elasticity: A small angle X-ray study

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ABSTRACT

The coupling of fullerene (C_{60}) to the structure and elasticity of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine bilayers has been explored by synchrotron small angle X-ray scattering. Multilamellar vesicles were loaded with 0, 2 and 10 mol.% of C_{60} and studied in a temperature range from 15 to 65 °C. The addition of C_{60} caused an increase in the bilayer undulations (~20%), in the bilayer separation (~15%), in the linear expansion coefficient and caused a drop in the bending rigidity of the bilayers (20–40%). Possible damaging effects of fullerene on biomembranes are mainly discussed on the basis of altered bilayer fluidity and elasticity changes.

1. Introduction

Fullerenes C_{60} are one of the most studied carbon nanomaterials due to their extraordinary material properties comprising their small size, spherical geometry, hydrophobicity, electronic configurations and photo-excitation states (Bakry et al., 2007). Moreover, the possibility for derivatization and functionalization makes fullerene a highly attractive material for various applications. In particular their carbon-based cage structure with delocalized π-molecular orbital electrons and diameter of about 1 nm makes them a promising candidate for medical diagnostic or therapeutic agents by entrapping desired material in the cage (Dellinger et al., 2013). Due to their unique structure and strong electronic properties, fullerenes C_{60} can also be used as radical scavengers, antioxidants, antiviral agents or enzyme inhibitors (Bakry et al., 2007; Dellinger et al., 2013; Rossi et al., 2013). Furthermore, the group of Ikeda successfully introduced the use of liposomes as solvents for fullerenes in order to deliver them into cells (Ikeda et al., 2012).

On the other hand the unique characteristic of C_{60} can also provoke undesired biological effects (Sayes et al., 2004, 2005). In a recent review, Rossi et al. (2013) pointed out that lipid membranes most likely mediate a mechanism of fullerene toxicity. There is still no clear consensus on the cytotoxicity of C_{60}, but is presumably related to biomembrane structure and functionality (Dellinger et al., 2013). In order to guarantee efficient and safe applications of C_{60}, it is of paramount importance that C_{60}-membrane interactions get understood (Monticelli et al., 2009; Rossi et al., 2013).

Computer simulation studies demonstrated that C_{60} may provoke formation of micropores or holes in phospholipid membranes, which then would contribute to membrane leakage (Chang and Lee, 2010; Qiao et al., 2007; Monticelli et al., 2009). Larger aggregates of C_{60} adhere on the surface of the bilayer membrane, whereas individual C_{60} molecules or small nano-agglomerates can penetrate into the lipid bilayer by means of passive transport through transient micropores in the membrane (Bedrov et al., 2008; Qiao et al., 2007). Once incorporated in the membrane, various studies specify that fullerenes are homogeneously dispersed in the centre of the bilayer membrane (within a 1–2 nm regime) (Li et al., 2008; Qiao et al., 2007; Wong-Ekkabut et al., 2008), and it is commonly anticipated that this incorporation causes an overall thickening of the membrane. Wong-Ekkabut et al. (2008) have additionally shown that a slight

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bilayer thickness increase is accompanied by a quite significant softening of the membrane. However, in their studies no bilayer rupture, micellization or formation of pores was seen, and they conclude that fullerene toxicity cannot be attributed to mechanical damage of the membrane alone, but elastic property changes of the bilayer have also to be taken into account.

Spectroscopy studies (Bensasson et al., 1994; Hungerbühler et al., 1993) provided first experimental evidence on successful incorporation of C_{60} fullerenes into vesicular and micellar membranes in aqueous environment. More recently, investigations on phospholipid model membranes have shown that both pristine and water soluble derivatives of C_{60} not only induce changes in the structural and elastic properties of the lipid bilayer, but also do change the phase behaviour (Chen and Bothun, 2009; De Maria et al., 2006; Jeng et al., 2003, 2005). Based on both, experimental and simulation studies, it has been further predicted that the partitioning of fullerene into lipid membranes is thermodynamically highly favourable (over $30k_BT$) (Rossi et al., 2013). We note though that the hydrophilic addends of functionalized C_{60} have a strong tendency to intercalate into the phospholipid bilayer keeping the C_{60}s attached to the membrane interface, whereas pristine C_{60} molecules, when solubilized in the liposomal POPC bilayers, tend to aggregate in the bilayer interior and cause strong reorganization of the phospholipid bilayer chains (De Maria et al., 2006).

In vitro studies revealed that nanoscale aggregates of water soluble C_{60} derivatives caused cellular damage, which was provoked through lipid peroxidation (Sayes et al., 2005). On the other hand, the protection of lipid membranes from radical induced-lipid peroxidation was found to be higher with pristine, liposoluble C_{60}, than its water soluble derivatives (Wang et al., 1999). Thus, fullerene's toxicity depends on the type and degree of functionalization; it has been shown that toxicity is seven orders of magnitude higher with pristine fullerenes in comparison to highly soluble functionalized derivatives (Sayes et al., 2004). When interpreting results of both *in vitro* studies, and studies on model membrane systems, it is therefore important to keep in mind the different behaviour of pristine fullerene C_{60} as compared to diverse fullerene derivatives.

In our previous study, we demonstrated by small angle X-ray scattering (SAXS) measurements that the up-take of fullerene-aggregates has the potential to disturb significantly the integrity of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) liposomes. In this case, the up-take of fullerene-aggregates (or smaller nano-agglomerates) from the aqueous phase was induced by several freeze and thaw cycles between room and liquid nitrogen temperature. Without destroying the integrity of the liposomes (no freeze and thawing applied), the fullerene-aggregates adhere to the outside of the vesicles and stabilize them, as observed in the increased stacking order of the bilayers (Zupanc et al., 2012). This latter observation is in agreement with De Maria et al. (2006), who also suggested that the presence of C_{60} increases the stability of POPC liposomes.

The aim of this study was to experimentally assess the interactions between fully dissolved pristine C_{60} and POPC multilamellar vesicles (MLVs) (no remaining C_{60} clusters in the excess of water phase). We explored their temperature dependent interaction by synchrotron SAXS. Our results are presented with respect to (i) the observed structural changes of the bilayer, and (ii) to the determined bilayer separation and membrane fluctuations, (iii) followed by a discussion on changes of the membrane elasticity. (iv) A comparison with the outcome on other model membrane systems is given, and (v) finally we discuss different scenarios for the interaction of fullerenes with biomembranes.

2. Materials and methods

2.1. Sample preparation

A stock solution of C_{60} nanoparticles (black crystalline powder, with estimated nominal purity >99.5%, Sigma–Aldrich, Steinheim, Germany) in chloroform ($CHCl_3$; Merck KGaA, Darmstadt, Germany) was prepared with a final concentration of 0.16 g/L. This is the solubility limit of C_{60} in chloroform at room temperature (Ruoff et al., 1993) and complete solubility was obtained by using 4 mg of powder C_{60} in 25 mL of $CHCl_3$ and applying water bath sonication for 3 h at 30 °C (note, after sonication the suspension appears clear and purple; even after 12 h the entire solution remains transparent clearly precluding the existence of aggregates, which would otherwise accumulate as sediment). A lipid stock solution was prepared by dissolving POPC powder (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, Avanti Polar Lipids, Inc., Alabaster, AL, USA) in $CHCl_3$ (50 mg POPC/mL). The pure POPC (control) as well as the POPC samples with 2 and 10 mol.% of C_{60}, respectively, were prepared from appropriately weighted amounts of the stock solutions (C_{60} mol. %:=C_{60} moles/(C_{60} moles plus POPC moles) × 100). After solvent ($CHCl_3$) evaporation for 12 h under vacuum conditions, MLVs were prepared by rehydrating the dry thin films with 0.1 mL of distilled water and subsequent vortexing of the dispersions was applied (each sample was vortexed intermittently five times at room temperature for 2 min). The readily prepared dispersions were subjected to a light stream of nitrogen and stored at −20 °C in sealed vials until usage for several days (note, no signs of C_{60} sediment in the form of black deposit on the bottom of the recipients was observed).

2.2. Small angle X-ray scattering experiments

Temperature resolved small angle X-ray scattering (SAXS) experiments were carried out at the Austrian SAXS beamline situated at the Synchrotron Trieste, Italy (Amenitsch et al., 1998; Bernstorff et al., 1998), using a wavelength of $\lambda = 1.54$ Å. Diffraction profiles were detected utilizing a Mar300 image-plate detector (Marresearch GmbH, Norderstedt, Germany) and calibrated using a powder sample of silver behenate (CH_3 $(CH_2)_{20}$—COOAg: *d*-spacing 58.38 Å) (Huang et al., 1993). The lipid dispersions were measured in a thin-walled 1 mm diameter quartz capillary in a steel cuvette (Anton Paar, Graz, Austria), which was inserted into a brass block. This sample holder block was in thermal contact with a water circuit, *i.e.* it was connected to a water bath with a freely programmable control unit (Unistat CC, Huber, Offenburg, Germany). In order to avoid air convection at the capillary the entrance and exit windows of the block have been covered with a thin polymer film. The temperature was measured in the vicinity of the capillary in the sample holder block with a Pt-element (100 Ω). Prior to measurement each sample was equilibrated for a minimum of 10 min at a predetermined temperature with an uncertainty of ±0.1 °C. The exposure time was set to 120 s. Scattering patterns were integrated using the program FIT2D (Hammersley, 1997). Background scattering originating from water, the capillary and air was subtracted, and data sets were normalized using the transmitted intensity, which was measured by a photodiode placed in the beamstop. Background corrected SAXS patterns were analysed by the application of the modified Caillé theory (see Supplementary material). The technique and underlying premises have been described previously in detail (Pabst et al., 2003, 2000b; for a review see Rappolt and Pabst, 2008)). The bilayer model used and its applications have been presented elsewhere (Rappolt, 2010). From the fits to the scattered intensities $I = S(q)|F(q)|^2/q^2$ ($S(q)$: structure factor; $F(q)$: form