Short Communication

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Evidence for nuclear internalisation of biocompatible [60]fullerene¹⁾

Abstract: A [60]fullerene derivative has been synthesised that demonstrates the ability to enter the nuclear volume of a variety of mammalian cells. This property is unique and has not been previously observed for other types of fullerene derivatives. This ability may have important implications in its use for biomedical applications, such as non-viral gene therapy and cancer chemotherapy where delivery of active agents to the cell nucleus is desired.

Keywords: colocalisation; confocal microscopy; fluorophore; fullerene; nucleus.

^aElectronic Supplementary Information (ESI) available: Experimental methods, Figures S1–S3, and Movies S1 and S2. See DOI: 10.1039/b000000x/.

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Background

Many types of nanoparticles (NPs) have been shown to internalise within mammalian cells (1), but only a few have been observed to internalise within the cell nucleus—most likely due to the tightly-regulated nuclear membrane (2). Internalisation of NPs into the nucleus is desirable for several reasons, including their use as 1. transfection agents (3), 2. drug delivery platforms for drugs that act on DNA (4), and 3. hyperthermia-inducing agents for cancer therapy using non-invasive stimulation by radiofrequency irradiation (5), magnetic-field cycling (6), or photonic

activation (7). For example, derivatised NPs, including protein-functionalised quantum dots (8) and peptide-functionalised gold NPs (9), have been shown to internalise into the nucleus. For underivatised NPs, single-walled carbon nanotubes (SWNTs), have been observed by direct transmission electron microscopy (TEM) imaging to also localise in the nucleus of human macrophage cells with dose-dependent cytotoxicity (10). Fullerene C_{60} is another classic carbon-based NP, however it was not been shown to enter the cell nucleus until recently. In particular, a water soluble derivative of C_{60} fluorescently labelled with a small molecule fluorophore was shown to enter cell nuclei through nuclear pore complexes in liver cancer cells (11). Here, we validate the nuclear internalisation ability of the C_{60} derivative in several other cell types, further supporting the unique intracellular biodistribution property of this specific fullerene compound.

Results and Discussion

We present further evidence for nuclear internalisation of the fluorescently-labelled, water-soluble [60] fullerene derivative, 3'-ethoxycarbonyl-3'-({2-[6-(2-{3-[2-*tert*-butyl-7-(ethyl-{3-sulfopropyl}-amino)-chromen-4-ylidene]-prop-1E-enyl]-3,3-dimethyl-5-sulfonato-3*H*-indolium-1-yl)-hexanoylamino]-ethoxy} carbonyl)-3'',3'',3'',3'',3''',3''',3''',3''',3''''-deca-(2-hydroxy-1-{hydroxymethyl} ethylcarbamoyl)-3'H,3'H,3''''H,3''''H,3''''H,3''''H-hexacyclop[1,9:16,17:21,40:30,31:44,45:52,60](C₆₀-I_h)[5,6]fullerene (abbreviated as C₆₀-serPF, structure shown in Figure 1). This observation is consistent with the fact that a similar unlabelled compound, C₆₀-ser (12), can be used as a transfection agent (13), as can other C₆₀ derivatives, both in vitro (14) and in vivo (15). The C₆₀-serPF conjugate is non-cytotoxic at the doses examined here (≤ 0.1 mg mL⁻¹) for HeLa (cervical cancer), SNU449 (liver cancer), SKOV3 (ovarian cancer), and HEK293T (immortalised fibroblast) cells. These cell types were chosen for study because (i) they are widely

Figure 1 The structure of C_{60} -serPF.

used (e.g., HeLa and HEK293T), allowing for easy standardised testing in the future with other fullerene derivatives; (ii) SNU449 is another liver cancer cell line, which may further support previous findings in liver cancer cells; and (iii) results in SKOV3 ovarian cancer cells may reveal the possibility of using the fullerene derivatives in other cancer applications.

Our interest in the study of subcellular localisation of fullerenes led to the search for a fullerene derivative that can be used to establish the distribution of fullerenes in mammalian cells. Our previous work with C_{60} -ser (12, 16) established the compound to be highly water-soluble and biocompatible; therefore, we adapted the same fullerene scaffold for derivatisation with an appropriate fluorophore for intracellular trafficking studies. We settled on the PF633 fluorophore (PF), as shown in Figure 1, because (i) its absorption and emission peaks are at wavelengths longer than 600 nm, which avoids the C_{60} -ser absorption range in the visible spectrum, (ii) it is one of the more efficient fluorescent dyes with a strong absorption band and a high quantum yield for the emission band, (iii) it is more resistant to singlet oxygen production [from C_{60} -ser under visible light (17)] than other fluorescent labels that were tested, such as Fluorescent Red 610, Alexa Fluor 610, and Atto 635 (data not shown). The detailed synthesis and characterisation of C_{60} -serPF is published elsewhere (11). The absorption and emission spectra for C_{60} -serPF are shown in Figure S2B. Results of MTT viability assays (Figure S1) show no significant cytotoxicity (p -values > 0.05) for the four cell lines examined due to exposure to the C_{60} -serPF concentration and longest incubation time used in this study. This observed biocompatibility for C_{60} -serPF is in sharp contrast to the behaviour of a less well-characterised colloidal fullerene material called “ nC_{60} ” which has been reported to be cytotoxic (18). Other well-characterised, water-soluble [60]fullerene derivatives such as the dicarboxyfullerenes, which concentrate in the mitochondria (19), or in

the endosome- or lysosome-like vesicles (20) (but not in the nucleus), have been reported to be biocompatible like C_{60} -serPF.

The intracellular distribution of C_{60} -serPF in HeLa cervical cancer cells was investigated with confocal microscopy. Cells were incubated with C_{60} -serPF for 4 or 24 h, fixed, stained with DAPI, and imaged with confocal microscopy. Cellular uptake of C_{60} -serPF was observed in the majority of the cells at both timepoints (Figure S2). Since individual C_{60} -serPF molecules are only ~ 3 nm in diameter, the images in Figure 2 must be of fullerene aggregates. Remarkably, 3-dimensional z-stacks obtained from confocal imaging indicate nuclear internalisation of C_{60} -serPF aggregates, as seen in the three orthogonal cross-sections (Figure 2A) and the 3D rendering model reconstructed from a z-section scan (Figure 2B, Movies S1 and S2). Interestingly, some fullerene aggregates appear associated with nucleoli found inside the nucleus (Figure 2A). Studies with the other cell lines showed similar findings (Figure S3). Further careful examination comparing fullerene intracellular distribution among various cell types may reveal important cell-type dependencies. Quantification of co-localisation indicated that approximately 27% and 21% (difference is not statistically significant), of the intracellular fullerene aggregates are within the nucleus after 4 and 24 h, respectively.

C_{60} -serPF appears to enter cell nuclei through nuclear pore complexes (11). Since C_{60} -serPF is observed in the majority of cells after 4 h of incubation, breakdown of the nuclear envelope during mitosis is likely not a requirement since only a small percentage of cells would have undergone this phase of the cell cycle during such a short period of time. Future studies need to investigate the intracellular location of fullerenes that are not in the nucleus. It is presently unclear if the cytoplasmic fullerene aggregates are associated with other organelles or are membrane-free. Lastly, fluorescently-labelled C_{60} -serPF may have a different intracellular distribution compared to unlabelled C_{60} -ser, so

Figure 2 C_{60} -serPF aggregates imaged in 3-dimensions demonstrate intranuclear localisation. (A) Orthogonal cross-sections were reconstructed from a set of z-stack scans of C_{60} -serPF aggregates in a HeLa cell. Coloured arrows in the x-z (green and blue arrows) and y-z (yellow arrow) orthogonal planes correspond to intranuclear C_{60} -serPF aggregates (red) visible in the x-y plane. Nucleus is in blue. Scale bar is 5 μm . (B) 3D rendering of the z-stacks shows C_{60} -serPF aggregates (red) inside and outside the nuclear profile (blue). The nucleus is cut in two orthogonal planes (top and bottom panels) to display C_{60} -serPF aggregates within the nuclear volume. Scale bar is 5 μm .

care must be taken when extrapolating the data presented here to the behaviour of unlabelled C_{60} -ser or other types of functionalised [60]fullerenes. Fullerenes of different structure might exhibit variable nuclear localisation behaviour. Comparison of different fullerene derivatives may yield useful structure-function relationships that can be used to design other bioactive fullerene-based materials.

Conclusion

We have compelling evidence for the nuclear internalisation and accumulation of a water-soluble, fluorescently-labelled [60]fullerene. Further studies are necessary to

uncover the biological mechanisms responsible for the observed intracellular distributions. The ability of C_{60} -serPF to enter the nuclear volume may have important implications in their use for biomedical applications, such as non-viral gene therapy and cancer chemotherapy, where delivery of active agents to the cell nucleus is desirable.

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Lon J. Wilson has been a Professor of Chemistry at Rice University for over 30 years. He has held NSF and NIH-sponsored fellowships and has published over 150 manuscripts and book chapters. Professor Wilson's research program involves bringing carbon nanotechnology to the fields of biology and medicine. The nanoparticle "building blocks" of this program are fullerenes (C_{60}), endohedral metallofullerenes ($M@C_{60}$), and ultra-short single-walled carbon nanotube capsules (US-tubes). Nanoengineered materials promise great advances in medicine, and, working with colleagues at various medical centers, his goal is to bring key, high-performance materials to the clinic as soon as possible.

Junghae Suh received her B.S. in Chemical Engineering at MIT and Ph.D. in Biomedical Engineering at Johns Hopkins School of Medicine. Before joining the Rice University department of Bioengineering in 2007 as an assistant professor, she completed a two-year postdoctoral fellowship in the Laboratory of Genetics at the Salk Institute for Biological Studies. Currently, Professor Suh works at the interface of chemistry, virology, biophysics, molecular biology, and protein engineering to investigate and create novel nanomaterials for various biomedical applications. In 2010, she received the National Science Foundation CAREER Award for her work.