Short Communication

Feiran Huang, Yuri Mackeyev, Erin Watson, Matthew A. Cheney, Lon J. Wilson*
and Junghae Suh*

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/.

*Corresponding authors: **Lon J. Wilson**, Department of Chemistry, Rice University, 6100 Main Street, Houston, 77005 TX, USA, E-mail: Durango@rice.edu; and **Junghae Suh**, Department of Bioengineering, Rice University, 6100 Main Street, Houston, 77005 TX, USA, E-mail: jsuh@rice.edu

Feiran Huang and Erin Watson: Department of Bioengineering, Rice University, 6100 Main Street, Houston, 77005 TX, USA

Yuri Mackeyev and Matthew A. Cheney: Department of Chemistry, Rice University, 6100 Main Street, Houston, 77005 TX, USA

Lon J. Wilson and Junghae Suh: R. E. Smalley Institute for Nanoscale Science and Technology, Rice University, 6100 Main Street, Houston, 77005 TX, USA

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-hexacyclopropa[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.

Acknowledgments: We thank Drs. Mary Dickinson and Tegy Vadakkan at Baylor College of Medicine for technical assistance with the image processing software. This work was supported by the National Institutes of Health (RC2GM092599) and the Welch Foundation (C-0627).

Received October 4, 2012; accepted February 8, 2013; previously published online March 18, 2013

References

1. Hussain SM, Hess KL, Gearhart JM, Geiss KT, Schlager JJ. In vitro toxicity of nanoparticles in brl 3a rat liver cells. *Toxicol in Vitro* 2005;19:975–83.
2. Terry LJ, Shows EB, Wente SR. Crossing the nuclear envelope: hierarchical regulation of nucleocytoplasmic transport. *Science* 2007;318:1412–6.
3. McBain SC, Yiu HH, El Haj A, Dobson J. Polyethyleneimine functionalized iron oxide nanoparticles as agents for DNA delivery and transfection. *J Mater Chem* 2007; 17:2561–5.
4. LaVan DA, McGuire T, Langer R. Small-scale systems for in vivo drug delivery. *Nat Biotechnol* 2003;21:1184–91.
5. Gannon CJ, Cherukuri P, Yakobson BI, Cognet L, Kanzius JS, Kittrell C, et al. Carbon nanotube-enhanced thermal destruction of cancer cells in a noninvasive radiofrequency field. *Cancer* 2007;110:2654–65.
6. Cai D, Mataraza JM, Qin ZH, Huang ZP, Huang JY, Chiles TC, et al. Highly efficient molecular delivery into mammalian cells using carbon nanotube spearing. *Nat Methods* 2005;2:449–54.
7. Carp O, Huisman CL, Reller A. Photoinduced reactivity of titanium dioxide. *Prog Solid State Ch* 2004;32:33–177.
8. Lowe AR, Siegel JJ, Kalab P, Siu M, Weis K, Liphardt JT. Selectivity mechanism of the nuclear pore complex characterized by single cargo tracking. *Nature* 2010;467:600–U126.

9. de la Fuente JM, Berry CC. Tat peptide as an efficient molecule to translocate gold nanoparticles into the cell nucleus. *Bioconjugate Chem* 2005;16:1176–80.
10. Porter AE, Gass M, Muller K, Skepper JN, Midgley PA, Welland M. Direct imaging of single-walled carbon nanotubes in cells. *Nat Nanotechnol* 2007;2:713–7.
11. Raoof M, Mackeyev Y, Cheney MA, Wilson LJ, Curley SA. Internalization of c60 fullerenes into cancer cells with accumulation in the nucleus via the nuclear pore complex. *Biomaterials* 2012;33:2952–60.
12. Wharton T, Kini VU, Mortis RA, Wilson LJ. New non-ionic, highly water-soluble derivatives of C-60 designed for biological compatibility. *Tetrahedron Lett* 2001;42:5159–62.
13. Sitharaman B, Zakharian TY, Saraf A, Misra P, Ashcroft J, Pan S, et al. Water-soluble fullerene (C-60) derivatives as nonviral gene-delivery vectors. *Mol Pharmaceutics* 2008;5:567–78.
14. Nakamura E, Isobe H, Tomita N, Sawamura M, Jinno S, Okayama H. Functionalized fullerene as an artificial vector for transfection. *Angew Chem-Int Edit* 2000;39:4254–7.
15. Maeda-Mamiya R, Noiri E, Isobe H, Nakanishi W, Okamoto K, Doi K, et al. In vivo gene delivery by cationic tetraamino fullerene. *Proc Natl Acad Sci USA* 2010;107:5339–44.
16. Wharton T, Wilson LJ. Highly-iodinated fullerene as a contrast agent for X-ray imaging. *Bioorgan Med Chem* 2002;10:3545–54.
17. Lee J, Mackeyev Y, Cho M, Li D, Kim JH, Wilson LJ, et al. Photochemical and antimicrobial properties of novel c-60 derivatives in aqueous systems. *Environ Sci Technol* 2009;43:6604–10.
18. Andrievsky G, Klochov V, Derevyanchenko L. Is the c-60 fullerene molecule toxic? *Fullerenes Nanotubes and Carbon Nanostructures* 2005;13:363–76.
19. Foley S, Crowley C, Smaih M, Bonfils C, Erlanger BF, Seta P, et al. Cellular localisation of a water-soluble fullerene derivative. *Biochem Bioph Res Co* 2002;294:116–9.
20. Li W, Chen CY, Ye C, Wei TT, Zhao YL, Lao F, et al. The translocation of fullerenic nanoparticles into lysosome via the pathway of clathrin-mediated endocytosis. *Nanotechnology* 2008;19:145102–14.

Feiran Huang received his Ph.D. in physics from Rensselaer Polytechnic Institute in 2003. Since then he has worked in the field of optical physics, biophysics, and nanomedicine. Dr. Huang investigated the electric effect on function of cell membrane transporters at the University of South Florida in 2004–2009. He then used real-time particle tracking with confocal imaging on nanocarriers as a post-doctoral fellow at Rice University in 2009–2011. Dr. Huang is currently a seismic imager at CGG.

Yuri Mackeyev received his Ph.D. from Lomonosov Moscow State University, Russia, with his dissertation on physical chemistry of fullerene fluorides. At present he is a Senior Research Scientist in Dr. Lon Wilson's Lab at Rice University, TX, focusing on development of a new group of fullerene-based, biocompatible organic compounds with potential for medical application. These include fullerene derivatives for a novel noninvasive radiofrequency (13.56 MHz RF) field treatment of tumors. He has published over 30 manuscripts cited in leading international publications.

Erin N. Watson received her B.S. in Bioengineering from William Marsh Rice University in 2012. There she worked on intracellular tracking of nanoparticles. She is currently pursuing a M.S. and Ph.D. in mechanical engineering from Stanford University, and is working in the field of medical robotics.

Matthew A. Cheney received his B.S. in 2005 and his M.S. in 2007, both in chemistry, from the University of Texas at El Paso. There he worked on the synthesis, resolution, and diastereoselectivity of a chiral auxiliary. He went on to receive a M.A. in chemistry and is currently working on his Ph.D. degree in chemistry at Rice University. For his doctoral degree, he is working on nanoparticle-assisted radiofrequency-induced hyperthermia of cancer.

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.