Synthesis of ^{14}C-Labeled C_{60}, Its Suspension in Water, and Its Uptake by Human Keratinocytes

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Buckminsterfullerene is so unlike other molecules that the question of how it will interact with living systems is a particularly compelling one. Soon after the bulk synthesis of fullerenes was achieved,[1] it was discovered that C_{60} could convert oxygen from the triplet to the singlet state[2] and it was suggested that this could present some potential health risks.[3] These concerns were further justified with the recent discovery that functionalized C_{60} derivatives exhibited photoinduced DNA scission properties,[4] that they can be incorporated into artificial lipid bilayers, and that fullerenes can serve as excellent photoinduced electron transport moieties.[5] Perhaps most surprising of all is the report of substituted fullerenes being able to inhibit HIV-1 protease.[6] We describe how, in order for us to determine whether unfunctionalized parent C_{60} had any biological activity, we synthesized ^{14}C-labeled C_{60}, developed a method to produce a fine aqueous suspension of the labeled material, and then monitored the uptake of the labeled C_{60} by human cells. The C_{60} became rapidly cell-associated, though it did not affect the proliferation of human keratinocytes or human fibroblasts, indicating that the rapid accumulation of C_{60} in human cells does not result in acute toxicity.

Buckminsterfullerene is soluble in aromatic solvents and carbon disulfide, but virtually insoluble in water.[7] Since aromatic solvents can be disruptive to biological systems, we devised a method for producing fine aqueous suspensions of C_{60} which are suitable for uptake and toxicity studies using cultured human cells. We made the suspensions by starting with a saturated solution of C_{60} (0.15 mg) in benzene (100 μL) and adding it to THF (10 mL) at room temperature. The resulting light purple-colored solution was added dropwise to rapidly stirred acetone (100 mL). To the predominantly acetone solution was slowly added water (150 mL). After the addition of the first 50 mL of water, C_{60} began to precipitate as a fine mustard yellow suspension. Upon complete addition of the water, the organic solvents, and some water, were removed by distillation to a final volume of 100 mL, which corresponds to a 2.1 μM suspension of C_{60} in water. The resulting mustard yellow suspension did not settle on standing for more than 3 months, but the particles were mostly retained on a 0.22 μm cellulose acetate filter (Costar). UV/vis spectra of the aqueous suspensions showed the characteristic C_{60} peaks at 227, 280, and 360 nm (broadened due to the suspension properties). Peaks for small amounts of residual organic solvents were negated by filtering the suspension and using the filtrate as the blank. This further confirmed that the spectrum resulted from the suspended C_{60} particles. Scanning electron micrographs of the suspensions showed roughly spherical particles of a rather uniform size, with an average diameter of 0.3 μm (95% of the particles were between 0.25 and 0.35 μm). A 3-month-old sample of the C_{60} suspension, prepared and stored in air, was checked for its stability. Remarkably, liquid–liquid extraction of the aqueous suspension for 12 h using toluene afforded no C_{60} in the toluene extract. However, filtration of the suspension and dissolution of the particles in toluene yielded the typical purple-colored C_{60}-containing solution. HPLC analysis (silica gel, hexane) showed that >97% of the sample was C_{60} with one other peak of 2.6% intensity eluting shortly after the C_{60} band. This could be $C_{60}O$, a possible first oxidation product that is indicating suspended particle oxidation after prolonged storage in air.[8] Suspensions used for the biological assays were only a few hours to 3 days old. No decomposition was detected over these shorter periods.

The synthesis of ^{14}C-labeled fullerenes was then undertaken. The Krätschmer–Huffman method[1] produces large amounts of soot, so a self-contained disposable apparatus (made from common laboratory glassware) was necessary to minimize contamination and facilitate disposal of radioactive waste.[9] We then devised a method to make ^{14}C-enriched graphite. Methods for the isotopic enrichment of graphite with ^{13}C are available. This is done by producing graphite rods from ^{13}C-enriched amorphous carbon using pressure and heat,[10] or by packing a cored graphite electrode with ^{13}C amorphous carbon.[11] ^{14}C amorphous carbon is not commercially available, although its synthesis from $Ba^{14}CO_3$ is known.[12] However, we deemed the method to be exceedingly laborious and hazardous. We also felt that the manipulation of the resulting ^{14}C amorphous carbon powder required for either of the two established carbon enrichment methods would be cumbersome and hazardous. Thus, after several trial optimizations for ^{13}C fullerene enrichment, we developed a new solution-based method for "doping" graphite electrodes with either ^{13}C or ^{14}C (eq 1). Ba^*CO_3 (0.40 g, 2.0 mmol, 100 mCi for the ^{14}C-labeled material) was dissolved in a molten 1:1 mixture of $PbCl_2$ (2 g) and AgCl (2 g) to evolve the $^*CO_2$, which was passed, via a sealed glass manifold, through a solution of 2-lithiofuran (prepared from 8 mmol of furan and 4.0 mmol of *n*-butyllithium in 10 mL of ether and 0.6 mL of TMEDA) to afford the labeled furoic acid (52% radioactive yield). Aqueous workup followed by reduction with $LiAlH_4$ (0.4 g, 10 mmol) in Et_2O (20 mL) yielded the labeled furfuryl alcohol. Aqueous workup of the alcohol followed by polymerization[13] at 150 °C for 1 h using

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(8) The HPLC conditions for $C_{60}O$ detection were kindly provided by Professor A. B. Smith III and Dr. D. M. Cox, personal communication. For a report of $C_{60}O$, see: Creegan, K. M.; Robbins, J. L.; Robbins, W. K.; Millar, J. M.; Sherwood, R. D.; Tindall, P. J.; Cox, D. M.; Smith, A. B., III; McCauley, J. P., Jr.; Jones, D. R.; Gallagher, R. T. *J. Am. Chem. Soc.* **1992**, *114*, 1103.
(9) CAUTION: ^{14}C is radioactive, and extreme care should be used when working with compounds that contain this isotope, including use of a radiation approved exhaust hood. We also used two particulate filters to isolate the guide arm assembly from the manifold of the plasma discharge reactor. See: Scrivens, W. A.; Tour, J. M. *J. Org. Chem.* **1992**, *57*, 6932.
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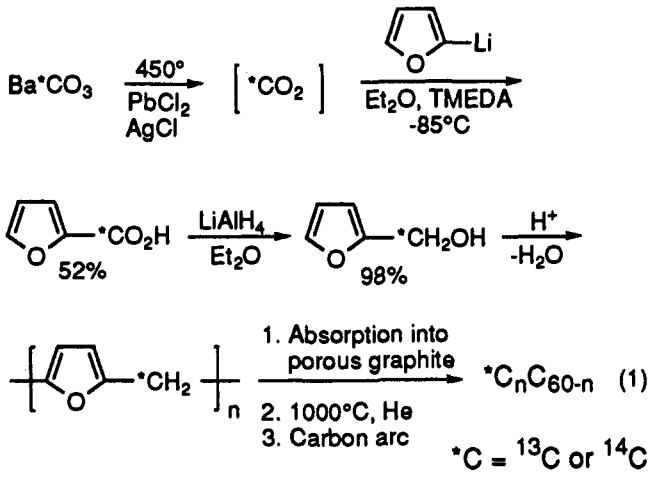

p-toluenesulfonic acid catalysis (13 mg, 70 μmol) in 1,2-dichlorobenzene (3 mL) afforded soluble poly(furfuryl alcohol). A porous graphite rod[14] (1 g, 4 cm long, $^3/_{16}$-in. diameter) was placed vertically in the poly(furfuryl alcohol) solution such that the bottom 3 cm of the rod was immersed for 2 min. The rod was then withdrawn from the solution and heated to 150 °C under a nitrogen stream for 1 h to remove the excess solvent. The absorption of the poly(furfuryl alcohol) solution into the rod was then repeated eight more times, which resulted in all of the polymer solution being absorbed into the rod. The polymer was cross linked within the graphite rod upon pyrolysis at 1000 °C under a static He atmosphere to produce amorphous carbon that was impregnated in the graphite rod. The resulting *C-impregnated graphite electrode was then treated in a glass plasma discharge reactor[9] and purified[15] to afford *C-labeled C_{60}. This procedure afforded ^{14}C-enriched C_{60} (14.6 mg, 227 μCi, 0.23% radioactive yield) with a specific activity of 11.2 mCi/mmol which is approximately ^{12}C:^{14}C = 200:1. Using HPLC (silica gel, hexane), a sample of unlabeled C_{60} was spiked with the ^{14}C-labeled C_{60}. The labeled C_{60} cochromatographed with the unlabeled material, and no other peaks containing radioactivity were detected.

We then proceeded to study the interaction of C_{60} with human cells. An aqueous suspension of ^{14}C–labeled C_{60} in serum–free MCDB153-LB culture medium was added to immortalized human keratinocytes, and the uptake of the label was measured at various times. As shown in Figure 1, the C_{60} rapidly became cell-associated in a time-dependent manner. After 6 h of incubation, approximately 50% of the applied radioactivity was taken up by the keratinocytes. No further cellular accumulation of label occurred over the next 9 h. Association of the label with control dishes not containing cells was about 10-fold less than that in dishes containing cells, thus ruling out a nonspecific interaction of the fullerene with the plastic dishes. To determine if C_{60} remained cell-associated following uptake, we incubated keratinocytes for 4 h with the labeled fullerene, the medium was then removed, and the cells were washed and refed with fresh

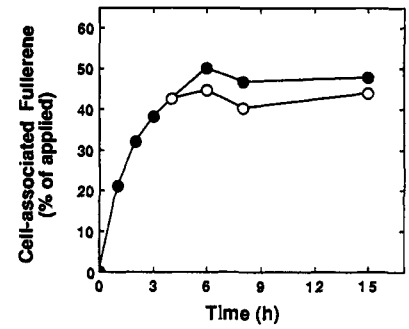

Figure 1. Uptake of ^{14}C-labeled C_{60} by human keratinocytes. Human keratinocytes immortalized by human papillomavirus type 16 DNA were cultured in 35-mm dishes in serum–free complete MCDB153-LB medium. Both the establishment of the cell lines and the culture methods have been described in detail previously.[16] To measure the uptake of ^{14}C-labeled C_{60} by the human keratinocytes, we followed a protocol previously established by us to study the uptake of another lipophilic molecule, *all-trans*-retinol (vitamin A), by mouse and human keratinocytes.[17] Briefly, basal MCDB153-LB medium containing ^{14}C-labeled C_{60} (32 000 dpm, 1.3 μM) was added when cells reached approximately 75% confluence. For the uptake, incubation was carried on at 37 °C in the absence of light and duplicate dishes were removed at the indicated times for measuring cell-associated ^{14}C-labeled C_{60} (closed circles). The medium was aspirated, and the cells were washed three times with 2 mL of Dulbecco's phosphate buffered saline. Distilled water (1 mL/dish) was then added, the cells were lysed by freeze–thaw, and radioactivity was measured in aliquots of the cell lysates. To measure the release of cell-associated ^{14}C-labeled C_{60} (open circles), cells were exposed to ^{14}C-labeled C_{60} for 4 h, then refed with fresh medium without C_{60}, and incubated at 37 °C. At the indicated times of chase, duplicate dishes were removed, and cell-associated radioactivity was measured as described above.

medium. At various times, cell-associated radioactivity was determined. As shown in Figure 1, C_{60} remained cell-associated during the 11-h chase. In addition, no increase of ^{14}C-labeled fullerene in the medium was observed during the chase period (not shown). Note that the thickness of the cells' plasma membrane is only 5–7 nm, and since the uptake was so facile, it is unlikely that the cells engulf whole particles. Moreover, since C_{60} could not be toluene-extracted from the aqueous suspensions, there are probably few wettable particles or dissolved C_{60} molecules. The C_{60} particles may be associated with the cell surface followed by diffusion of molecular C_{60} or small particle sections of the material. Further studies are underway to resolve this point.

We have also performed studies aimed at investigating whether C_{60} had any effect on the proliferation rate of cultured human keratinocytes and human fibroblasts. Aqueous suspensions of unlabeled C_{60} in MCDB153-LB (20 nM to 2 μM) were applied to human keratinocytes or human fibroblasts cultured in 24-well plates and incubated for 48 h. Cells were then incubated with [^3H]thymidine (0.5 μCi/well) for 16 h in media containing C_{60}, and thymidine incorporation was determined. We found no effect of the various C_{60} concentrations on thymidine incorporation by either human keratinocytes or fibroblasts.

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