Publications:

Manuscripts:

Abstracts

Funding Obtained
NIH R21

Results:

1. Characterization of polyhydroxylated fullerene by Fourier Transform Infra-Red spectroscopy (FTIR)

![FTIR spectra](image)

Figure 1. FTIR spectra of polyhydroxylated fullerene isolated by dialysis (a); and methanol precipitation (b).

Polyhydroxylated fullerene (also named C60) was synthesized using a typical two-phase reaction in the presence of tetrabutylammonium hydroxide, and isolated by two different methods: dialysis and methanol precipitation. The FTIR spectra of both products were generally similar, with peaks at 1049 cm\(^{-1}\), 1392 cm\(^{-1}\), 1645 cm\(^{-1}\) and 3425 cm\(^{-1}\), which in the FTIR spectrum are assigned to C-O stretching vibration, -OH in-plane bending vibration, C=C double bond vibration, and -OH stretching vibration, respectively.

2. Radical scavenging activities of aqueous fullerene nanoparticles

In the present experiments, fullerol, a water soluble form of fullerene, was dissolved in distilled water and the resultant nanoparticle suspension is characterized by UV-Vis spectrometry, transmission electron microscopy (TEM), size distribution and zeta-potential analyses. The absorption peak of
Fullerol is 293 nm with the UV-Vis spectrum measurement (Fig. 2A). The particle size is between 25 nm and 45 nm (Fig. 2B). The nanoparticles are negatively charged, and the average potential of the particles is between -40 mV and -60 mV (Fig. 2C). In addition, the preparation is stable for up to 1 month at room temperature in the dark, and thus quite suitable for biological assays.

Figure 2. Characterization of nano-fullerol. Analysis of UV-Vis spectrum (A), size distribution (B) and surface charge (C) indicated that the aqueous nano-particles of fullerol had a characteristic absorption peak at 293 nm, a narrow size range of 25 nm-45 nm and a negative surface charge from -40 mV to -60 mV. Insert (C) illustrates the chemical structure of fullerol. The nano-fullerol was also proven able to scavenge various reactive oxygen species including superoxide anion (D), hydroxyl radical (E) and nitric oxide (F). The scavenging activities of various suspensions against superoxide anion were determined by a pyrogallol auto-oxidation method (Fig. 2D). Superoxide dismutase (SOD) is a biologically-derived anti-oxidative enzyme that elicits a strong scavenging activity against superoxide anion radical. The nano-fullerol suspension eliminates the radical in a dose-dependent manner, with its activity at 50 μM approximately equivalent to that of SOD at 25 U/ml. Similarly, hydroxyl radical assay using the Fenton-type reaction revealed that nano-fullerol was able to eliminate hydroxyl radical, with its activity at 25 μM approximately equivalent to that of mannitol between 1-10 mM (Fig. 2E). In addition, using a commercial Griess Reagent System, nitric oxide production was inhibited by the nano-fullerol suspension in a dose dependent fashion (Fig. 2F).
3. Fullerol has little cytotoxicity in human NP cells and mice bone marrow stem cells.

To test the cytotoxicity of fullerol in the cells, we performed LDH assay in human NP cells and mice bone marrow stem cells (BMSCs). As shown in Figure 3, Fullerol has little toxicity in these cells up to 10 μM.

4. Fullerol is taken up into mouse bone marrow stem cells.

Passage 3 mouse BMSCs exposed to 1 μM fullerol were visualized by transmission electron microscopy (TEM). Specimens for TEM were prepared by fixing the cells in 1.2% glutaraldehyde for 1 h at 4ºC and 1% OsO4 solution for 1 h at 4ºC, dehydration in ethanol, and embedding in epoxy resin. The resulting samples were cut into ultrathin sections with a diamond-knife suitable for TEM observations. TEM observations were carried out using a Hitachi H-7000 TEM (Hitachi, Japan) with an acceleration voltage of 75 kV. At days 3 and 6, C60 aggregates were found inside the cells (Figure 4).

3. Fullerol antagonizes overloaded oxidative stress induced by IL-1β and H2O2.

To evaluate the anti-oxidative stress effect of nano-fullerol (also Name C60), passage 3 mouse BMSCs were cultured in 6-well plates with growth medium (GM, LG-DMEM+10% FBS+100 μg/mL streptomycin+100 U/mL penicillin), GM+1μM C60, GM+10ng/mL IL-1, or GM+1μM C60+10ng/mL IL-1. At day 3, culture medium was removed and cells were incubated with the specific fluorescence probe H2DCFDA (10μM; Invitrogen, Eugene, OR) at 37ºC in dark for 30 minutes. The ROS signals were visualized with fluorescence microscopy. The cells were trypsinized, collected by centrifugation and washed twice with PBS, and then the median fluorescence intensity (MFI) was measured by flow cytometry. As shown in Figure 5A, the positively stained (green color) cells increased in the IL-1β treatment group, but decreased in the C60 group compared with the non-treatment group. Quantification with cell flow cytometry confirmed the significance (Figure 5B). C60 supplementation to IL-1β treated cells significantly decreased the ROS activity compared the group treated with IL-1β alone. Similar results were also obtained in human NP cells treated with H2O2 (Figure 6). These results showed that C60 reduced ROS activity and counteracted overloaded oxidative stress induced by IL-1β and H2O2.
Figure 5. fullerol (C60) reduced ROS activity and counteracted overloaded oxidative stress induced by IL-β. Mice BMSC were treated with 10ng/ml IL-β, 1µM C60 or both for 3 days and stained with 10 µM H2DCFDA, a fluorescent cellular reactive species indicator (A); scale bar=100µm. Fluorescence signal was increased in IL-β treated cells compared with the control group, but this increase was attenuated by administration of C60, while the lowest fluorescence signal was found in the presence of C60 alone (B). The median fluorescence intensity was measured by flow cytometry, which was consistent with fluorescence signal image. *: p<0.05.
Figure 6. Reactive oxygen species detection in human NP cells. Human NP cells were cultured in alginate beads and stained with YOYO-1 probing cell cytotoxicity (A-C) and H$_2$DCFDA dye for reactive oxygen species (D-F) after treated with H$_2$O$_2$ (500 µM) alone or together with fullerol. Fullerol was able to inhibit H$_2$O$_2$-induced cell damage as well as increase in cellular oxidative level.

6. Fullerol promotes gene expression of anti-oxidative enzymes

We used real-time RT-PCR to investigate the effect of C60 on SOD and catalase mRNA levels in D1 cells, a cloned mouse BMSC cell line (Figure 7A and B). In the presence of 10mM dexamethasone (DEX), both SOD and catalase mRNA levels were lower than the untreated control cells ($p<0.05$ for SOD and $p<0.01$ for catalase). Compared with DEX treatment alone, the addition of 1.0 µM C60 to DEX caused a significant increase in the mRNA level of both SOD and catalase, ($p<0.05$). Combined treatment of DEX with 0.1 µM C60 or 10µM glutathione (GSH) markedly increased the level of mRNA for SOD ($p<0.05$), but not catalase.

Figure 7. C60 promotes gene expression of antioxidative enzymes. D1 cells were seeded at a density of 3.0×10^4 cell/well in a 24-well plate and cultured in the presence of dexamethasone (DEX) and/or either glutathione (GSH) or C60. After 7 days in culture, cells were harvested and the expression of the antioxidative enzymes SOD (A) and catalase (B) was analyzed by real-time RT-PCR with 18s as an internal control. Data are expressed as the mean ± SD. *$p<0.05$, **$p<0.01$ versus DEX group; # $p<0.01$ versus control group; n = 3
7. Nano-fullerol protects matrix destruction induced by hydrogen peroxide or IL-1β

We examined the effects of nano-fullerol on extracellular matrix destruction caused by oxidative stress or inflammatory cytokines in human NP cells.

The GAG level was significantly decreased by the treatment of H$_2$O$_2$. However, co-treatment with nano-fullerol reversed the adverse effect of H$_2$O$_2$ (Fig. 8A). By immunofluorescence, the signal intensity of aggrecan was decreased upon treatment of H$_2$O$_2$, which could be reversed by nano-fullerol (Fig. 8B, upper panel). Similar results were obtained with type II collagen staining (Fig. 8B, lower panel).

The gene expression of aggrecan, type I and II collagen was examined by real-time RT-PCR. In the presence of H$_2$O$_2$ alone, mRNA levels of three genes decreased significantly, co-treatment with nano-fullerol had no effect with type I and II collagen (Fig. 8C). The mRNA level of aggrecan was actually decreased after treatment with fullerol and H$_2$O$_2$. However, while H$_2$O$_2$ caused a significant elevation in mRNA levels of MMP3, MMP9 and ADAMTS5, use of nano-fullerol did abolish the increase (Fig. 8C). Either H$_2$O$_2$ alone or together with fullerol didn’t affect gene expression of the tissue inhibitor of metalloproteinase TIMP1 (Fig. 8C).

We further investigated the protective effects of fullerol on IL-1β—an extensively studied proinflammatory cytokine—induced degeneration of NP cells. Human NP cells were treated with 10 ng/mL of IL-1β for 3 or 7 days in the presence or absence of fullerol. IL-1β caused an increase in nitric oxide production in the culture medium (Fig. 9A), a decrease in cellular proteoglycan content (Fig. 9B), and a robust decrease in mRNA levels of aggrecan, type I and II collagens at 3 and 7d (Fig. 9D-4F). Fullerol elicited a significant counteraction against the IL-1β activity (Fig. 9A-4B). Similar results were obtained with proteoglycan production at 7 d (Fig. 9C). In addition, with nano-fullerol treatment alone, the expression of aggrecan was significantly up-regulated, while the expression of type II and type I collagen remained unchanged (Fig. 9D-4F).
Figure 8. Fullerol rescues the impairment of extracellular matrix production by over-loaded oxidative stress. Human NP cells in alginate bead were treated with \( \text{H}_2\text{O}_2 \) (500 µM) alone or together with fullerol (Ful, 1 µM) for 4 h and cultured for additional 60 h. A, Cellular proteoglycan determined by a colorimetric method using the dimethylmethylene blue (DMMB) reagent. B, Immunofluorescence with anti-aggreca (Agg) and type II collagen (Col II) antibodies. Bar=50 um. C, Gene expression of genes related to extracellular matrix production by real-time RT-PCR. a, p<0.05 vs control; b, p<0.05 vs \( \text{H}_2\text{O}_2 \) group.

Figure 9. Fullerol inhibits nitric oxide production and extracellular matrix destruction by a pro-inflammatory cytokine. Human NP cells in alginate bead were treated with IL-1 (10 ng/mL) alone or together with fullerol (Ful, 1 µM) for 3 d (A-B) or 7 d (C-F). A, Nitrite level in culture medium was tested by the Griess reagent. B-C, Cellular proteoglycan was determined by a colorimetric method using DMMB. D-F, Gene expression of extracellular matrix proteins was assessed by real-time RT-PCR. * p<0.05; ** p<0.01.

8. Anti-inflammatory effects of fullerol on neuronal DRGs
The levels of TNF-\( \alpha \) are shown to be elevated in animal models of inflammatory response or neuropathic pain around the spinal cord and at the site of injury. Several clinical trials showed that application of the TNF-\( \alpha \) inhibitor etancercept onto the spinal nerve produced pain relief (Shimizu et al, 2010; Pillay et al, 2010; Kawakami et al, 2000). We treated freshly isolated DRGs from mice L3/L4 with TNF-\( \alpha \) and fullerol (0, 0.1,1,10 µM) for 24 h. The supernatant were collected for PGE2 and IL-6.
measurement with ELISA kits (Thermo Scientific and eBioscience). As shown in **Fig. 10**, TNF-α significantly increased both IL-6 and PGE-2 contents, while fullerol alleviated the inflammatory responses in a dose dependent manner. The maximum effects were observed in 1-10µM of fullerol group that bring the expression of IL-6 and PGE-2 to basal level. Our goal is to confirm and validate these anti-inflammatory effects with an *in vivo* model.

![Graph showing IL-6 and PGE-2 contents](image)

**Figure 10: Fullerol decreases the contents of IL-6 and PGE2 in DRG explants induced with TNF-α.** DRGs from mice L3/4 were cultured in 24-well plates with F-12 + 10% FBS, 100 U/ml penicillin/streptomycin and 10 ng/ml NGF (nerve growth factor) for 2 d. The explants was treated with 10 ng/mL TNF-α + 0, 0.1, 1, or 10 µM fullerol for 24 h in serum free medium. The supernatant was collected to detect PGE2 and IL-6 contents with ELISA kits. Wells without cells were used as a blank control. **p<0.01 compared with TNF-α group, # p>0.05 compared with the control group.

9. Nano-fullerol retards disc degeneration in a rabbit annulus puncture model

In our pilot study we investigated the therapeutic effects of nano-fullerene on annulus puncture-induced disc regeneration. In 5 New Zealand white rabbits, the anterior surfaces of lumbar IVDs were exposed under general anesthesia. Selected discs were punctured with an 18G needle and 25 µL of a nano-fullerene suspension, 1x10^5 adipose stem cells, or PBS, were injected into the disc. Four weeks post-operation, the rabbit spines were X-rayed first and then a T2 weighted MRI study was performed. The images from a morphological study and the X-ray and MRI scans showed that discs punctured and then injected with PBS showed a blurred AF/NP junction, narrowed disc space and a lower T2 weighted MRI signal indicative of disc degeneration. However, discs punctured and then treated with either fullerene or an intra-disc injection of adipose stem cells showed a distinct AF/NP junction, improved disc height and strong T2 weighted MRI signals (**Fig. 11**). We did not find any toxic effects of fullerol in this study.

![Images showing disc degeneration and repair](image)

**Figure 11: Nano-fullerene protects intervertebral discs from annulus puncture-induced degeneration.** Lumbar discs from 5 New Zealand White rabbits were punctured with an 18G needle and then 25 µL of a nano-fullerene suspension (Nano-C60), 1x10^5 adipose derived stem cells or PBS was injected into each disc. Representative images of lumbar discs (white arrows) at 4 weeks following the procedure are shown. (A) Morphology; (B) X-ray; (C) MR