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Cellular localisation of a water-soluble fullerene derivative

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Abstract

Fullerenes are a new class of compounds with potential uses in biology and medicine and many insights were made in the knowledge of their interaction with various biological systems. However, their interaction with organised living systems as well as the site of their potential action remains unclear. In this work, we have demonstrated that a fullerene derivative could cross the external cellular membrane and it localises preferentially to the mitochondria. We propose that our finding supports the potential use of fullerenes as drug delivery agents as their structure mimics that of clathrin known to mediate endocytosis.

In 1985, the third allotropic form of elemental carbon after graphite and diamond was discovered by Kroto et al. [1]. The preparation of fullerenes in workable quantities [2,3] and the synthesis of fullerene derivatives have led to their application in both chemical and engineering processes and numerous photophysical electrochemical studies [4–10]. However, the relationship between fullerene and biology has only recently become a realistic target for scientific research due to the increase in organic functionalisation of the highly hydrophobic C_{60} moiety, by covalent attachments of hydrophilic addends [11–13], thus, strongly improving their solubility in biological media.

Thus, the fullerenes have attracted considerable research interest partly for the intrinsic interest in their unique structures but also because once suitably dissolved, they have displayed a diverse range of biological activity. They have been shown to be particularly promising in the fields of neuroprotection [14] and antiviral investigations [15]. The potential thus exists that they can be used as drugs or carriers for other biological molecules. However, to determine whether fullerenes will ever be used in such a capacity, it is necessary to explore their interaction at the cellular level. In this paper, we report the first data to our knowledge for the distribution of a water-soluble fullerene derivative $C_{61}(CO_2H)_2$ in a sub-cellular environment using the techniques of immunofluorescence and differential centrifugation using a radioactive-labelled fullerene.

Two possible strategies exist for synthesising radioactive-labelled fullerenes. Conceptually, the simplest requires the labelling of the C_{60} core carbons with ^{14}C. However, this route is practically difficult to perform [16,17], since it involves a several-step elaboration of ^{14}C enriched graphite, which in turn requires the handling of large quantities of radioactive soot from which the labelled C_{60} can eventually be extracted. The second route involves the attachment of a labelled carbon fragment to the fullerene moiety and thus it can be applied to fullerenes other than C_{60}.

The very low quantum yields of fluorescence for the fullerenes have been well documented in the literature [18–20] and thus direct fluorescence in vitro cannot be easily achieved. Immunofluorescence is a technique, which is invaluable for locating specific molecules in cells by fluorescence microscopy using antibodies, labelled with high quantum yield fluorescent dyes. The discovery that the mouse immune repertoire is diverse enough to recognise and produce antibodies specific for fullerenes [21] has allowed the indirect determination of the distribution of the fullerene within the cellular environment.

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The properties of a sub-cellular particle that can be used for separation are extremely limited. They include, mass volume and density. The majority of investigations within this field use differential centrifugation. The separation of sub-cellular particles by differential centrifugation is based on the differences in the sedimentation coefficients of these particles. The sedimentation coefficient is a complex function of the volume, shape, and density of the particle, which also considers the viscosity and density of the medium [22]. The combination of these approaches leads us to propose that a water-soluble fullerene derivative could cross the cellular membrane barrier and target the mitochondria.

Experimental

Synthesis of $C_{61}(CO_2H)_2$. C_{60} (0.31 mmol) was dissolved in toluene (200 cm^3) and stirred under argon and vacuum at 140 °C for 24 h. The 2-diethyl malonate (0.463 mmol) was added along with carbon tetrabromide (0.31 mmol) and the solution was stirred for several minutes. DBU (1,8-diazabicyclo[5,4,0]undec-7ene (0.93 mmol) was added in two equal portions and the solution was stirred continuously for 18 h at room temperature.

The compounds in solution were separated by chromatography on silica gel (500 mm × 20 mm) first eluting with a solution of hexane:toluene [1:1] to elute C_{60} and then with toluene:ethylacetate [98:2] to elute the mono-ester. The mono-ester was evaporated and dried under low pressure (1 mm Hg) at room temperature for 24 h.

The mono-ester obtained as a 46.75% yield was dissolved in 150 cm^3 dried toluene and NaH (60% w/w suspension in mineral oil, 210 mg) which was added in five parts. The solution was stirred under argon at 70 °C for 24 h. Then, 30 cm^3 MeOH was added and the solution was cooled in air for 15 min prior to cooling in an ice bath for 1 h.

The resulting mixture was filtered using a porosity frit 3 under gravity and completed under vacuum. The resulting residue was washed twice with 25 cm^3 aliquots of hexane and twice with 25 cm^3 aliquots of MeOH.

The dried product was further washed using 20 cm^3 25% HCl solution and filtered. The product was further washed with Mili-Q plus grade water and dried under vacuum for 24 h. The resulting product was dissolved in THF and rotary evaporated before drying to reach a constant weight at 50 °C and 1 mm Hg, yielding 80.7 mg $C_{61}(CO_2H)_2$.

The ^{13}C MAS-NMR spectrum obtained on this product was measured at room temperature and 100.6 MHz using a Bruker ASX400 spectrometer. A high-power decoupling (DEC) pulse sequence was used with a recycle delay of 30 s, a 90° ^{13}C pulse (4 μs) and 7600 scans. The chemical shifts were quoted in parts per million from the external reference tetramethylsilane (TMS) with a spectral width equal to 250 ppm. The high-power setting was verified by ^{13}C DEC on adamantine. The FID processing used a 5-Hz line broadening. The fullerene powder was put into zirconia rotors that were spun at 10 kHz.

Cell culture. HS 68 human fibroblast (CRL-1635) and monkey kidney COS-7 (CRL-1651) cell lines were from the American Type Culture Collection. All cells were grown at 37 °C in a DMEM (Dulbecco's modified Eagle's medium), supplemented with 10% foetal calf serum (FCS), penicillin (100 IU/cm^3), streptomycin (100 μg/cm^3), and L-glutamine (2 mM) in a 95% air–5% CO_2 humidified incubator.

Indirect immunofluorescence microscopy. HS 68 cells growing on 12-mm glass coverslips and incubated for 24 h with 10 μM $C_{61}(CO_2H)_2$ were fixed in −20 °C methanol for 10 min before rehydrating in PBS containing 1% BSA and 0.05% NaN_3. Cells were incubated for 60 min at 37 °C with the mouse monoclonal anti-fullerene diluted 1:50 and/or with a rabbit anti-tubulin (T3526 Sigma) diluted 1:100.

After a brief washing in PBS, incubation was continued for a further 60 min, following the addition of secondary antibodies (Rockland) anti-mouse coupled with FITC and anti-rabbit coupled with rhodamine. Both were used at 1:100 dilution. After a further brief wash in PBS, incubation was further continued for 15 min in the presence of Hoechst solution diluted 1:100.

Differential centrifugation and the distribution of $C_{61}(CO_2H)_2$. The distribution of the labelled fullerene was studied using COS-7 or HS68 cells which were cultivated under the same conditions as described in the previous section and incubated for 24 h. The ^{14}C labelled compound (10 μM) dissolved in DMSO was added into the cell medium and any non-dissolved material was removed by centrifugation. This supplemented medium was then added to the cells and the radioactivity was measured using a liquid scintillation counter at frequent intervals during incubation.

For a typical experiment, the fullerene-supplemented medium was added to three 175 cm^2 half confluent flasks. After 24 h, the cells were collected by trypsinisation followed by a low-speed centrifugation (2000*g*, 10 min) and the medium was subjected to a further radioactive count. From there, the cells were washed twice with 50 ml PBS, then resuspended in 10 ml PBS. The cellular structure was disrupted by repetitive passage through a Tenbroeck homogenizer. The homogenate was then submitted to a 3000*g*, 10-min centrifugation to remove unbroken cells, plasmic membrane, and nucleus. The resulting supernatant was centrifuged at 15,000*g*, 20 min and the mitochondria-rich preparation was obtained as the pellet. Finally, this last supernatant was centrifuged at 105,000*g* for 60 min and a microsomal pellet and soluble cytosolic proteins were recovered. Each fraction was then subjected to a radioactive count and protein content determination. The protein level for each cellular component was determined using the BCA Assay (Pierce) with bovine serum albumin as standard.

Results and discussion

Characterisation of $C_{61}(CO_2H)_2$

Nuclear magnetic resonance provides a powerful tool for the study of C_{60} and doped fullerenes. The icosahedral structure for the C_{60} molecule makes that every carbon site is equivalent to every other site. Such a structure is consistent with a single sharp line in the NMR spectrum [23]. The ^{13}C NMR line for C_{60} is known to be at 143.7 ppm (parts per million frequency shift relative to tetramethylsilane). Through measurements of the chemical shift of ^{13}C, NMR can be used to get detailed information about the accomplishment of bridging reactions [24].

As the addition of functional groups to C_{60} induces a modification in the icosahedral structure, carbon atoms located near the bridge present a slight variation in chemical shift.

The integrated intensities of the peaks provide a characterisation of the sample stoichiometry and are also indicative of the chemical shift anisotropy of the ^{13}C NMR line due to the slow molecular reorientation of the C_{60} ions.

Fig. 1 presents the spectrum recorded on $C_{61}(CO_2H)_2$. It shows two lines at 143.7 and 144.4 ppm in a 57:3

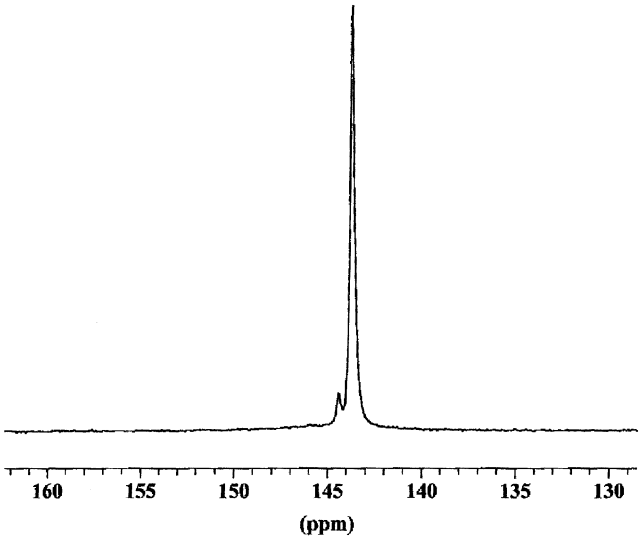

Fig. 1. 13-C MAS-NMR spectrum measured at room temperature and 100.6 MHz (chemical shift reference: TMS).

integral ratio indicative of two different site symmetries on the molecule. Thus, this spectrum demonstrates the accomplishment of the bridging.

Immunofluorescence microscopy reveals the intracellular distribution of $C_{61}(CO_2H)_2$. Cells were double-stained using anti-tubulin (rhodamine staining, Figs. 2A, B) and anti-fullerene (FITC staining, Figs. 2C, D) antibodies along with Hoechst to determine the location of the cell nuclei. Figs. 2B, D, and F show the fluorescence images obtained using cells for which no fullerene had been injected. The anti-tubulin antibodies stained the dense microtubule network of the cell with a clearly identified nucleus as determined by the Hoechst staining. No significant fluorescence could be detected with respect to the anti-fullerene antibodies.

Figs. 2A, C, and E illustrate those images obtained for the cells which have been incubated for 24 h with 10 μM $C_{61}(CO_2H)_2$. The microtubule staining remains identical whereas an FITC fluorescence could be easily detected as punctated dots surrounding the nucleus (Fig. 2C). The presence of this fluorescent signal is indicative for the presence of the fullerene $C_{61}(CO_2H)_2$ inside of the cell, instead of a plasmic membrane association and its colocalisation with a definite organite.

The above results have shown that $C_{61}(CO_2H)_2$ does locate in a cellular environment and thus we further wanted to confirm the intracellular location for the fullerene. This has been achieved using the radioactive labelling of one of the $C_{61}(CO_2H)_2$ carbon atoms with ^{14}C incubation with cells and the recovery of various fractions by differential centrifugation. Under the incubation condition we used, >90% of the 10 μM fullerene added was recovered with the cells. The distribution of the captured radioactive compound within the various cellular compartments is presented in Table 1. The

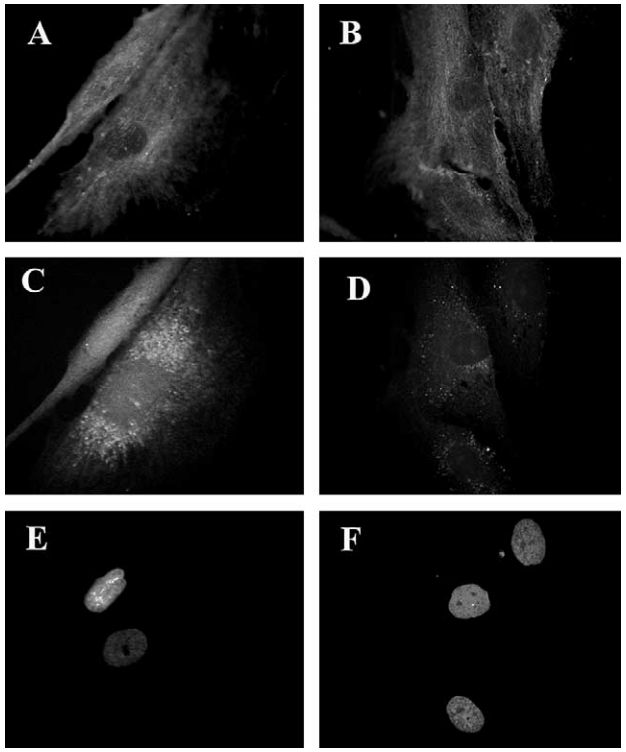

Fig. 2. Cellular localisation of $C_{61}(CO_2H)_2$ in Cos-7 cells: indirect immunofluorescent staining of microtubule network (A) and fullerene (C) and Hoescht labelling of DNA (E) of cells incubated with 10 μM of fullerene compared to identical staining (B: microtubules, D: fullerene, F: DNA) of untreated cells.

Table 1
Distribution of the [^{14}C] radioactivity in various cellular compartments after the cells were incubated for 24 h with 10 μM [^{14}C]–$C_{61}(CO_2H)_2$

Sub-cellular particle	Counts per minute ^{14}C/mg protein
Cytosolic fraction	544
Membranous fraction	5180
Mitochondria	6727
Microsomes	3013

breakdown for the number of radioactive counts obtained for each fraction shows that, in spite of its water solubility, the $C_{61}(CO_2H)_2$ fixes to cell membranes. Under the microscope, the fraction described as the membranous fraction appears as a mixture of both heavy membranes (including some mitochondria) and unbroken cells. The remarkable result is the association of the fullerene in the mitochondria-rich fraction. Moreover, this finding is consistent with the punctuated distribution of mitochondria typically obtained in COS-7 cell line [25].

Conclusions

To our knowledge, this study is the first to report the intracellular distribution of a water-soluble fullerene

derivative. We present evidence first by fluorescence microscopy that the fullerene derivative $C_{61}(CO_2H)_2$ is able to cross the cell membrane and second by radioactive labelling that the fullerene preferentially binds to the mitochondria. The generation of oxygen-free radical in cells comes from the leakage of electrons from the mitochondrial electron transport chain. The finding that fullerene locates close to this organelle suggests that the protective effects of fullerene described by others [14,26,27] with respect to oxygen radical species could be a result of this localisation. The demonstration that the water-soluble fullerene derivative we used is able to cross the cell membrane could be paralleled with the structural analogy between the fullerene cage and that of the clathrin-coated vesicles [28]. These last structures are known to be essential in the endocytosis pathway [29]. This observation strengthens the proposed use of fullerenes to mediate the delivery of other drugs through membranes and to the targeting of tissue [30].

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References

[1] H.W. Kroto, J.R. Heath, S.C. O'Brien, R.F. Curl, R.E. Smalley, Nature 318 (1985) 162–163.
[2] W. Krätschmer, L.D. Lamb, K. Fostiropoulos, D.R. Huffman, Nature 347 (1990) 354–358.
[3] W. Krätschmer, K. Fostiropoulos, D.R. Huffman, Chem. Phys. Lett. 170 (1990) 167–170.
[4] M.S. Dresselhaus, G. Dresselhaus, P.C. Eklund, Science of Fullerenes and Carbon Nanotubes, Academic Press, San Diego, 1996.
[5] Y.-P. Sun, J.E. Riggs, Z. Guo, H.W. Rollins, in: J. Shinar, Z.V. Vardeny, Z.H. Kafafi (Eds.), Optical and Electronic Properties of Fullerene and Fullerene-Based Materials, Marcel Dekker, New York, 1999.
[6] D.M. Guldi, K.-D. Asmus, J. Phys. Chem. A. 101 (1997) 1472–1481.
[7] Y.-P. Sun, R. Guduru, G.E. Lawson, J.E. Mullins, Z. Guo, J. Quinian, C.E. Bunker, J.R. Gord, J. Phys. Chem. B 104 (2000) 4625–4632.
[8] S. Foley, M.N. Berberan-Santos, A. Fedorov, D.J. McGarvey, C. Santos, B. Gigante, J. Phys. Chem. A. 103 (1999) 8173–8178.
[9] P.V. Kamat, D.M. Guldi, K.M. Kadish (Eds.), Recent Advances in the Chemistry and Physics of Fullerenes and Related Materials, Electrochemical Society, Pennington, NJ, 1999.
[10] R.V. Bensasson, E. Bienvenue, C. Fabre, J.-M. Janot, E.J. Land, S. Leach, V. Leboulaire, A. Rassat, S. Roux, P. Seta, Chem. Eur. J. 4 (1998) 270–278.
[11] M. Brettreich, A. Hirsch, Tetrahedron Lett. (1998) 2731–2734.
[12] S. Schergna, T. Da Ros, P. Linda, C. Ebert, L. Gardossi, M. Prato, Tetrahedron Lett. (1998) 7791–7794.
[13] A.H. Kurz, C.M. Halliwell, J.J. Davis, A.O. Hill, G.W. Canters, J. Chem. Soc., Chem. Commun. (1998) 433–434.
[14] L.L. Dugan, D.M. Turetsky, C. Du, D. Lobner, M. Wheeler, C.R. Almli, C.K.F. Shen, T.-Y. Luh, D.W. Choi, T.-S. Lin, Proc. Natl. Acad. Sci. USA 94 (1997) 9434–9439.
[15] S.H. Friedman, D.L. DeCamp, R. Sijbesma, G. Srdanov, F. Wudl, G.L. Kenyon, J. Am. Chem. Soc. 115 (1993) 6506–6509.
[16] W.A. Scrivens, J.M. Tour, K.E. Creek, L. Pirisi, J. Am. Chem. Soc. 116 (1994) 4517–4518.
[17] T. Tsuchiya, Y.N. Yamakoshi, N. Miyata, Biochem. Biophys. Res. Commun. 206 (1995) 885–894.
[18] S.-K. Lin, L.-L. Shiu, K.-M. Chien, T.-Y. Luh, T.I. Lin, J. Phys. Chem. 99 (1995) 105–111.
[19] C.S. Foote, Top. Curr. Chem. 169 (1994) 347–363.
[20] Y.-P. Sun, in: V. Ramamurthy, K.S. Schanze (Eds.), Molecular and Supramolecular Chemistry, Marcel Dekker, New York, 1997.
[21] B.-X. Chen, S.R. Wilson, M. Das, D.J. Coughlin, B.F. Erlanger, Proc. Natl. Acad. Sci. USA 95 (1998) 10809–10813.
[22] De, C. Duve, J. Berthet, H. Beaufay, Progr. Biophys. Biophys. Chem. 9 (1959) 325.
[23] R.D. Johnson, G. Meijer, J.R. Salern, D.S. Bethune, J. Am. Chem. Soc. 113 (1991) 3619.
[24] R. Taylor, J.P. Hare, A.K. Abdul-Sada, H.W. Kroto, J. Chem. Soc. Chem. Commun. 20 (1990) 1423.
[25] M. Yasuda, P. Theodorakis, T. Subramanian, G. Chinnadurai, J. Biol. Chem. 273 (1998) 12415–12421.
[26] I.C. Wang, L.A. Tai, D.D. Lee, P.P. Kanakamma, C.K. Shen, T.Y. Luh, C.H. Cheng, K.C. Hwang, J. Med. Chem. 42 (1999) 4614–4620.
[27] L.L. Dugan, E.G. Lovett, K.L. Quick, J. Lotharius, T.T. Lin, K.L. O'Malley, Parkinsonism Relat. Disord. 7 (2001) 243–246.
[28] B.I. Shraiman, Biophys. J. 72 (1997) 953–957.
[29] K. Takei, V. Haucke, Trends Cell Biol. 11 (2001) 385–391.
[30] D.W. Cagle, S.J. Kennel, S. Mirzadeh, J.M. Alford, L.J. Wilson, Proc. Natl. Acad. Sci. USA 96 (1999) 5182–5187.